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Effect of Dietary Inclusion of Conjugated Linoleic Acid on Quality Indicators of Aged Pork Loin

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Abstract

The purpose of this study was to examine the effect of dietary inclusion of conjugated linoleic acid (CLA) in hog feed on quality indicators of pork loins. A total of twenty barrows (PIC 380 x Cambrough 1025) were randomly paired, housed in pens, and fed 1% soybean oil or 1% CLA diet for 6 weeks. There were no significant differences in proximate composition of the pork loins ($P > 0.05$). Muscle pH increased and there were color changes over time ($P < 0.05$) which was independent of diet ($P < 0.05$). Shear force decreased over time ($P=0.017$), with CLA loins having lower shear force than the control ($P = 0.032$). These results indicate that CLA supplementation of finishing diets resulted in pork quality similar to that of traditional diets.

Keywords: conjugated linoleic acid, pork loin, muscle pH, pork quality

1. Introduction

Meat marbling is defined as the intramuscular fat located in perimysial connective tissue between the muscle fiber bundles. Marbling is associated with increased quality, tenderness, and acceptability of meat (Aberle, Forrest, Gerrard, & Mills, 2001). Leaner hogs have less intramuscular marbling which effects product quality (Lonergan et al., 2007); therefore, there is interest in the swine industry for strategies that would improve intramuscular fat deposition and increase marbling.

Soybean oil is a typical dietary source of fat in hog feed; however, in an effort to improve pork quality, the inclusion of various feed additives in the finishing diets have been examined, like conjugated linoleic acid (CLA) (Wiegand, Sparks, Parrish Jr., & Zimmerman, 2002; Dugan, Kramer, & Aalhus, 2004). CLA is known to have anticarcinogenic, antidiabetic, and altered lipid composition when supplemented in mammals (Brandebourg & Hu, 2005). CLA is comprised of positional and geometric conjugated isomers of linoleic acid and are also shown to effect proliferation and differentiation of adipocytes (Jiang et al., 2010). Adipocyte differentiation is regulated by many transcription factors, and is strongly linked to peroxisome proliferator-activated receptor (PPARγ) expression (Wolins et al., 2006). PPARγ is the main gene responsible for regulating the adipocyte specific genes and is required for adipose differentiation in mice (Rosen et al., 1999). Although the complete mechanism behind this gene expression regulator is not fully understood, various ligands (such as conjugated linoleic acid) have shown to decrease the activity of this gene, decrease subcutaneous preadipocyte formation, and increase intramuscular preadipocyte formation, or marbling (Moya-Camarena, Vanden-Heuvl, Blachard, Leesnitzer, & Belury, 1999; Zhou et al., 2007).

Dietary inclusion of CLA in finishing feed is important to swine producers because it has been reported to reduce backfat and increase subjective marbling scores (Dugan, Aalhus, Jeremiah, Kramer, & Schaefer, 1999; Dugan, Aalhus, Schaefer, & Kramer, 1997; Wiegand, Larsen, Baas, Parrish, & Swan, 2001). A positive relationship exists between intramuscular lipid content and sensory attributes of pork such as texture, tenderness, flavor and juiciness (Lonergan et al., 2007); therefore, increasing marbling scores may positively impact fresh pork quality. Inclusion of unsaturated fatty acids in animal feed may cause meat to be softer and less shelf-life stable due to a greater predisposition to fatty acid oxidation (Larsen, Swan, Sparks, Wiegand, & Parrish, 2009); however, CLA, also an unsaturated fatty acid, will improve growth rates of finishing hogs and increase the firmness of the meat (Larsen et al., 2009; Wiegand et al., 2002). Barnes, Winslow, Shelton, Hlusko and Azain (2012) showed that the loins of CLA-fed hogs had a greater proportion of saturated fatty acids and a slight increase of marbling when compared to

non-CLA-fed hogs. More intramuscular saturated fat and increased marbling may improve meat palatability traits such as tenderness and flavor (Barnes et al., 2012).

In response to consumer demand for meats containing less saturated fat, the meat industry has produced hogs with lower hot carcass weights as well as less overall fat content. Leaner hogs have less intramuscular marbling, which negatively affects product quality (Lonergan et al., 2007); therefore, there is interest in the swine industry for strategies that would improve intramuscular fat deposition. Therefore, the objective of this study was to determine if the inclusion of CLA in finishing diets would affect quality indicators of pork *Musculus longissimus dorsi* muscle.

2. Method

2.1 Animals and Diets

All animal procedures were carried out according to the Animal Care and Use Committee Guidelines of West Virginia University (WVU) and are reported elsewhere (Barnes et al., 2012). Briefly, twenty barrows (PIC 380 x Cambrough 1025) of similar body weights (53 ± 5.61 kg) and ages (~3 months) were housed at the WVU livestock farm for the duration of this study. Barrows were blocked by weight and assigned to pens of two hogs per pen (pen as experimental unit). Pens were randomly assigned diets (Table 1) containing either 1% soybean oil (SBO diet; control) or 1% CLA oil (LUTA-CLA, 60% CLA isomers, 50:50 *cis*-9, *trans*-11 : *trans*-10, *cis*-12; BASF, Offenbach/Quiech, Germany) for a finishing period of 6 wks (Barnes et al., 2012).

Table 1. Diet formulation calculated (as-fed basis) of diets shown below. Both control and CLA diets only differed in the 10 g kg^{-1} oil additive

Ingredient	**Inclusion, g kg^{-1}**
Corn	849.9
Soybean meal	108.3
Meat and bone meal	21.1
Oil[a]	10.0
Limestone	6.5
Vitamin and trace mineral pre-mix[b]	2.5
Salt	1.7
Lysine	0.05
Calculated analysis	
Crude protein, %	135.5
ME, kcal/kg	3266
Lysine, %	6.0
Calcium, %	5.0
Phosphorus, available %	1.9

[a] Soybean oil or CLA-60 (60% CLA isomers, 50:50 c9, t11:t10, c12; provided by BASF) in their respective diet treatments.

[b] Supplied per kilogram of diet: vitamin A, 7,716 IU; thiamin, 2.20 mg; riboflavin, 6.61 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; vitamin B_6, 1.38 mg; biotin, 0.03 mg; folic acid, 0.69 mg; vitamin B_{12}, 0.01 mg; vitamin D_3, 2,133 ICU; vitamin E, 16.53 IU; choline, 386 mg; menadione, 0.83 mg; copper, 0.0025%; iodine, 0.003%; iron, 0.01%; manganese, 0.02%,; selenium, 0.00003%; and zinc, 0.02% (Barnes et al., 2012).

Hogs were allowed *ad libitum* access to feed and water for six weeks prior to slaughter, and were weighed weekly to monitor growth. Barnes and others (2012) reported no differences in average daily gain and average daily feed intake. Following the six-week feeding study, all hogs were slaughtered at a commercial facility. Hot carcass weights were measured (81.4 ± 4.97 kg), and carcasses were chilled for 24 h at 3-4 °C. Pork loins were removed from the chilled carcass, packaged in non-gas permeable bags and transported on ice to the meats laboratory.

Pork chops, located at the 10th and 11th thoracic vertebrae, were collected to score marbling and determine intramuscular, fatty acid content, results of which are reported elsewhere (Barnes et al., 2012). Loins (beginning with 3rd thoracic vertebra) were divided into thoracic and lumbar portions, deboned, immediately placed into non-permeable vacuum bags, sealed, and stored at -20ºC. After 24 h, frozen loins were cut with a band saw into 2.54 cm thick pork chops. Pork chops were repackaged in non-gas permeable vacuum sealed bags, randomly assigned for lab analyses and stored at -20°C until analyses were conducted to minimize any change in loin quality over time (Hansen, Juncher, Henckel, Karlsson, & Bertelsen, 2004). Length of frozen storage prior to these analyses did not exceed 120 days.

2.2 Sample Preparation and Thermal Processing

Prior to all analyses, designated pork chops were removed from the freezer, and held at 4°C for 14 days in dark storage to simulate vacuum aging before distribution to retail stores. Pork chops remained in the vacuum package during the aging process, and were then placed on Styrofoam trays, wrapped with oxygen-permeable, polyvinyl chloride film and refrigerated at 4°C for a maximum of 7 days. Proximate composition was confirmed on day 1, and pork quality indicators were measured on days 1, 3, 5, and 7.

Pork chops used for proximate composition were cooked 1 day after transfer to Styrofoam trays. Full chops were placed in individual glass baking pans and cooked in 118 ml water. All chops were cooked at 165°C for approximately 45 min or until reaching an internal temperature of at least 70°C. A Beckman Industrial Data logger (Model 205; Beckman Instruments, Fullerton, CA) was used to monitor internal chop temperatures during the cooking process; thermometer probes were placed in the center of the chops.

2.3 Sample Analyses

2.3.1 Proximate Composition

Proximate composition (crude fat, crude protein, moisture, and ash) of cooked loin samples was determined on day 1 and conducted according to Association of Official Analytical Chemists methods (1995). Results are expressed as g/100g (wet basis). Crude fat of raw muscle was also determined. Fat was measured using the Soxhlet extraction method, and protein was measured using the Kjeldahl assay. Moisture was determined by weight differences of fresh and dry samples after 16 h of drying at 70°C at a partial vacuum pressure of 20 mm Hg. Ash content was determined by weight differences in samples incinerated at 550°C for 24 hours in a muffle furnace.

2.3.2 Loin pH

The pH of the pork loin was assessed on days 1, 3, 5, and 7 prior to and after cooking to determine initial loin pH and changes in pH over time. Each chop was divided into four quadrants; pH assessments were taken at the center portion of each quadrant using a pH probe (Model 350, Corning Inc.; Corning NY, USA).

2.3.3 Texture Analysis

Shear force was conducted on cooked samples following 1, 3, 5, and 7 days of storage. After cooking, muscle sections were cored (1.27 cm dia.) using a uniform coring instrument and measured for shear force. The Warner-Bratzler attachment on a texture analyzer (Model TA-HDi, Texture Technologies Corp., Scarsdale, NY) was used to determine the peak force required to shear one core as a measure of tenderness.

The texture analyzer parameters were as follows: pre-test speed = 2 mm/sec; test speed = 5mm/sec; post test speed = 5 mm/sec; distance = 25mm; force = 100 g; time = 5 sec; load cell = 50 kg.

2.3.4 Lipid Oxidation

Lipid oxidation of pork loin was measured by the thiobarbituric acid reactive substances (TBARS) test. Samples were measured in triplicate for lipid oxidation after cooking on days 1, 3, 5, and 7 using a modified method described by Yu and Sinnhuber (1957). Absorbance was read at 535 nm using an UV/Vis spectrophotometer (model DU530, Beckman Instruments, Fullerton, CA), and the concentration of TBARS was calculated using the molar absorptivity of malondialdehyde (MDA;156,000 M^{-1} cm^{-1}). Relative MDA was calculated for comparison to the control Day 1 pork sample mean.

2.3.5 Instrumental Color Analysis

Meat samples (raw and cooked) were tested for color change at days 1, 3, 5, and 7 using a standard chromameter (8-mm aperture, Model CR-300; Minolta Camera Co. Ltd., Osaka Japan.) Evaluations were conducted at the center of each muscle section prior to and after cooking. The following values were recorded: lightness (L*-scale: 0-100), yellowness (b*-scale: -60 - +60), and redness (a*- scale: -60 - +60).

2.3.6 Statistical Design

The experimental unit for all analyses was pen (n = 5 per diet). The evaluations for the storage study included pH, color, texture, and TBARS, which were all completed in triplicate. The main effects of diet (CLA vs. SBO) and day (days 1, 3, 5 and 7) were analyzed by analysis of variance (ANOVA) along with their interactions (diet*day). Student's t-test was used to separate means of statistically-different parameters and interactions. All data were analyzed at a 5% level of significance using JMP 10 software (SAS Institute, Cary, NC, USA) and reported as mean values ± standard error of the mean, unless otherwise indicated.

3. Results and Discussion

3.1 Proximate Composition

Proximate composition of cooked pork loin chops from control and CLA hogs revealed no significant differences ($P > 0.05$) in moisture, ash, protein, or fat (Table 2). We hypothesized that fat concentration in the control and CLA chops would be different because others have observed that CLA increased marbling and that CLA may alter fat deposition through PPARγ gene expression when supplemented in finishing hog feed; however, the fat content of CLA loin samples in this current study were not significantly different from the control. Our results are in agreement with Barnes et al. (2012) results where relative gene expression for intramuscular adipocyte markers of differentiating adipocytes (PPARγ) and mature adipocytes were measured on rib and loin meat from the same animals used in this current study and did not show significant differences ($P > 0.05$). However, the expression of these genes was numerically greater in CLA-fed hogs than in SBO-fed hogs; this trend may be indicative of greater intramuscular adipose tissue compared to subcutaneous adipose tissue which was decreased in our CLA-fed hogs (Barnes et al., 2012). In addition, Wiegand et al. (2002) reported increased fat deposition with increasing weight gain of CLA-fed hogs. It is likely that this increase was because CLA was included at 1.25% (*vs*. 1.0% in this current study) and feeding duration was based on weight gain before slaughter (Wiegand et al., 2002). It is possible that increasing the sample size and feeding duration of this present study would have yielded significant results.

Table 2. Proximate composition [a] (g/kg sample) of cooked pork loin chops from SBO control and CLA pigs

	SBO	**CLA**	**P-value**
Moisture, cooked	589.4 ± 3.1	574.5 ± 8.6	0.143
Protein, cooked	345.4 ± 9.3	342.4 ± 7.9	0.810
Ash, cooked	6.5 ± 0.9	7.6 ± 0.7	0.356
Fat, cooked	53.9 ± 7.1	73.0 ± 11.4	0.193
Fat, raw[b]	28.1 ± 4.8	37.9 ± 4.1	0.163

[a] Values are given as mean scores ± SEM (n=5 per diet).

[b] For comparison, fat composition of raw samples was also determined.

3.2 Loin pH

Values for raw and cooked loin pH over a storage period of 7 days are presented in Table 3. For the SBO control and treatment groups, means ranged from 5.69 to 6.56, and all values were considered within acceptable pork pH ranges (Bendall & Swatland, 1988). Raw and cooked loin samples showed an increase in loin pH between days 3, 5, and 7 ($P < 0.001$), independent of diet type. Loin pH is commonly used in the pork industry as a primary indicator of loin quality and is correlated to attributes such as color and water-holding capacity (Huff-Lonergan et al., 2002). At higher pH values, pork tends to have better textural quality; however, a higher pH may also reduce shelf-life due to color changes and a predisposition to support the growth of spoilage organisms (Holmer et al., 2009). The initial loin pH measurement is a reflection of slaughter procedures and stress response evident during the perimortem period. As the meat ages, protein breakdown produces free amino acids that lead to an increase in pH. The elevation of pH during storage may also be due to the natural spoiling effect that occurs within muscle.

3.3 Shear Force

Pork loin tenderness is an important attribute in relation to consumer acceptability. Tenderness within the loin was measured by the Warner Bratzler shear force test, which is a measurement of the amount of force needed to shear a 1.27-cm dia. core (Table 3). The higher the instrumental shear force value is, the tougher the meat product is likely to be. Changes in texture over time ($P = 0.017$), with texture scores improving with storage time, is

consistent with reported studies (Huff-Lonergan et al., 2002). The shear force necessary to cut CLA samples were significantly less (P = 0.032) than control samples; indicating that the CLA samples were more tender. The CLA loins appeared to contain more lipid (3.8 *vs*. 2.8 g/100 g sample) and tended to have greater marbling scores (P = 0.069) (Barnes et al., 2012). Some studies have shown that there is a negative relationship between marbling scores and shear force (Huff-Lonergan et al., 2002).

Table 3. Mean values of pH (raw and cooked), texture by shear force (cooked) and lipid oxidation by relative malondialdehyde (MDA)/kg sample) test (cooked) on pork loin chops from SBO control and CLA pigs over a 7 d storage period

	pH (raw)	**pH (cooked)**	**Shear Force (N)**	**Relative MDA**
SBO	6.06 ± 0.08	6.19 ± 0.05	22.26 ± 0.74[a]	0.92 ± 0.06
CLA	6.02 ± 0.07	6.21 ± 0.06	20.52 ± 0.44[b]	0.89 ± 0.05
Day 1	5.69 ± 0.02[d]	5.98 ± 0.06[c]	23.50 ± 1.09[a]	0.99 ± 0.06[a]
Day 3	5.83 ± 0.04[c]	6.00 ± 0.02[c]	20.77 ± 0.60[ab]	1.04 ± 0.05[a]
Day 5	6.21 ± 0.07[b]	6.25 ± 0.04[b]	21.41 ± 0.93[b]	0.87 ± 0.09[ab]
Day 7	6.42 ± 0.05[a]	6.56 ± 0.04[a]	19.89 ± 0.52[b]	0.72 ± 0.09[b]
P-value day	<0.001	<0.001	0.017	0.017
P-value diet	0.486	0.602	0.032	0.264
P diet*day	0.114	0.782	0.352	0.258

Values are given as mean scores ± SEM (n=5 per diet) for each treatment on each treatment day.

[a,b,c,d] Values with different letters within columns are significantly different (P < 0.05).

3.4 Lipid Oxidation

Dietary inclusion of unsaturated fatty acids (UFA) in finishing hog feed may increase the UFA profile of the meat, and an increase in UFAs may affect the shelf-life stability of the meat due to a greater predisposition to lipid oxidation (Cardenia et al., 2011). CLA has been used to combat increased UFA in meat as it inhibits stearoyl-CoA desaturase (Park et al., 2000) and decreases the iodine value of pork (Corino, Magni, Pastorelli, Rossi, & Mourot, 2003). Therefore, the TBARS test was used to screen samples for lipid oxidation by measuring MDA (Table 3). Results showed that there were changes in relative MDA over time (P = 0.017); however, changes in relative MDA were independent of diet (P = 0.264). Wiegand and others (2002) reported that hogs fed CLA diets produced chops that had lower TBARs values than hogs fed a traditional diet. They attributed this improvement to the reduction of polyunsaturated fatty acids and the increase in saturated fatty acids of the CLA chops because UFA are more susceptible to oxidation (Wiegand et al., 2002). Fatty acid profile of samples used in this study were reported elsewhere (Barnes et al., 2012); however, fatty acid analysis confirmed that the CLA pork samples used in this current study had significantly more saturated fatty acids than the control samples (P = 0.006), but there was not a significant reduction in polyunsaturated fatty acids (P = 0.500) (Barnes et al., 2012).

3.5 Color

Color provides a direct indication of overall pork quality as well as spoilage. Consumers determine the freshness and quality of the loin by evaluating the amount of discoloration. Oxidation of myoglobin (indicates pork quality), the main protein responsible for protein color, is evident as an increase in the L* value (lightness), and a decrease in a* (redness) and b* (yellowness) values within the muscle (Mancini et al., 2005). Color for raw and cooked loin samples over time and results are presented in Table 4. Significant (p < 0.001) color changes were seen in raw and cooked lightness (L*) and yellowness (b*) values over time regardless of diet. Lightness decreased over time for raw and cooked samples; whereas, yellowness decreased in raw samples and increased in the cooked samples as storage time increased. Lightness is influenced by water retention in the samples (Sanchez del Pulgar, Gazquez, & Ruiz-Carrascal, 2012); it is likely moisture was lost from the samples over the storage period resulting in a reduction of L* values. Lightness is also influenced by pH and storage time, with darker products forming as pH and time increase (Huff-Lonergan et al., 2002). Wiegand and others (2002) reported that hogs fed CLA diet had

higher b* values than hogs fed the control diet, which is indicative of a more yellow product. In our study, b* value of the raw CLA chops was numerically greater than the control diet, but was not different (P = 0.290). There was a significant reduction in redness (a*) and yellowness (b*) (P = 0.027 and P >0.001, respectively) of the raw chops over the storage period which is associated with the increase in pH typical during aging and simulated retail display (Holmer et al., 2009; Mancini & Hunt, 2005). Thiel et al. (1998) suggested that dietary CLA may protect meat color; however, results of this study indicate that loin color was not affected by diet.

Table 4. Raw and cooked L*, a*, and b* values of pork loin chops from SBO control and CLA pigs through a 7-d storage period

		*L**	*a**	*b**
Raw	**SBO**	56.99 ± 0.82	9.67 ± 0.33	9.85 ± 0.27
	CLA	56.48 ± 0.74	10.09 ± 0.26	10.23 ± 0.34
	Day 1	61.37 ± 0.84[a]	10.71 ± 0.34[a]	11.03 ± 0.39[a]
	Day 3	56.04 ± 0.68[b]	8.95 ± 0.45[a,b]	10.77 ± 0.29[a]
	Day 5	54.31 ± 0.75[b]	9.97 ± 0.36[a,b]	9.22 ± 0.42[b]
	Day 7	55.22 ± 0.35[b]	9.90 ± 0.39[b]	9.14 ± 0.24[b]
	P-value day	<0.001	0.027	<0.001
	P-value diet	0.480	0.287	0.290
	P diet*day	0.953	0.376	0.593
Cooked	**SBO**	67.33 ± 0.70	5.75 ± 0.17	11.64 ± 0.21
	CLA	66.21 ± 0.61	6.01 ± 0.12	11.34 ± 0.20
	Day 1	70.45 ± 0.66[a]	6.27 ± 0.18[a]	10.90 ± 0.10[b]
	Day 3	66.56 ± 0.57[b]	5.78 ± 0.15[a,b]	10.79 ± 0.17[b]
	Day 5	64.84 ± 0.59[b]	5.93 ± 0.16[a,b]	11.93 ± 0.29[a]
	Day 7	65.23 ± 0.68[b]	5.54 ± 0.27[b]	12.35 ± 0.20[a]
	P-value day	<0.001	0.101	<0.001
	P-value diet	0.085	0.223	0.151
	P diet*day	0.876	0.964	0.680

Values are given as mean scores ± SEM (n=5 per diet) for each treatment on each treatment day.

[a,b] Values with different letters within columns are significantly different (P < 0.05).

In conclusion, the results of this study showed that CLA supplementation of finishing diets results in pork quality similar to that of traditional diets. Because addition of CLA to finishing hog feed did not affect product quality indicators, it is difficult to justify the added expense of CLA supplementation to the diet. However, there may still be times when adding CLA to the diet is beneficial (i.e. to increase firmness of meat, especially pork belly cuts). It is possible that increasing the sample size of the study would yield more significant differences in quality parameters.

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Soil Properties Affecting Rainfall Water Use Efficiency (RWUE) in Wheat Dry-Farming Lands, NW Iran

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Abstract

Effective use of rainfall water is a key issue in agricultural development in the arid and semi-arid regions since rainfall water is a precondition for crop production there. This study was conducted in a semi-arid agricultural region with 900 km^2 in area in Hashtroud, northwest of Iran to determine the relationship between rainwater use efficiency (RWUE) and soil properties. Winter wheat yield and soil properties were determined at 108 plots (40.41 m^2 in area) installed in thirty six dry-farming lands. RWUE of each plot obtained from the ratio of crop dry matter per unit of abstracted rainfall water volume (ARWV). ARWV was computed from deduction of the rainfall and runoff volume during a two-growth period. Runoff data for each land was obtained from field measurements at the plots under natural rainfalls. Analysis of rainfalls uniformity using four rain gauge stations data showed that spatial distributions of rainfalls were homogeneous in the area. The RWUE values in the lands were ranged from 0.35 kg m^{-3} to 1.49 kg m^{-3} with an average of 0.84 kg m^{-3}. Soil properties which considerably affected either the infiltration capacity or the available water controlled the RWUE in the study area. Multi-regression analysis indicated that the RWUE significantly related to silt, organic matter and lime (R^2=0.82, $p < 0.001$). Maintaining crop residues and incorporating with the soil can be proper techniques and sustainable strategies to improve the soil properties and enhance the RWUE in the dry-farming lands.

Keywords: available water, infiltration capacity, semi-arid region, natural rainfall, winter wheat

1. Introduction

Precipitation is one of the most important factors affecting agricultural productions, especially in the arid and semi-arid regions. Water from precipitation must be captured and retained in soil and used efficiently for optimum yield production (Morell et al., 2011). Effective use of rainfall water is a key issue in agricultural development in the arid and semi-arid regions since rainfall water is a precondition for crop production there. RWUE is the ratio of crop dry matter per unit of abstracted rainfall water volume (ARWV). The abstracted rainfall water volume is a part of precipitation that could be stored in soil. The RWUE is often considered an important determinant of yield under stress and even as a component of crop drought resistance. It has been used to imply that rainfed plant production can be increased per unit water used, resulting in ‘‘more crop per drop’’ (Blum, 2009).

A major research challenge is to investigate methods that maximize wheat yield and the RWUE. It has been concluded that most of the agronomic options for improving RWUE in rainfed agricultural systems decrease water losses by declining soil evaporation, runoff, through flow, deep drainage, and competing weeds, thereby making more water available for increased water use by the crop (Asseng et al., 2001; Turner, 2004). Soil properties are one of the most important factors influencing crop water availability due to their effects on the water holding capacity, evaporation, and runoff generation. Rainfall water use efficiency (RWUE) decreases when both plant water supplying in the soil decreases and runoff generation increases. The supplied water for the plant (available water, AW) is that portion of water held in soil that can be absorbed by plant roots (Richards & Wadleigh, 1952). AW is the amount of water between the field capacity (FC) and the permanent wilting point (PWP) (Veihmeyer & Hendrickson, 1927) that may strongly affected by some soil properties particularly texture and structure. Soil properties also affect on runoff rate in the land. Runoff occurs only when the rate of rainfall on a surface exceeds the rate at which water can infiltrate the soil (Schwab et al., 1993). Runoff more commonly occurs in the arid and semi-arid regions, where rainfall intensities are high and the soil infiltration capacity is

reduced because of surface sealing, or in paved areas. The rate of infiltration of water into the soil depends on several soil properties, particularly physical characteristics of the soil (Ghawi & Battikhi, 1986).

Almost 39 percent of Iran (642797 km^2) has a semi-arid climate condition, with an annual precipitation between 200 and 500 mm. East-Azarbijan province located in north west of Iran is one of the typical semi-arid regions, with an mean annual precipitation of 300 mm (Modarres, 2006). On average, precipitation has a nonuniform annual distribution, with the major part occurring in early spring (37%), middle autumn (26%) and early winter (31%) and little precipitation in summer (6%) (Anonymous, 2011). The arable area is estimated to be about 1220980 ha (27 percent of total surface area). Farming is mostly done in rainfed condition (813119 ha) and water is the principal limiting factor for agriculture development (Shefaat, 2006). Wheat is the main crop in the region with a mean yield of 650 kg ha^{-1} (Iranian Agriculture Ministry, 2009). It is widely adopted as a monoculture crop, with the growing period between March and July, and October and July for spring and winter-sown, respectively.

Improving water use efficiency has been an urgent issue in the region as ecological water demand has been increasingly concerned. Determining factors affecting the RWUE and quantification their effects value are important to model the RWUE and predict crop yield in the area. Some authors have investigated various factors influencing the RWUE, which include cropping systems (Kar et al., 2006; Rao, 2008), fertilization (Rao et al., 2010), mulch (Rehman et al., 2009) and tillage (Ronner, 2011), previously. Up to now, the effect of soil properties on the RWUE has not been quantitatively investigated; therefore, the objective of this work was to quantify the influence of soil properties on the RWUE and model it in dry-farming lands of the semi-arid region.

2. Materials and Methods

2.1 Study Area

The study was carried out in a semi-arid area of northwest of Iran located in Hashtroud township (southern part of East Azarbyjan province) from March 2005 to March 2006. The study zone was 900 km^2 in area located between 37° 18' 49" and 37° 35' 0" N latitude, and 46° 46' 5" and 47° 6' 5" E longitude (Figure 1). The climate is semi-arid with an average annual precipitation of 322 mm, mostly falling as snow in the winter and autumn and as rain in the spring, and a mean annual temperature of 13°C. Agricultural soils located mostly in 5-15% slopes (Hakimi, 1986) and mainly are utilized for wheat production under rainfed condition. Soils according to USDA Soil Taxonomy classification system (Soil Survey Staff, 1975) were classified as calcixerepts (Banaee, 1999). Soils were mostly tilled and planted in slope direction. So, as noted by Blanco and Lal (2008) surface runoff rapidly concentrates in furrows and immediately flows up- and down-slope direction.

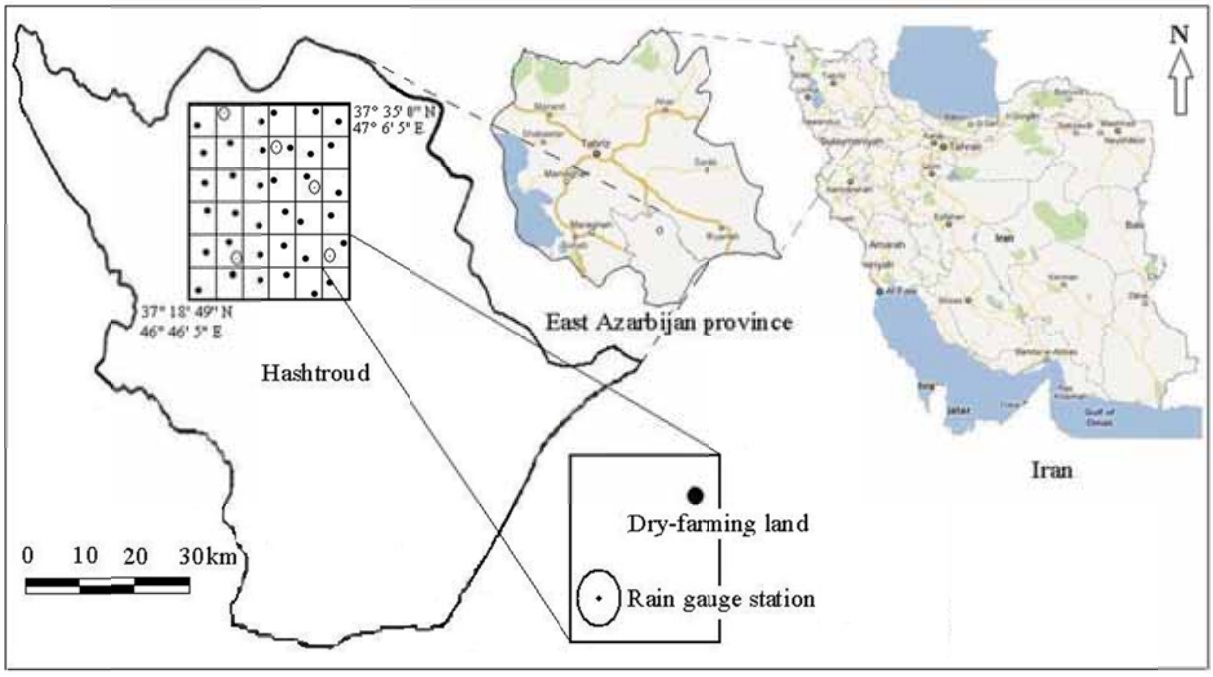

Figure 1. Location of the study area, dry-farming lands and rain gauge stations

2.2 Determination of the RWUE

Based on the water use efficiency definition that refers to the ratio of economic yield to water consumed by the crop (Katerji et al., 2008), rainwater use efficiency (RWUE) obtained from the ratio of crop dry matter (CDM) per unit of the abstracted rainwater volume (ARWV) as fallowing:

$$RWUE = \frac{CDM}{ARWV} \quad (1)$$

where RWUE was in kg m^{-3}, CDM was in kg and ARWV was in m^3. The abstracted rainfall volume (ARWV) obtained from differentiation of the rainfall volume (m^3) and runoff volume (m^3) during a two-growth period.

2.2.1 Installation of the Crop and Runoff Plots

Thirty six dry-farming lands were considered in the study area to installation of crop plots and runoff plots (Figure 1). The crop plots and runoff plots were separately installed in a 200 m^2 area at three replications beside together in each dry land at the same time. In fact crop yield and runoff volume were separately determined at 108 plots during a two-growth period for a two-study period (2005-2007). The crop and runoff plots were established based on USLE standard/unit plots (Wischmeier & Smith, 1978) with 22.1 m length in slope direction and 1.83 m width and a buffer bed about 1.2 m between two plots. The plots were plowed and accordingly disked up to down slope at middle October 2005. For providing similar conditions between the crop plots and runoff plots, was avoided from fertilizer application to enhance crop yield in the planted area.

2.2.2 Determination of Wheat Yield

The Sardary winter wheat variety, normally grown for bread, was planted at the crop plots by a drill in depth of 4-6 cm, with 20 cm row spacing and 5 cm plant spacing right after plowing at last October 2005. Length of growing period of the winter wheat was about eight months and on July 25, the crop was harvested for determining grain yield. Plant samples were randomly taken from three 1 m^2 locations from each plot area by clipping the plants at the soil surface and accordingly mean grain yield of each plot was computed. Mean grain yield and mean dry matter of each dry-farming land (kg ha^{-1}) were calculated from averaging the yield and biomass values of its three plots, respectively. The mean wheat yield and dry matter for a two-year study period were computed based on the yield and biomass values of the first and second year (kg ha^{-1}).

2.2.3 Determination of Surface Runoff Volume

Surface runoff caused by natural rainfalls was measured at the lower parts of the runoff plots during a two-growth period for a two-study period (2005-2007). The plots were surrounded using 30 cm ridges and runoff-collecting installations consisted of gutter pipes, pipes and 70-1 tanks were established at their lower parts. After each natural rainfall event producing runoff at the plots, total contents (runoff-sediment) mass in the collecting tank was measured. Then, the tanks contents were mixed thoroughly and a 0.5 kg homogeneous sample was taken to determination of runoff mass. In the laboratory, the samples were weighed and evaporated on a hot plate then weighed again to determine runoff mass. Water loss of each plot was determined based on multiplying total contents mass of the tank by mass percentage of water in its sample. Annual surface runoff was also computed from summation of total surface runoffs produced in different rainstorms for a two-growth period.

2.2.4 Determination of Rainfall Volume

Rainfall volume (m^3) was calculated from multiplying the rainfall depth (m) and plot area (40.44 m^2). Rainfall data were taken from five rain gauge stations located in the study area (Figure 1). Four standard rainfall gauges located in the grids 2, 10, 27 and 30 were used to manually measure the depth of rain after occurring the runoff at the plots. An automatic rain gauge station located in the grid 17 was also used to determine intensity of rainfall events. Rainfall data for a two- growth period was also used to determine spatial variations of the rainfall amounts in the study area.

2.3 Determination of Soil Properties

To determine soil properties in each dry-farming land, soil samples (0-30 cm depth) were taken randomly from three locations within each plot before plowing. Then, the samples were mixed together to provide a representative sample from each plot. After being dried, the soil samples were grounded to pass a 2 mm sieve and stored in sealed polyethylene bags in a cool and dry place until the chemical analysis in the laboratory. The particle size distribution consisted of sand (0.05-2 mm), silt (0.002-0.05) and clay (<0.002 mm) was determined by the Robinson's pipette method (SSEW, 1982). Gravel (2-8 mm) was determined using the weighting method (Gee & Bauder, 1980). The total soil organic carbon was measured by the Walkley-Black wet dichromate oxidation method (Nelson & Somers, 1982) and converted to organic matter through multiplying it by 1.724. To

determine soils carbonates (lime), the total neutralizing value (TNV) on the basis of calcium carbonate was measured using acid acetic volume consumed to neutralization of carbonates (Goh, Arnaud, & Mermut, 1993). The aggregate stability was determined using the wet-sieving method based on the mean weight diameter (MWD) as proposed by Angers and Mehuys (1993). The water-stable aggregates were determined by placing 100 g soil surface aggregates with diameter larger than six mm on the top of sieves set and moved up to down in a water cylinder for one minute. Soil infiltration capacity was determined by measuring the one-dimensional water flow into the soil per unit time by double-ring infiltrometer (Bouwer, 1986) at four to six replications at the plots during dry period (in July 2005). Available water (AW) for each soil obtained from difference of mass soil moisture contents between the holding capacity (FC) and permanent wilting point (PWP). Soil moisture content by mass at FC (-30 kPa matric potential) and PWP (-1500 kPa matric potential) were measured using a pressure plate and pressure membrane apparatus, respectively (Hillel, 1982).

2.4 Statistical Analysis

Soil, rainfall and runoff data were assessed for normality using the Kolmogorov-Smirnov test before analysis. Differences in rainfall amounts among rain gauge stations were analyzed using one-way ANOVA. Relationship between runoff and rainfall was extracted using the different equations based on the highest determination coefficient (R^2). Runoff and RWUE difference among the plots was analyzed using Duncan's parametric test. Soil properties influencing the RWUE were extracted based on bivariate Pearson's correlation matrix. A stepwise multiple regression analysis was applied to develop a relationship between the RWUE and the effective soil properties. SPSS 18 software was used, and the significance level was 95% ($p < 0.05$) in all statistical analyses.

3. Results and Discussion

3.1 Rainfall Characteristics

Annual rainfall amount in the first and second study year was 249.3 mm and 159.5 mm, respectively. Thirty six and twenty seven natural rainfall events occurred in the study area during the growth period in the first and second study year, respectively. Table 1 shows the statistical characteristics of the rainfall events in the first and second study year. Rainfall intensity in the first year varied from 0.1 to 13.78 mm h^{-1} with an average of 3.25 mm h^{-1}. Rainfall intensity in the second year was between 0.31 to 8.20 mm h^{-1} with an average of 2.57 mm h^{-1}. Total rainfall height during the growth period in the first and second year was 151.41 mm and 93.95 mm, respectively. There was no significant difference among the rainfall depth values in different rain gauge stations ($F = 0.03$, p-value = 0.99). In fact, spatial variations of the rainfall events were uniform in the study area.

Table 1. The statistical characteristics of the rainfall events in the growth period in the first and second study year

Growth Periods	Height (mm)		Intensity (mm h^{-1})	
	Mean	StD.	Mean	StD.
2005	4.21	4.52	3.25	2.81
2006	3.48	2.84	2.57	1.99

3.2 Runoff and RWUE

Nineteen rainfall events and thirteen rainfall events produced runoff at the plots in the first and second growth period, respectively. Table 2 shows rainfall height and runoff depth in the rainstorms in the first and second growth period in the study area.

Mean surface runoff during the growth period in the first study year varied from 2.15 lit to 49.37 lit with an average of 17.13 lit. It was between 1.79 lit and 27.67 lit with an average of 10.63 lit in the second growth period. The abstracted rainfall water volume (ARWV) values were ranged from 74.36 lit to 696.83 lit and from 127.38 lit to 485.95 lit in the first and the second growth period, respectively.

Runoff depth significantly affected by rainfall height ($R^2 = 0.70$, $p < 0.001$). With an increasing rainfall height, runoff remarkably increased (Figure 2). Rainfall having a height of 17.1 mm had the highest potential to generate runoff in the study area. The highest productive runoff had a height of 17.1 mm that could produce 0.8 mm runoff in the study plots. Rainfalls that had a height value lower than 1.7 mm did not had any potential in runoff production in the study area. The abstracted rainfall significantly correlated with the rainfall ($R^2 = 0.99$, $p < 0.001$).

Table 2. Rainfall height and runoff depth in the rainstorms in the first and second growth period

Growth Periods					
2005			2006		
Date	Rainfall (mm)	Runoff (mm)	Date	Rainfall (mm)	Runoff (mm)
April 2	2.5	0.053	March 29	5.3	0.130
April 3	3.65	0.106	April 5	4.2	0.082
April 15	13.7	0.803	April 7	6.7	0.093
April 16	2.7	0.103	April 17	12.7	0.684
April 17	4.8	0.231	April 24	4.2	0.168
April 18	3.7	0.181	April 25	3.3	0.150
April 26	17.8	0.620	April 26	5.6	0.369
April 27	2.8	0.314	may 3	8.1	0.499
May 3	8.3	0.412	May 4	4	0.350
May 4	2	0.085	May 5	3.4	0.193
May 5	2.5	0.234	May 6	4.8	0.268
May 6	4.2	0.302	May 10	6.8	0.385
May 14	11.9	0.982	June 25	4.1	0.044
May 15	12.4	1.221			
May 16	8.1	0.698			
May 19	12.5	0.557			
May 20	10.4	0.717			
May 31	3.5	0.365			
June 2	1.9	0.061			

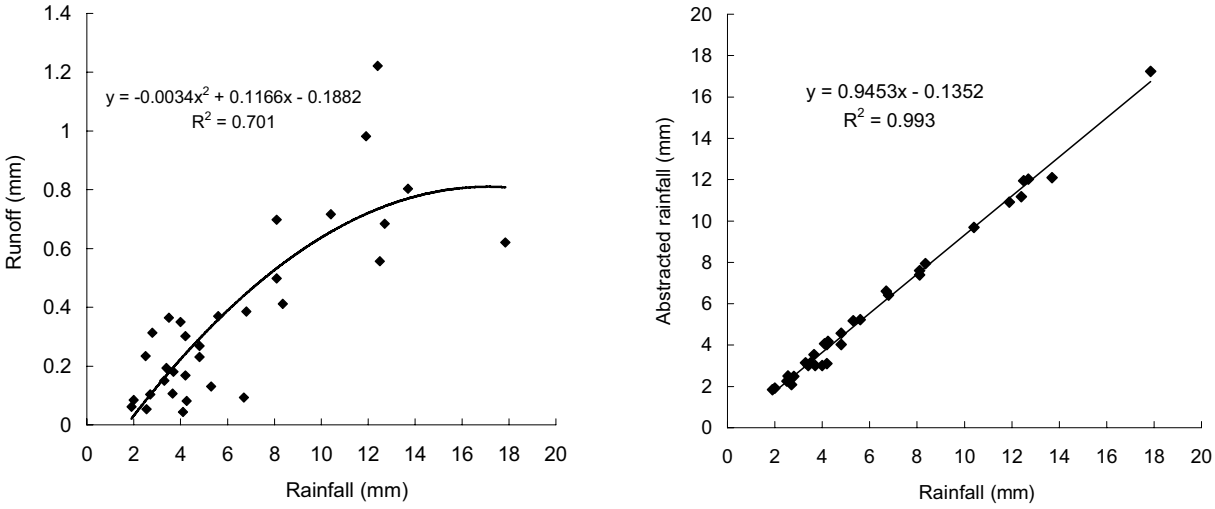

Figure 2. Relationship between runoff and abstracted rainfall, and rainfall during a two-growth period

Both runoff and the abstracted rainfall water volume (ARWV) significantly ($p < 0.001$) varied among the plots installed in 36 dry farming lands (Table 3). Since the spatial distribution of the rainfalls were uniform in the study area, differences of the runoff and ARWV among the dry-farming lands directly related to soil properties.

Table 3. Analysis of variance of mean runoff and ARWV in the dry-farming lands

Variable	Sum of squares	DF	Mean square	F	Significant level
Runoff	421190.955	35	12034.027	45.531	0.000
ARWV	609780.425	35	17422.298	62.609	0.000

3.3 Wheat Yield and RWUE

Mean annual wheat grain yield values in the dry-farming lands were ranged from 801.4 kg ha^{-1} to 3484.3 kg ha^{-1} with an average of 1937.8 kg ha^{-1}. Mean annual rainfall water use efficiency (RWUE) values were between 0.72 and 3.13 kg m^{-3}. Table 4 shows mean annual wheat grain yield and RWUE in 36 dry farming lands. Both wheat yield and RWUE considerably ($p < 0.001$) varied among the dry farming lands (Table 5). Difference of the yield among dry-lands was only due to variations of the soil properties in dry-farming lands.

Table 4. Mean wheat grain yield and RWUE in 36 dry farming lands in the study area

Land No.	Wheat yield (kg ha^{-1})	RWUE (kg m^{-3})	Land No.	Wheat yield (kg ha^{-1})	RWUE (kg m^{-3})	Land No.	Wheat yield (kg ha^{-1})	RWUE (kg m^{-3})
1	801.4	0.72	13	1559.0	1.41	25	1376.3	1.24
2	1125.4	1.01	14	2118.6	1.92	26	2970.3	2.66
3	834.6	0.75	15	1445.4	1.31	27	3476.5	3.13
4	2540.3	2.28	16	3263.7	2.81	28	1187.5	1.07
5	1130.3	1.02	17	3396.0	2.91	29	1846.7	1.66
6	2641. 7	2.35	18	1724.3	1.48	30	1202.7	1.09
7	1256.7	1.12	19	1234.5	1.07	31	2340.8	2.14
8	3484.3	3.12	20	2443.3	2.14	32	3316.4	3.02
9	1265.7	1.13	21	3470.9	3.03	33	1484.5	1.33
10	1176.9	1.05	22	1757.8	1.53	34	2174.4	1.89
11	1364.5	1.22	23	1017.8	0.88	35	1446.6	1.25
12	3017.7	2.67	24	1543.7	1.33	36	1323.2	1.15

Table 5. Analysis of variance of wheat grain yield and RWUE in the dry-farming lands

Variable	Sum of squares	DF	Mean square	F	Significant level
Wheat yield	$5.711*10^7$	35	1631653.076	71.413	0.000
RWUE	$56.394*10^7$	35	1.611	67.591	0.000

3.4 Soil Properties

Soil physicochemical analysis indicated that the soils were mainly clay loam having 36.7% sand, 31.6% silt and 32.0% clay (Table 6). Soils had inherently low amount of organic matter (1.1%) due to low plant growth caused by water stress and frequency cultivation without considering fallow condition. Soils were calcareous/limy with a relatively high value of carbonates (about 13% equivalent calcium carbonate /lime). Soil aggregates were mainly granular and mean wheat diameter of the water-stable aggregates was very low (1.13 mm). The soils based on the SCS method (USDA, SCS, 1991) were mostly classified in C hydrological grope with a mean infiltration capacity of 3.5 cm h^{-1}.

Table 6. Soil properties in the study area

Soil property	Mean	St.D.
Sand	36.72	6.69
Silt	31.59	7.12
Clay	31.69	5.75
Gravel	9.89	2.37
Organic matter	1.09	0.25
Carbonates/lime / (%)	12.66	5.25
Porosity	0.46	0.06
Aggregate stability in Water, (mm)	1.13	0.44
Infiltration capacity (cm h^{-1})	3.56	1.17
Available water (%)	8.31	2.70

3.5 Relationship between the RWUE and Soil Properties

As shown in Table 7, the RWUE significantly correlated with silt ($r = -0.62$, $p < 0.01$), clay ($r = 0.52$, $p < 0.01$), pH ($r = 0.44$, $p < 0.01$), organic matter ($r = 0.68$, $p < 0.01$), Nitrogen ($r = 0.28$, $p < 0.05$), aggregate stability ($r = 0.61$, $p < 0.01$), infiltration capacity ($r = 0.68$, $p < 0.01$) and available water ($r = 0.41$, $p < 0.05$). With an increasing in clay, pH, organic matter, Nitrogen, aggregate stability, infiltration capacity and available water, the RWUE remarkably improved. Aggregate stability, infiltration capacity and available water were the dependent soil variables that were affected by some independent soil properties. Aggregate stability positively correlated with clay, organic matter and lime, whereas sand as resulted by Moreno-de and Heras (2009) negatively affected it. Clay and organic matter as cementation collides encouraged soil particles to stick together and form the stable aggregates in the soils. Presence Ca^{2+} ion in the limey soil matrix also stimulated flocculation of soil colloids (Charman & Murphy, 2000) and increased the aggregate stability. In some dry-lands, presence of the stable aggregates in the soil surface decreased runoff generation, and probably roots aeration due to enhancing resistance of the aggregates against the impact of raindrops and finally soil crusting. Other authors also found significant negative relationships between the aggregate stability and susceptibility to runoff (Reichert & Norton, 1994; Amezketa, Singer, & Le Bissonnais, 1996). Cantón et al. (2009) showed that the stability of topsoil aggregates can be a valuable indicator of field assessed runoff of sandy loam range soils under semiarid conditions. Infiltration capacity had the highest correlation with the RWUE because of its direct influence on the runoff generation. This result was in accord with Gómez et al. (2001), who found that approximately 50% of variability of runoff in fallow plots, can be explained by the final infiltration rate. Infiltration capacity increased with an increase in sand, organic matter and lime. Despite presence sand particles in soil decreased soil porosity, it caused more large pores (macropores) which allowed rapid entry of water into the soil. Organic matter increased water infiltration rate in the soil due to promoting aggregates formation and increasing macrospores proportion in the soil. Studies by Brakensiek and Rawls (1994) and Maestre and Cortina (2002) also indicated that spatial variability of the soil infiltration capacity is related to the high spatial variability of soil properties (organic matter content, structure) that affect the runoff generation in the hillslopes. The presence of organic matter in the soil also improved the soil-water availability due to increasing the water holding capacity (FC) of the soil as a result of the aggregates formation. In many studies, the effect of organic matter in improving the physical properties of soil, such as soil porosity, structure and water-holding capacity were well known (Oades, 1984; Lal, 1986; Lavelle, 1988). While findings of Katerji and Mastrorilli (2006) showed that the WUE was reduced significantly when crops (potato, corn, sunflower, and sugar beet) were grown in clay soil, in the present study clay positively affect on the RWUE. This result was due to strong role of clay in enhancing the stability of soil structure. While findings by Xiaoyan et al. (2002) showed that surface gravel mulch could negatively affect the runoff generation, gravel presence in soil matrix had no significant effect on the soil physical properties and in consequent the RWUE. Nevertheless, in some studies (Li et al., 2000) benefits of Nitrogen (N) was a dependent element to organic matter which improves the crop yield and the RWUE. Deng et al. (2006) also reported that the use of nitrogen fertilizers was one of reasons of increasing the water use efficiency (WUE) in China from 1949 to 1996. Lime has been recognized as an important factor controlling runoff in the soils because Ca^{2+} cations could bind soil particles and improves the aggregates stability (Pepper & Morrissey, 1985).

Table 7. The correlation matrix of the RWUE and physicochemical soil properties in the study area

	Gr	Sa	Si	Cl	F	pH	EC	OM	Li	N	K	AS	If	AW	RWUE
Gr	1														
Sa	0.02	1													
Si	0.02	-0.68**	1												
Cl	-0.06	-0.38*	-0.41*	1											
F	-0.04	-0.39*	-0.09	0.61**	1										
pH	-0.02	-0.16	0-.21	0.44**	0.09	1									
EC	-0.07	-0.35*	0.41*	-0.11	-0.09	-0.03	1								
OM	0.16	0.06	-0.23	0.21	0.29*	0.06	0.02	1							
Li	-0.03	-0.27	0.17	0.03	0.02	0.47**	0.36*	0.05	1						
N	0.13	-0.03	-0.09	0.24	0.55**	-0.08	-0.06	0.60**	-0.24	1					
K	0.09	-0.08	-0.18	0.31*	0.16	0.19	-0.15	0.06	-0.09	-0.07	1				
AS	-0.09	-0.46**	-0.12	0.70**	0.48**	0.56**	0.24	0.29*	0.48**	0.22	0.22	1			
If	0.09	0.57**	-0.55**	-0.07	-0.16	0.26	-0.14	0.54**	0.29*	0.11	0.08	0.13	1		
AW	0.22	0.26	-0.18	-0.07	0.27	-0.01	-0.04	0.55**	-0.10	0.55**	-0.08	0.10	0.33*	1	
RWUE	0.04	0.19	-0.62**	0.52**	0.23	0.44**	-0.03	0.68**	0.30*	0.28*	0.16	0.61**	0.68**	0.41*	1

Gr: gravel; Sa: sand; Si: silt; Cl: clay; F: porosity; pH: potential of hydronium ions; EC: electrical conductivity; OM: organic matter; Li: lime (carbonates); N: nitrogen; K: potassium; AS: aggregate stability; If: infiltration capacity; AW: available water; RWUE: rainfall water use efficiency.

The stepwise multiple regression analysis of the relationship between the RWUE and soil properties showed that the RWUE significantly ($R^2 = 0.85$, $p < 0.001$) related to silt, organic matter and lime (Table 8). Organic matter and lime contrary to silt improved the RWUE in the dry-farming lands. These properties considerably enhanced either the soil infiltration capacity or the soil available water. As well known by Hartanto et al. (2003) and Zhang et al. (2007b), organic matter was the most important binding and bridging agent in enhancing the soil's structural stability, infiltration capacity, and in consequence reducing runoff in the study area. Besides this, organic matter was only effective factor influencing the soil available water and plant growth (Zhang et al., 2007a). Since the soil organic matter is strongly affected by tillage methods such as crop residues and cultivation systems, it can be considered the only management soil factor influencing the RWUE in the study area. Thus, adding organic matter to the soil through maintaining crop residues is a proper technique and sustainable strategy to improve the soil properties (Shaver, 2010), prevention of the excessive soil water evaporation (Howell et al., 1990), decline runoff (Freebairn & Boughton, 1985) and enhance the RWUE in the dry-farming lands. Contour farming is another effective method to prevent runoff generation (Blanco & Lal, 2008; Gebreegziabher et al., 2009) and soil nutrients loss and promote soil physicochemical properties in order to the effective use from rain waters in the sloped dry-farming lands.

Table 8. The multi-regression analysis of the relationship between RWUE and some dependent soil properties

Model variable	Unstandardized coefficients		Standardized coefficient	t-level	p-level
	Model coefficients	Standard error			
Constant	-2.563	0.316		-8.101	p< 0.001
1/Silt	-56.458	6.754	0.588	8.360	p< 0.001
Organic matter	1.582	0.210	0.523	7.523	p< 0.001
Lime	0.054	0.010	0.367	5.370	p< 0.001

A regression equation was developed based on the relationship between the RWUE and the effective soil properties:

$$RWUE = -56.458/Silt + 1.582\ OM + 0.054\ Lime \quad (2)$$

where the RWUE was in kg m^{-3}, silt, OM and lime were in percent.

4. Conclusions

The study indicated that the RWUE (Rainfall water use efficiency) in rainfed conditions remarkably affected by soil properties in the dry-farming lands. Soil properties which considerably enhanced either the water infiltration rate into the soil or the water availability to plant could also improve the RWUE in the study area. The RWUE significantly ($R^2 = 0.85$, $p < 0.001$) related to silt, organic matter and lime. Organic matter and lime positively affected on the soil structure, water infiltration rate and water-holding capacity, while silt inversely affected these soil physical parameters and in consequence the RWUE in the study area. A regression equation was developed based on theses soil properties to predict the RWUE in the study area. Organic matter was only the most important management factor influencing the physic-chemical properties and controlling the RWUE in the study area. Therefore, adding organic matter to the soil through maintaining crop residues is a proper technique and sustainable strategy to improve the soil physic-chemical properties and enhance the RWUE in the dry-farming lands. Contour farming is another effective approach to prevent runoff generation, conserve soil and nutrients, and promote soil physicochemical properties to the effective use of rains in the sloping fields.

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Comparative Analysis of Follicle Stimulating Hormone Beta-Subunit Gene in Mammals

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Abstract

Follicle stimulating hormone (FSH) is a glycoprotein hormone expressed by gonadotropes in the pituitary gland that regulated the development and function of gonads and is necessary for normal propagation functions in mammals. In this study, FSHβ revealed a high degree of conservation by contrasted with homologous fragment from serviceable mammals. These results suggested an insight into the characteristics of FSHβ. Using the software of RepeatMasker and Alignment, the content and distribution of SINE/tRNA-Glu and LINE/L1 in pig FSHβ subunit were detected. These insertion elements did not emerged in other artiodactylous FSHβ. It is probable positive selection during the evolutionary process of pig in artificial selection.

Keywords: mammals, evolution, FSHβ, analysis

1. Introduction

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), chorionic gonadotropin (CG) and thyroid-stimulating hormone belong to the family of glycoprotein hormones, and the chorionic gonadotropin (CG) has been found only in the placenta of equine species and primates. These hormones are heterodimers consisting of non-covalently linked common α and hormone-specific β subunits. FSH is responsible for development and survival of follicular somatic cells, and inducting of ovarian estrogen in women. In males, FSH regulates spermatogenesis by binding to the Sertoli cells (McGee & Hsueh, 2001; Plant & Marshall, 2001). Because of its necessary for unique mammalian reproduction function, FSHβ has possess evolutionarily conserved (Wallis, 2001). The FSHβ gene is characterized by excess of multiformity with allele frequencies and low genetic variation (Grigorova et al., 2007). Rearrangement sequence study identified a density of polymorphisms of 3 SNPs/1 kb in three human populations (European Estonians, Chinese Han and African Mandenkalu). Majority of these were common polymorphisms located in non-coding regions and were shared by three human populations. Although many FSH models were exist to deliberate the effects of gonadotropins on testicular functionand and testosterone as well as its role in spermatogenesis (Zheng et al., 1998). Sun et al. (2006) showed that FSH has a direct role in causing hypogonadal bone loss. Little attention has been paid to the interspecific evolution of FSH. In this study, we focused on nucleotide variation in order to demonstrate molecular evolution of the analyzed species and to provide useful data for studying the relationship of nucleotide variation in interspecific FSHβ gene.

2. Materials and Methods

In the present research, mRNA sequences of FSHβ in 20 species and DNA fragments in 15 sequences were downloaded from GenBank. All the sequences were aligned before the phyletic evolution tree was conducted. To avoid redundant sequences, the aligned nucleotide sequences were classified into different haplotypes using DNASP 4.0. The polymorphism site, the number of total mutations (M), the synonymous nucleotide diversity[p(s)], number of haplotypes (h), nonsynonymous nucleotide diversity [p(a)], the average number of nucleotide differences (Tajima, 1983) were calculated by DNASP 4.0. The Molecular Evolutionary Genetic Analysis (MEGA version 4.0) package (Tamura et al., 2007) was used to complete deletion of gaps. The Neighbor-Joining (NJ) method through bootstrap analysis (1000 pseudoreplicates) was used to construct two phylogenetic trees using the computer program MEGA. Repeats sequences were analyzed in repeat masker

(http://repeatmasker.org/).

3. Results and Discussion

Through analysis it was found that the full coded sequences of FSHβ ranges from 1827 (Ovis aries) to 1984 bp (Ornithorhynchus anatinus). Whereas, FSHβ identity varied from 100% for *Macaca fascicularis*-*Macaca mulatta* comparisons to 70% homology between Cervus nippon and *Ornithorhynchus anatinus* at the nucleotide level; this represents 100 and 65% similarity at the amino acid level, respectively. Primates had the lowest diversity compared to the average variation among all of the mammals. And the average nucleotide differences and nucleotide diversity are 0.172 and 0.043, respectively. Hence, the exogenous hormone of related species hormone or homogeneous hormones should be more conducive to captive breeding success.

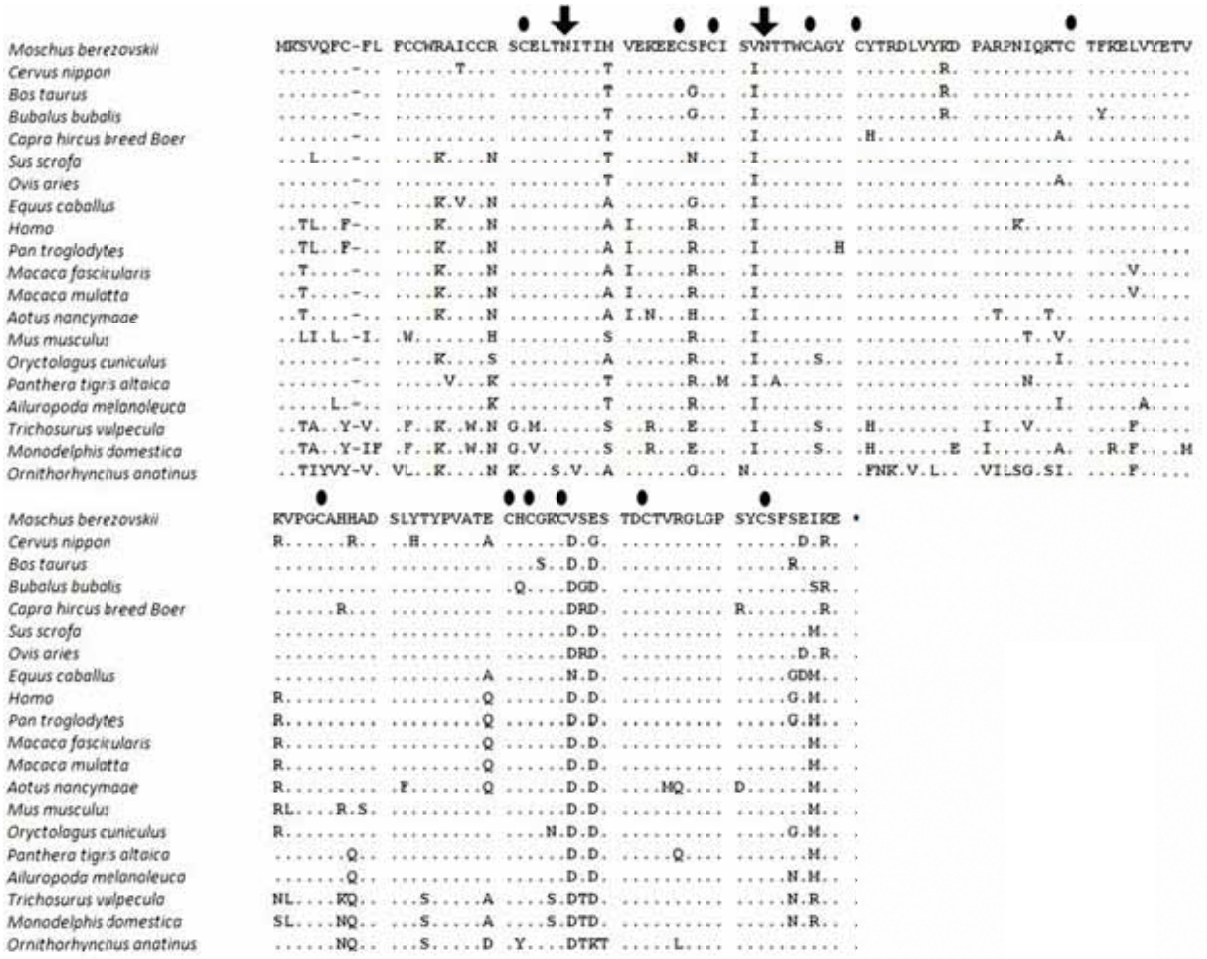

Figure 1. Analyses of amino acid sequence of FSHβ in mammals

"→" means potential N-linked glycosylation sites, "●"means cysteine residues.

There was a highly conserved among the species in the deduced amino acid sequence. Through these multiple sequence alignments, some common features of FSHβ in the amino acid sequences were demonstrate. Firstly, within the mature protein region, the same positions of 12 half Cys residues of FSHβ were the same in the mammals. Six pairs of disulfide bonds of Cys residues are essential not only for the function of heterodimer FSH molecule but also for the correct folding of β subunit. Secondly, two putative N-linked glycosylation sites presented at the 25th and 42th amino acid residue in the all FSHβ subunit (Figure 1); one is located between the first and second Cys residues, while the other is located between the third and fourth Cys residue. Glycosylation of FSHβ is essential for full bioactivity and protein biosynthesis, especially in protein secretion and folding, safeguard from enzymolysis in recurrence, and the signal conduction pass by receptors-ligands binding (Ulloa-Aguirre et al., 1999). These substantial similarities suggest a common ancestral origin. It was earlier proposed that all members of the glycoprotein gene family (common-α, LHβ, FSHβ and TSHβ) evolved from a

single ancestor through gene duplications. The first duplication produced a-subunit and b-subunit and was followed by a second duplication of the ancestral β-subunit to yield the LH-β subunit gene and the ancestor of the TSHβ and FSHβ subunits which eventually gave rise to the genes for these two latter β-subunits (Li & Ford, 1998).

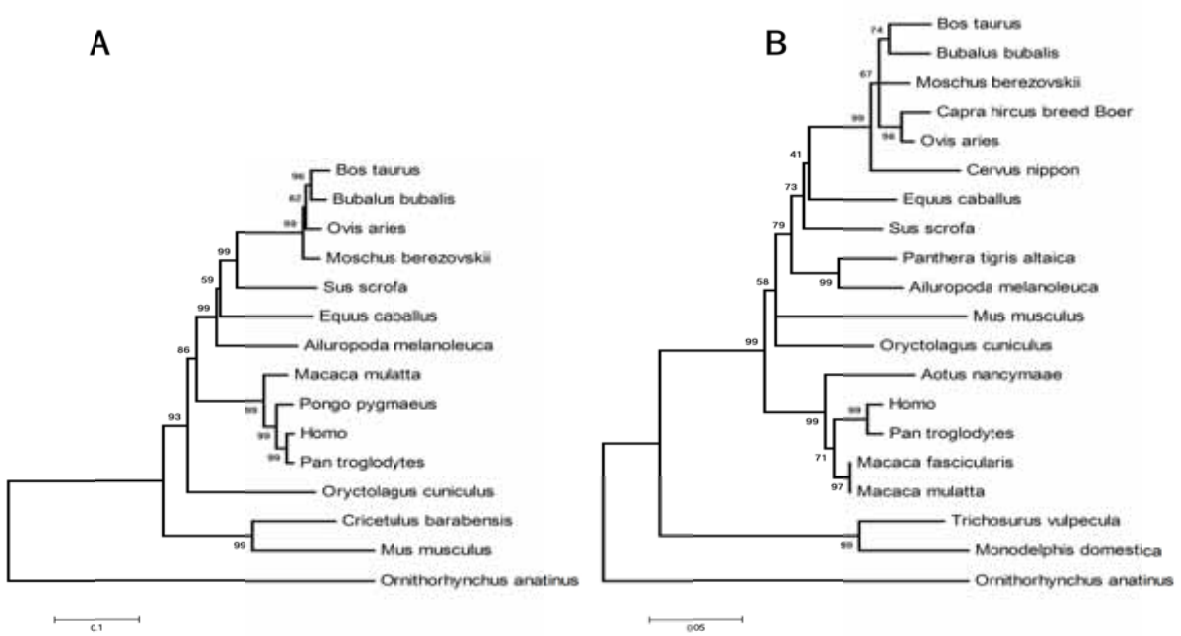

Figure 2. The NJ-tree of mammals based on FSHβ gene. The node number are Bootstrap values. A was inferred from DNA data and B from mRNA data

The constructed system evolution tree of FSHβ of families and species was basically conform to taxology of NCBI except for Sus scrofa, which was clade root of ungulate rather than clustered artiodactyla (Figure 2B). It provided that there was a difference evolution speed of FSHβ molecule among evolution of amniotic vertebrates (Wallis, 2001).

Using RepeatMasker and sequence alignments of FSHβ gene identified that porcine FSHβ subunit exhibits a remarkable different with those of other mammals (Figure 3). The sequence alignments of FSHβ gene among mammals indicated two microsatellite sites obviously in artiodactyls. However pig FSHβ contain (CT)n repeats, (AT)n repeats is absent. Whether this additional microsatellite sequence present in artiodactyls FSHβ is related to litter size and other reproductive traits requires further investigations.

Table 1. The analysis results of repeatmasker (http://repeatmasker.org/) of domestic pig (GenBank: D00621)

position in query			matching repeat		position in repeat			
begin	end	(left)	repeat	class/family	begin	end	(left)	ID
5997	6256	(3916)	Pre0 SS	SINE/tRNA-Glu	1	260	(0)	14
7054	7092	(3080)	AT_rich	Low_complexity	1	39	(0)	15
7368	7439	(2733)	MIR3	SINE/MIR	(55)	153	82	16
8055	8075	(2097)	AT rich	Low_complexity	1	21	(0)	17
8817	8990	(1182)	L1-2 SSc	LINE/L1	(1)	6015	5843	18
9117	9223	(949)	L2b	LINE/L2	3246	3405	(21)	19
9885	9917	(255)	AT_rich	Low_complexity	1	33	(0)	20
10031	10172	(0)	PRE1 SS	SINE/tRNA-Glu	1	142	(116)	21

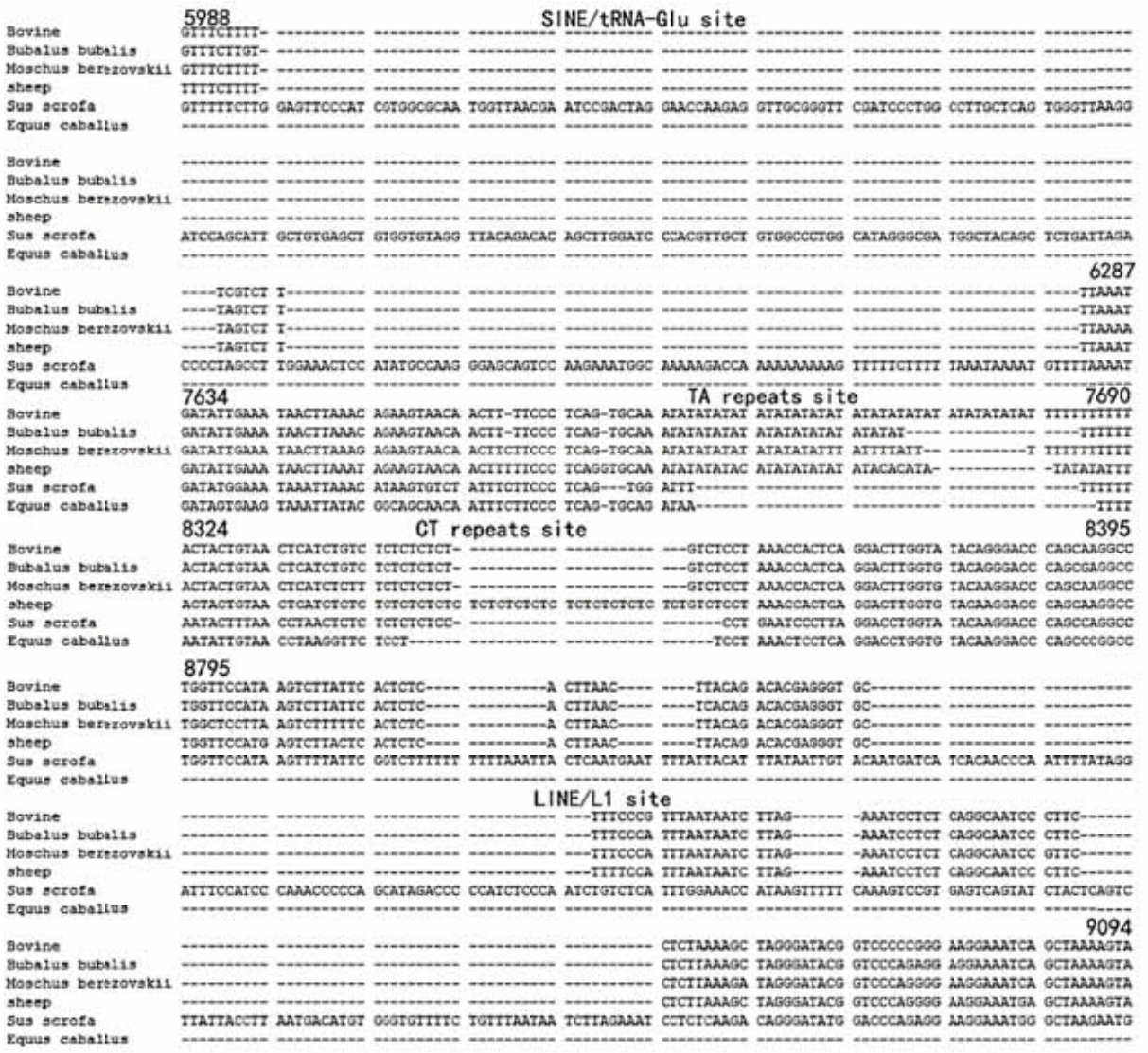

Figure 3. Alignment of FSHβ gene in Artiodactyla. Repeats sites and short interspersed nucleotide elements (SINE) site, long interspersed nucleotide element (LINE) site are shown in figure

In a computer search for repeats sequences, two retrotransposons sequences short interspersed nucleotide elements (SINE) and long interspersed nucleotide elements (LINE) showed in intron1 region and exon3 region of porcine FSHβ gene (GenBank: D00621), respectively (Table 1). Zhao et al. (1999) found an extra SINE in intron 1 of porcine FSHB gene [7]. SINEs were considered to be genomic parasites and having no benefit to the organism (Batzer & Deininger, 2002). The SINE insertion, found in introns of animal gene, may modify the gene's transcription pattern and its presence is well known to affect the expression level (Kramerov & Vassetzky, 2005). The presence/absence of SINE resulted in allelic polymorphism of porcine FSHβ gene. The pig breeds with SINE− allele was correlated with higher litter sizes than the breeds with SINE+ allele (Shi et al., 2006). When comparing with two SINE indicated that the SINE finding by zhao was located downstream of the SINE in FSHβ gene (GenBank: D00621) and their similarity are 80%. The same high frequency SINE in pig due to probably positive selection during the evolutionary process of pig in artificial selection.

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Entomophagy: A Panacea for Protein-Deficient-Malnutrition and Food Insecurity in Nigeria

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Abstract

The paper posits that Nigeria like most developing countries is not immune to protein deficiency among its population because of the often prohibitive prices and overdependence on conventional animal protein source which are in limited supply. The consumption of edible insects is a way out of this predicament. Entomophagy is common practice in the tropics only varying with location, insect(s) consumed, and ethnic group. Again, it posits that insects as a source of protein is better than many conventional sources of protein; it juxtaposes the protein value of some edible insects with conventional animal protein sources like beef, pork, fish, eggs, and milk. Furthermore, many advantages that go with entomophagy are indicated. It identified some militating factors against entomophagy, and maintained that people are not eating insects because of prejudices and not because insects are inferior nutritionally. Finally, it offers practical recommendations that could lead to more people consuming insects.

Keyword: mini-livestock, edible insects, nutrition, protein, prejudice

1. Introduction

The global food crises have made scholars to reassess some unconventional approaches to food production, especially protein production (Durst & Shono, 2010). The health situation in Africa nay Nigeria is characterized by high rate of malnutrition and most common of these is protein deficiency (WHO, 1999). This has been aggravated by prohibitive prices and excessive dependence on conventional protein sources (Olaofe et al., 1998), which are limited in supply (Umoh & Bassir, 1977). Protein-energy malnutrition (lack of protein and energy food) is a range of mild, moderate to pathological conditions arising from a deficiency of protein and energy, and no other disease compares in importance with it (DeMaeyer, 1976). Protein-energy malnutrition is affecting over 100 million people in Africa and an additional 200 million are at risk (Maletnlema, 1992). It is a crucial public health problem for many developing countries (Dulger et al., 2002). Edible insect species mainly belong in the traditional and informal food systems (Roos, 2012). Insects constitute about 75% of all known animal species (Yoloye, 1998). Edible insects have played an important role in the history of human nutrition (Lyon, 1991; Bodenheimer, 1951). Archaeological evidences as well as analysis done on fossilised faeces reveals beyond doubt that the human race has evolved as an ant-eating species (Ramos-Elorduy, 2009). Though some insect species are poisonous (Adamolekun, 1993), most are not (Meyer-Rochow, 2010). Edible insect groups that provide nutrition for people in Africa are: ants, termites, caterpillars, water bugs, beetle larvae, flies, crickets, katydids, cicadas and dragon nymphs (Srivastava et al., 2009). Insects are classified under non-timber forest products (FAO, 1989). Insects are very adept in converting what they have eaten into tissues that can be consumed by others – about twice as chicken and pigs, and more than five times as efficient as beef – the food conversion efficiency of insects may be 20 times that of cattle (Durst & Shono, 2010). Furthermore, Insects constitute quality food for humans and emit low levels of green house gasses (van Huis, 2013). However, a lot of effort has been put into the elimination of insects rather than on its preservation and production (Ogunlabi & Agboola, 2007).

Entomophagy literally means the consumption of insect by humans. It is a term derived from the Greek word entomos (insect) and phagein (to eat), the practice is a well-established although a diminishing custom of many parts of the world (Sutton, 1998; DeFoliart, 1999), the practice seems to be culturally universal, only varying with

location, type of insect and the ethnic group involved (Johnson, 2010). Insect consumption is part of a population's cultural heritage, and that 1391 insect species are eaten worldwide, of which 524 are eaten in 34 countries of Africa representing 38% of all species consumed globally, she maintained that Mexico consumes 348 species which is the highest number recorded for a single country (Ramos-Elorduy, 1997). Jongema (2012) holds a contrary opinion and posits that about 1900 species of insects are eaten worldwide, mainly in the developing countries. Bodenheimer (1951) maintained that the people of Madagascar love to eat fried grasshoppers, Bahuchet (1990) posits that caterpillars are a very important food item for the Pigmies, Kitsa (1998) wrote that in a certain city in Southwest DR Congo 28% of its inhabitants eat insects, Roulon-Doko (1998) maintained that about 15% of the meat diet of the Gbaya people in Central African republic consist of insects. Adriaens (1951) reported that between 1954 and 1958 close to 300 tonnes of dried caterpillars were consumed yearly in the Kango district of DR Congo. Furthermore, some tribes in Colombia, Venezuela, and South Africa preferred certain insects to fresh meat (Quin, 1959). Though insects are seen as food for the poor and backward by some but they are sought after and served in the best restaurants in most countries of Asia, Australia, and Europe (Ramos-Elorduy, 2009; Yen, Hansboonsong, & van Huis, 2012). Robert (1989) indicated that a 10% increase in the world supply of animal protein through the mass production of insects for food can to a large extent reduce if not eliminate the malnutrition problems of the world and also decrease the pressure on conventional protein sources. According to Olaofe et al. (1998) one of the possible ways to counter protein energy malnutrition in Nigeria is to promote the utilisation of lesser known and cheaper source of animal proteins such as those from insects.

Scholars have written enormously on entomophagy among several tribes in Nigeria, however, most writers maintained that the practice is more prevalent among rural populations than urban population (Fasoranti & Ajiboye, 1993). There is a significant trade in, and consumption of edible insects among some ethnic groups in Nigeria. Akingbohungbe (1992) and Banjo et al. (2006) maintained that grubs of palm weevil are fried and eaten in several parts of Western Nigeria, Edo, and Delta state. Also and Adamolekun (1993) posits that larvae of *A. venata* are eaten among the people of South-western Nigeria. The larvae of the Saturnid Caterpillar (*Cirina forda*) known locally as *kanni* is widely eaten among the people of Kwara state, again. It is believed that this caterpillar is the most widely marketed edible insect in Nigeria and sells for about twice the price of beef and is used for vegetable soup. Other widely marketed insect in Nigeria include termites and dragon flies (Fasoranti & Ajiboye, 1993). The Gwari people of Niger state eat the larvae of dung beetle (*Aphodius rufipes*) and to them are a delicacy and serves as an alternative source of protein (Paiko, 2012). Ene (1963) maintained that a number of insects or their products were used as food in some parts of Nigeria and to a large extent eaten as titbits or exclusively by children.

'Ordinarily, insects are not used as emergency food during shortage, but are included as planned part of the diet throughout the year or when seasonably available' (Banjo et al., 2006). Banjo et al. (2006) further gave the following as the list of commonly eaten insects in South-western Nigeria; *Macrotermes bellicosus*, *Brachytrypes spp.*, *Cyrtacanthacris aeruginosa unicolor, trisfasciata (F.), Oryctes boas, Rhynchophorus phoenicis, Apis mellifera* (*oyin*), and *Cirina forda* (*ekuku*).The most abundant, most preferred, and most consumed insect by the people of Benue State, Nigeria is *Macrotermes natalensis* followed by *Brachytrupes membranaceus* and then *Cirina forda* (Agbidye et al., 2009). In Borno state, northern Nigeria, *Zonocerus variegatus* is widely eaten and seen as a delicacy; the grasshoppers are readily displayed in the market and sold like meat (Solomon et al., 2008). With the 'rising global population and limited pasture lands, production of animal protein is becoming ever unsustainable and one of the possible ways to overcome this problem is to adopt the entomophagy practice' (T. Abassi & S. A. Abassi, 2011). More so, whether insects are eaten or not does not depend on low nutritional value of insects, but often such is tied to customs, preference, or and prohibitions (van Huis, 2003), and in some societies it is just a taboo (Weiss & Mann, 1985; McElroy & Townsend, 1989). Awareness of entomophagy and its advantages are required as part of the solution to protein deficient malnutrition in the developing countries, Nigeria inclusive.

2. Protein Value of Insects in Human Nutrition

The nutritional benefit of insects is often overlooked, and some do not even know that insects are of any nutritional importance. Insects are good sources of essential nutrients which could help alieviate the nutritional problems among the populations that consume them (Ekpo, 2011) Documentation of the nutritional importance of insects to human diet is sparse (Roos, 2012). The prejudice against eating insects is not justified from nutritional point of view (van Huis, 2003). According to Meyer- Rochow (2010), Bodenheimer (1897-1959) was the first scholar to write about nutritional importance of insect in his book 'Insects as human food' in 1951. Insects are a source of protein for the improvement of human diet; they are an important source of protein for people who are malnourished. Some insects contain more protein than meat (Johnson, 2010). Bergeron et al. (1988) maintain that the crude protein of three aquatic insects eaten around the Lake Victoria Uganda to be 67%. Insects contain a high

amount of crude protein. In g/100g dry weight caterpillars contain 50-60, palm weevil larvae 23-36, Orthoptera 41-91, ants 7-25, and termites 35-65 (Bukkens,1997). According to Santos et al. (1976) 100 grams of caterpillar would provide 76% of an individual's daily protein requirement, and more than 100% of daily requirement for many of the vitamins and minerals. For example, the mean values in protein percentages of insects in their mature stages, and adult stages have been found to be 36, and 38 respectively, and some are as high as 72 and 69 (wet weight). Tsvangirayi (2013) posits that the Emperor moth (Mopane worm) contains three times the amount of protein as beef. The crude protein value of *Rhynchophorus* sp. is as high as 71.6% (Bride & Nwaoguikpe, 2011). In a study of 94 edible insects by Ramos- Elorduy and Pino Moreno (1990) they found out that 50% of the insects have higher caloric values than soyabeans, 63% were superior to beef, and 70% were better than fish and beans.

Many insects are low in cholesterol and fat (Srivastava et al., 2009), they are herbivores and have clean eating habits which makes them cleaner than chicken, pigs, and many other conventional protein sources, in fact , the grasshopper is one of the cleanest animals (Abbasi & Abassi, 2011). Most often than not, most insect consumed are harvested from the wild, or come from the wild and are gathered for food, hence, they are mostly free from pesticide and other chemical contaminant which abound in places where conventional source of protein are found (Durst & Shono, 2010). When insects feed on vegetation they are able to transform phytomass into zoomass much more efficiently than conventional livestock; more than 10 times more plant nutrients are needed in order to produce one kilogram of meat than one kilogram of insect zoomass (Nakagaki & DeFoliart, 1991; Taylor, 1979). Furthermore, when insect are generally eaten by most in the society insects especially those of them that are edible would be seen not as pest but as source of much needed protein. The use of pesticide and other chemicals which have adverse effect on heath and the environment would be brought to its barest minimal. More so, some locust species despised and considered dangerous to crops in tropical Africa are seen as delicacies by people of other land and culture (Owen, 1973).

3. Some Factors Militating Againts Entomophagy

Though the eating of insects has unquantifiable benefits, some factors have been hindering its acceptance. Some edible insects are very expensive and cost twice the price of beef. Also, in many circles people see eating of insect as a practice that is associated with poverty, hunger, unavailability of meat and fish and other prejudices (Owen, 1973). Seasonality of most edible insects have a negative impact on entomophagy; availability of most edible insects is dependent on some plant cycles, hence, these insects are not available all year round. Furthermore, some customs and religions forbid the eating of insects, or some certain species of insects (Fasonranti & Ajiboye, 1993). Some people who are educated and in the cities have come to see insects as been dirty and as pest and a nuisance, hence, not ideal as food. Also, modernisation and changing attitudes have had negative impact on the practice of entomophagy; those who have practiced entomophagy at one time or the other have had to jettison the practice because they now see it as a practice that belongs to the past.

4. Recommendation and Conclusion

Aside from the nutritional value of insect and the prospect in them in assuring food security, eating of insects can be enhanced through the promotion and adoption of modern food technology and standards to ensure that insects are safe and attractive for human consumption. When promoting insects as food they should be farmed like any other conventional protein source like chicken, beef, pigs, and fish instead of relying on natural harvesting; micro-livestock have the potential of increasing household protein consumption (Barwa, 2009). According to Fasonranti and Ajiboye (1993) this would allow a continuous supply. Raloff (2008) reported that this have proved successful in Northeast Thailand where agricultural extension agents have taught locals low-cost insect rearing techniques, and with this technique 400 families from two villages produced 10 metric tons of crickets in the summer of a single year. Nigeria can take a cue from this feat and initiate an insect rearing program on a national scale. Due to their high level of protein, edible insects especially those common in a locality could be used to fortify grains like sorghum (Nigerian Stored Products Research Institute is presently working on this) to counter protein-energy malnutrition in children. Srivasta et al. (2009) alluded to this when they maintain that in some part of the world flour made from a certain caterpillar is mixed to prepare pulp given to children to fight malnutrition.

There should be a concerted effort at all levels to make harvesting of insects as human food a better option and the use of insecticides should be deemphasised unless on extreme occasions (Ledger, 1987). Nigerian entomologists, in tandem with Agricultural extension agents, nutritionists and health workers should make a comprehensive list of all edible insects in the country and adjoining countries. There should be enlightenment campaigns at all levels as a matter of policy to disabuse the mind of people about the negativities associated with entomophagy which are often not true. People should be made to know that eating of edible insects is less taxing on the environment than eating beef, pork, and chicken; apart from fact that it takes far lesser leaves to produce insects than it does to

produce the same amount of beef (Fiala, 2008). Food consumption patterns have been known to be greatly influenced by the mass media (Ramos- Elorduy, 1990), hence, the mass media too has a great deal to do to see to it that edible insects are consumed by the Nigerian populace in general ,and consumption of it should not be restricted to the rural populace. Furthermore, because most edible insect species occur seasonally, and because harvest can be more than what can be consumed in a short time, more research is needed on storage and preservation of these edible insects. All factors militating against eating of edible insect in the country should be looked into and addressed accordingly. Conclusively, because insects have high nutritional value they are a good alternative to solving the PEM problems facing the third world countries, hence, efforts should be made to encourage and retain the practice of entomophagy (Ekpo, 2011).

Table 1. Crude protein content of some insects on a dry weight basis

	Common English name of insect	Scientific name of insect*	Crude Protein %
1	Leaf hoppers	*Graphocephala sp.*	56
2	Yellow mealworm beetle larvae	*Tenebrio molitor*	47
3	Darner larvae	*Aeshna umbrosa*	56
4	June beetle larvae	*Phyllophaga sp.*	42
5	Agave billbug larvae	*Scyphophorus acupunctatus*	55
9	Water boatman adults	*Notonecta glauca*	53
10	Leaf cutting ants	*Acromyrmex octospinosus*	58
11	Paper wasp pupae	*Polistes humilis*	57
12	Red-leg locust	*Melanoplus femurrubrum*	75
13	Corn earworms	*Helicoverpa zea*	41
14	White agave worms	*Aegiale hesperiaris*	30 - 35
15	Red agave worms	*Hypopta agavis*	45 - 46
16	Tree hoppers	*Ceresa taurina*	45 - 46

Source: Ramos – Elorduy (1988).* Content in this column is ours.

Table 2. Crude protein (nx6.25) of some commonly eaten dried insects in southwestern nigeria

	Insects (Scientific name)	Crude protein	Yoruba name*	English name*
1	*Macrotermes bellicosus*	20	Esusu	Termite
2	*Brachytrypes spp.*	6	Ire	Cricket
3	*Turrita (L.)*	12	Tata	Grasshoppers
4	*Zonocerus variegatus*	26	Tata	Short horn Grasshoppers
5	*Analeptes trifasciata*	29	Ipe	Rhinoceros beetle
6	*Anaphe infracta*	20	Ekuku	Caterpillar
7	*Anaphe recticulata*	23	Ekuku	Caterpillar
8	*Anaphe venata*	25	Ekuku	Caterpillar
9	*Cirina forda*	20	Ekuku	Caterpillar
10	*Apis mellifera*	20	Oyin	Honeybee
11	*Oryctes boas*	26	Ogongo	Scarab beetle
12	*Rhynchophorus phoenicis*	28	Munimuni	Snout beetle

Source: Banjo et al (2006). * Content in these Colums is ours.

Table 3. Protein value of some insects based on 100 grams serving

	Insect	Scientific name of insect*	Protein (grams)
1	Giant water beetle	*Lethocerus americanus*	19
2	Red ant	*Solenopsis geminata*	13
3	Silk worm pupae	*Bombyx mori*	9
4	Meal worms	*Tenebrio molitor*	20
5	Wax worm	*Galleria mellonella*	15
6	Super worms	*Zophobas morio*	17
7	Dung beetle	*Circellium bacchus*	17
8	cricket	*Brachytrypes ssp.*	21
11	June beetle	*Phyllophaga crinita*	13
13	Termite	*Reticulitermes sp.*	14

Source: Berenbaum (1996) *Content in column is ours.

Table 4. Protein value of some conventional protein sources (dry weight)

	Protein source	Protein %
1	Beef	17 - 19
2	Pork	15 - 17
3	Fish	19
4	Eggs	13
5	Milk	4

Source: www.ent.orst.edu.

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A Rapid and Efficient Method for the Isolation of Mitochondrial DNA From Wheat Crop

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Abstract

The mitochondrial DNA was isolated from wheat (*Triticum aestivum*) crop in combination of different centrifugation and density gradient ultracentrifugation, DNaseI enzyme treatment, sucrose sedimentation; lysis with SDS and potassium proteinase. Pure mitochondrial DNA was successfully obtained by phenol/chloroform/isoamyl alcohol extraction to remove protein, and digested with RNase phenol/chloroform extraction method. To detect mitochondrial DNA purity, specific primers were designed for nuclear (β-actin) and mitochondrial (COXII) gene. Isolated mitochondrial DNA is pure, suitable for PCR and genetic analysis.

Keywords: wheat, yellow etiolated shoots, mitochondrial DNA isolation

1. Introduction

Mitochondria play an important role in the development and reproduction of plant, encoding necessary proteins involved in the system of energy production. They occupy a specific evolutionary pattern relative to nuclear counterparts (Lynch et al., 2006). Numerous metabolic pathways at cellular site of mitochondria are essential for higher eukaryotic life (Logan, 2006; Reichert, 2004). Principal pathways are the tricarboxylic acid cycle, respiratory electron transfer and ATP synthesis (Logan, 2006; Barrientos, 2003). Isolation of mtDNA is particularly difficult and protocols developed for one species often do not work for other species (Hanson et al., 1986). This phenomenon is due to intact plant tissue may be the abundance of phenolic compounds which are known to exist in differentiated plant tissues. Moore and Proudlove (1983) reported that phenolic compounds bind strongly to mitochondrial membranes and are thought to be destructive to the integrity of the mitochondria. The protocol presented in this paper evolved from a protocol described by Li et al. (2007) for wheat crop and may serve as a guide for the development of other mtDNA isolation protocols.

2. Material and Method

2.1 Plant Material

In this investigation wheat (*Triticum aestivum*) line 1376 was used. The seed was soaked in water for 6 hours, Wheat yellow etiolated seedlings were grown in dark over a period of 7 to 10 days at 30°C in growth chamber and they meet their growth needs by on nutrients only supply sterile water. The plants were strictly protected from light. All the cultural practices were done in dark, prevent them to become green and get a high quality mtDNA which is free from chloroplast, genomic DNA and other impurities.

2.2 Mitochondria Isolation

We isolated mitochondria, ground 20 g of fresh etiolated yellow leaf, in high ionic-grinding buffer [50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol , 0.2 mol/L sucrose , 25 mmol/L EDTA pH=8, 0.1% bovine serum albumin (BSA), 0.6% Polyvinylpyrrolidone (PVP) and 0.1% β-mercaptoethanol], BSA, PVP and β-mercaptoethanol added before use; 10 ml/g tissue; filtered it with 6 layer of Mira cloth. Centrifuged at 3500 rpm for 10 minutes on 4°C discarded the chloroplast and nuclear DNA pellets. The supernatant was centrifuged at 10 000 rpm for 20 minutes on 4°C. Re-suspended the pellets in buffer solution (50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol , 0.2 mol/L sucrose , 0.1% BSA and 0.6% PVP) gently used soft paint brush, centrifuged at 3500 rpm on 4°C for 10 minutes discarded the nuclei pellets. Supernatant was centrifuged at 10 000 rpm for 20 minutes at 4°C. To eliminate nuclear DNA, the mitochondrial pellet was re-suspended in 10 ml of cold buffer (0.2 mol/L sucrose, 50 mmol/L Tris-HCl pH=8) added 100 µl $MgCl_2$ (1 mol/L), 8µl DNaseI (50 ng/ml) (Segma) enzyme and Incubated on ice for

90 minutes. The reaction was stopped by added EDTA-Na_2 to a final concentration was 0.2 mol/L. Washed the organelles from 25 ml buffer (50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol and 0.6 mol/L sucrose) and Collected the organelle pellets of mitochondria by centrifuged at 10 000 rpm for 20 minute, at 4°C.

2.3 Mitochondrial DNA Isolation

The mitochondrial pellets was re-suspended in lysis buffer (25 mmol/L Tris-HCl pH=8, 25 mmol/L EDTA , 5% SDS, 0.1 mol/L NaCl and 0.1% β-mercaptoethanol) and added 100 µg/ml potassium proteinase, incubated at 50°C and then 37°C on each temperature for 60 minutes. The mtDNA was cleaned with 2 mol/L NH_4-acetate, phenol/chloroform/isoamyl alcohol (25:24:1), pH 6.7 and centrifuged at 18 000g for 5minutes on 4°C. (Repeat the step). mtDNA was washed with Chloroform/isoamyl alcohol (24:1) and Centrifuge at 18 000g for 5 minute at 4˚C. RNA was removed from the samples with 10µg/µl DNase-free RNase enzyme (Sigma) and incubated at 37°C for 60 minutes. Purified the mtDNA by added ice cold anhydrous ethanol, $1/10^{th}$ of 3mol/L CH_3COONa and incubated at -70°C for 60 minutes. Pelleted mtDNA by centrifuged at 18 000g for 15minutes on 4°C. mtDNA was washed with ethanol 70% (2 times) and 96% (1 time). Dry at room temperature, re-dissolved in TE-buffer (10mmol Tris and 1mmol EDTA (pH 8.0) and store at -20°C.

2.4 Quality Test

mtDNA Quality were measured at absorbance ratio A260nm, A280nm and calculated optical density (OD) value, used UV spectrophotometer for purity analysis.

2.5 Polymerase Chain Reaction and Electrophoresis

PCR reactions were performed in 20 µl volumes with 50 ng mtDNA, 6 µl ddH_2o, 1 µl (forward and reverse) primer of each specific-gene (β-actin and COXII gene) and 10 µl of master mix. Performed in an Eppendorf Mastercycler using the following program: 94°C for 5 min followed by 5 cycles at 94°C for 1 min, 60°C for 30 sec and 70°C for 1 min, 35 cycles of 50 sec at 94°C, 1min at 60°C, and 50 sec at 72°C and final extension at 72°C for 6 min. PCR products were separated on 1% (w/v) agarose gels electrophorese and ethidium bromide staining.

3. Result and Discussion

The mtDNA was extracted from yellow etiolated shoots (20 g) of wheat crop (Scotti et al., 2001), used the method of Li et al. (2007) with some amendments. Removed the genomic, Plastid DNA by DNaseI enzyme treatment. Proteinase k was used to remove the protein. RNA was removed by RNase enzyme treatment. The mtDNA, OD values ratio of A260/A280 was in between 1.81 to 1.99, at A260/A230 1.90 to 2.0 and the concentration of mtDNA/µl was in between 559 to 590 (Table 1) which indicates the good quality mtDNA (Figure 1).

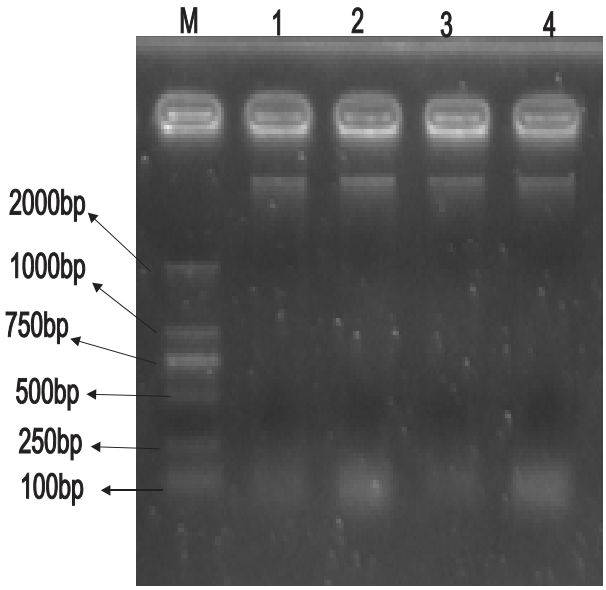

Figure 1. Results of mtDNA

M: Marker, Wheat line 1376 (1-4).

Purified mitochondrial pellets from genomic DNA organelles applied DNase I enzyme treatment. This DNase I enzyme concentration can be adjusted as needed for the different plant species until the nuclear DNA amplification is undetectable (Christine et al., 2004). For the mtDNA purity assessment, we analyzed samples through amplification of nuclear β subunit (β-actin) actin genes and the mitochondrial C oxidase subunit (COXII) gene. The products were separated on a 1% (w/v) agarose gel electrophoresis. Mitochondrial COXII gene 400bp was amplified and β-actin gene was not amplified, so the mtDNA was not contaminated by nuclear DNA, plastid DNA, RNA, protein, and was successfully used for PCR, cloning and southern blot analyses (Figure 2).

Table 1. Spectrophotometer values of mtDNA

Sr.No.	A260/A280	A260/A230	ng/µl
1.	1.99	2.00	559
2.	1.81	1.90	590
3.	1.92	1.99	585
4.	1.89	2.00	569

Values of Wheat line 1376 (1-4).

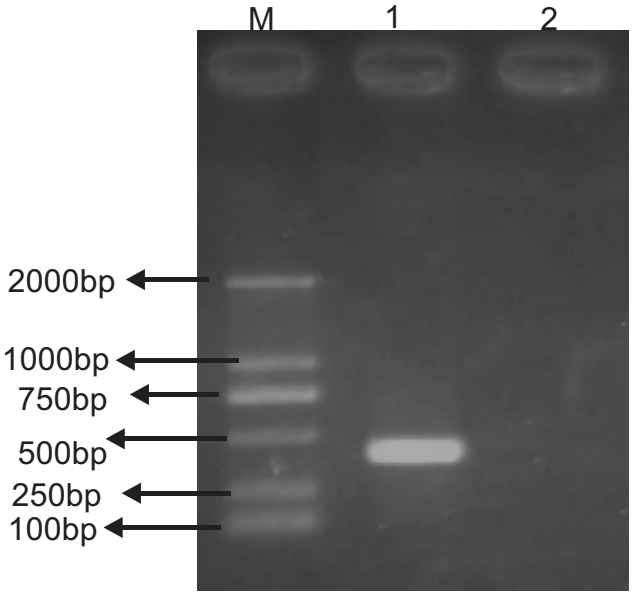

Figure 2. M. Marker 1. COXII gene 2. β-actin gene

M: Marker, Wheat line 1376 (1-2).

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The Effect of Market Liberalization on Maize Price Distributions in Nigeria

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Abstract

Market liberalization is a major provision of the structural adjustment programme .This paper examined the nature of maize price fluctuations following the introduction of the reform .Secondary data on average monthly prices of maize covering the period 1983-2000 were sourced from various publications. Data on monthly prices were deflated by consumer price index of food items to construct real price series for maize. The econometric model, Autoregressive Conditional Heteroskedastic in Mean (ARCH-M) was employed to determine the effect of the policy reform on the mean and volatility of maize prices.

The results of the ARCH-M model show that a sharp increase was observed in the first-order autocorrelation between the pre- liberalization and post- liberalization periods for both the mean (0.02 to 0.10) and variance (0.49 to 3.96). This implies that the long term changes in the price of maize due to free marketing are different from periods of administrative pricing. The variation was lowest in the pre- liberalization period relative to the post-liberalization period while the highest variability was experienced in the short term period immediately after the reform. The price of its close substitute, sorghum, and the border parity price affects the price of maize thus allowing for informal cross border trade in the post- liberalization period. The lagged prices of maize and seasonal changes reduce volatility in the price of maize between the pre and post liberalization periods. There are also differences in the volatility of maize price across regions. Regional differences show that volatility increased in Adamawa and Niger and decreased in Akwa Ibom in the post- liberalization period. Prices became more stable in the southern agro- climatic zones in the long run but were high in the northern savannahs. Policy makers can stabilize maize prices by disseminating information on price movements and keeping stock of maize for the dry season. Present efforts to increase productivity through improved access to inputs should be strengthened and effectively monitored to ensure increased output so as to increase maize stock. The conclusion is that the reform has increased the mean prices of maize and its volatility over the years, however, other factors have also contributed to price increase since Nigeria's maize are only traded across close borders and the country is yet to be integrated into the world's maize market.

Keywords: market liberalization, maize, price distributions, arch-m model, Nigeria

1. Introduction

Maize is a staple food crop for most sub-Saharan Africans including Nigeria. Despite the economic importance of maize to the teeming populace in Nigeria, its production has not met the food and industrial needs of the country and this could be attributed to low productivity of maize farms or that farmers have not adopted improved technologies for maize production (Onu et al., 2010). The demand for maize sometimes outstrips supply as a result of the various domestic uses (Akande, 1994). Additionally, other factors like price fluctuations, diseases and pests, poor storage facilities have been associated with low maize production in the country (Ojo, 2000). In a bid to increase food production in Nigeria over the years, several policy reforms have been put in place by successive governments and one of such policy reforms in time past is the Structural Adjustment Programme (SAP) introduced in July 1986. Olomola et al., (1996) pointed out that the Structural Adjustment Programme (SAP) was introduced in Nigeria in 1986 and the principal components include the improvement of public sector management and resource allocation, institutional reforms and market liberalization. Of these components, market liberalization appears to be the core strategy in the implementation of SAP.

Mesike et al. (2008) observed that prior to the inception of SAP in 1986, several policies aimed at providing support for the agricultural sector turned out to be regarded as anti-competitive. The policies were introduced in the past due to market failures in the allocation of resources and the need to achieve sustained growth and equitable development in the country. They included: price control (administered output prices for export commodities), guaranteed minimum price for grains, input subsidy, centralized marketing and export monopoly.

Market liberalization, an important component of structural adjustment programmes has been a dominant feature of economic reforms in developing countries and it is assumed that it will shift internal terms of trade in favour of farmers as the real prices received by farmers will improve with the dismantling of structures the government used to suppress food prices such as subsidies, price ceiling mechanism, territorial pricing and commodity boards (Timmer, 1986).

Trade liberalization is the major instrument geared towards the goal of global economic integration. It has been at the heart of World Trade Organisation (WTO) negotiations and agreements, and entails the removal of import quotas and other quantitative restrictions, abolition or reduction of the level and dispersion of import tariff rates, removal of export taxes, removal of protection for local industries and export subsidies, elimination of non-tariff barriers, and devaluation of the local currency (Olomola et al., 1996).

In Nigeria, attempts have been made by past governments to liberalize the market for food items. This was done through the abolition of import licences and scrapping of commodity boards in 1986 and consequently allowing prices to be determined through the market forces.

A common argument in literature is that, has market liberalization and deregulation of domestic markets in developing countries resulted in increased incentives for agriculture? What effect has this major policy reform had on the price of maize? This paper evaluates the effect of market liberalization policy on maize price distributions across four ecological zones of Nigeria.

2. Materials and Methods

2.1 Sources and Scope of Data.

The data analyzed were obtained from secondary sources. Data collected were the monthly price of maize and sorghum per kilogram, the local currency (Naira) official exchange rate to a US Dollar and the international market price of maize; covering the period 1983-2000. The choice of time period is predicated on data availability and the need to capture the pre-and post liberalization periods. The reference year for the post liberalization period is 1987. The data were obtained from various publications of Federal Office of Statistics (FOS), Federal Ministry of Agriculture and Rural Development (FMA&RD) and the World Bank. The monthly prices were deflated by the consumer price index (CPI); to construct the real price series for maize and sorghum. The border parity price (BP) was estimated as the international market price of maize converted into Naira at the nominal exchange rate and then deflated by the Nigerian CPI. The real exchange rate (ER) was calculated as the nominal bilateral Naira (₦) per US dollar rate deflated by their respective CPI.

2. 2 Analytical Procedure

The Augmented Dickey-Fuller (ADF) test and the Autoregressive Conditional Heteroskedastic in Mean (ARCH-M) model were employed in the analysis.

2.3 Test for Stationarity

The first step in carrying out a time series or panel data analysis is to check for the stationarity of the variables (price series in this case). A series is said to be stationary if the means and variances remain constant over time. It is referred as I (0), denoting integrated of order zero. Non stationary stochastic series have varying mean or time varying variance. The price series were initially tested for stationarity in order to avoid the problem of having a spurious regression. A stationary series tends to constantly return to its mean value and fluctuations around this mean value have broad amplitudes, hence, the effects of shocks are only transient. Other attributes of stationary and non-stationary data and their implications in econometric model are discussed by Adams (1992), Gujarati (1995) and Juselius (2006).

A variable that is non -stationary is said to be integrated of order *d*, written *I(d)*, if it must be differenced *d* times to be made stationary. Consequently, a variable that has to be differenced once to become stationary is said to be *I (1)* i.e., integrated of order 1. The augmented Dickey Fuller (ADF) was adopted to test for stationarity. This involves running a regression of the form:

$$\Delta P_{it} = \propto + \beta \Delta P_{it-1} + \gamma P_{it-1} + \sum_{i=2}^{m} Y_i \Delta P_{it} + \mu_t \quad (1)$$

Where:

P_{it} = Price of maize in region *i* in month *t;*

m = number of lagged differences;

μ_t = error term.

The null hypothesis that $\gamma = 0$ implies existence of a unit root in P_{it} or that the time series is non-stationary. The critical values which have been tabulated by Dickey and Fuller (1979), Engle and Yoo (1987) and Mackinnon (1990) are always negative and are called ADF statistics rather than t-statistics. If the value of the ADF statistics is less than (i.e. more negative than) the critical values, it is concluded that P_{it} is stationary i.e. $P_{it} \sim I(0)$.

When a series is found to be non-stationary, it is first-differenced (i.e. the series $\Delta P_{it} = P_{it} - P_{it-1}$ is obtained and the ADF test is repeated on the first-differenced series). If the null hypothesis of the ADF test can be rejected for the first-differenced series, it is concluded that $P_{it} \sim I(1)$. The price series for all the markets included in this study were investigated for their order of integration using the Augmented Dickey-Fuller test.

2.4 Autoregressive Conditional Heteroskedastic in Mean (Arch-M) Model

It is accepted that price uncertainty tends to influence retail prices and marketing margins of food commodities (Holt, 1993; Brorsen et al., 1985). The econometric model Autoregressive Conditional Hetroskedastic in Mean (ARCH-M) was employed to measure the effect of temporal variation in the conditional means and variances of the dependent variable (price per kilogram of maize) and at the same time permitting for evaluation, the simultaneous estimation of time-varying price risk on conditional mean price estimates. Gujarati (1995) observed ARCH-M methods are used to evaluate the determinant of prices reflected in the conditional mean and various regressors as well as factors that explain price risk as reflected in series conditional variance and the interactions between the mean and variances of food prices, that is, the price risk premium, prevailing in food market. Hence, the ARCH-M was adopted to evaluate the effect of liberalization on the price of maize and whether there were significant changes in the price of maize after the policy reform. With the assumption that errors are conditionally normally distributed, ARCH-M is estimated by maximum likelihood using iterative algorithms. Following Barrett (1997), the ARCH-M model empirical model can be written for the mean and variance estimations respectively as:

$$P_{it} = \beta_0 + \beta_1 P_{it-1} + \beta_2 BP_t + \beta_3 POS_{it} + +\beta_4 ER_t + \beta_5 S + \sum_{i=1}^{3} \varphi R_i + \beta_6 TREND + \delta h_{it}^{1/2} + \mu_{it} \quad (2)$$

Where $\mu_{it} \mid \psi t\text{-}1 \sim iid\ N(0, hit)$

$$h_{it} = \alpha_0 + \alpha_1 U_{it-1}^2 + P_{it-1} + \beta_2 BP_t + \beta_3 POS_t + \gamma_3 ER_t + \gamma_5 S + \sum_{i=1}^{3} \varphi_i R_i + \gamma_2 TREND \quad (3)$$

Where

P_{it} = Price of maize in Naira in region *i* in month *t;*

P_{it-1} = Price of Maize in Naira in region i the previous month;

BP_t = Border Parity Price in month t;

POS_t = Price of Sorghum in region *i* in month *t;*

ER_t = Real exchange rate in month t;

S = Seasonal Dummy 1 for rainy season; 0 for dry season;

R_i = Region specific dummy;

Trend = Time trend;

U = Error term.

The δ estimate reflects a risk premium with respect to the conditional standard deviation.

3. Results

3.1 Price Fluctuation in Pre-Liberalization Period (1983-1987)

Table 1 shows the pattern of maize prices for the four agro-climatic zones in the pre-liberalization period 1983-1987. The four agro-climatic zones include the Guinea Savannah (Adamawa), the Mangrove Swamp zone (Akwa Ibom), the Derived Savannah (Niger), and the Rain Forest (Osun).

Table 1. Regional prices of maize ₦/kg in the pre-liberalization period (1983-1987)

Regions	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
Adamawa	1.0748	0.50	1.76	0.4718	0.4390
Akwa-Ibom	1.1550	0.59	2.10	0.4746	0.4109
Niger	0.9712	0.58	1.48	0.3289	0.3387
Osun	0.530	0.22	1.30	0.3632	0.6853

Source: Computed from FOS Annual Price Data (Various Issues).

The result shows that the Akwa-Ibom region had the highest mean price of maize followed closely by Adamawa. Although Osun had the least mean price, it had the highest variation. The mean price in Niger is relatively lower than Akwa- Ibom and Adamawa and also had the least variability.

3.2 Price Fluctuation in the Post-Liberalization Period in the Short Term (1988-1992)

Table 2 gives the summary statistics of maize prices for the agro-climatic zones for the immediate post liberalization period between 1988-1992. As shown in the table, the mean prices increased in all the zones relative to the pre reform period. The disparities between the minimum and maximum prices for each year were wide in all the regions. Price variability was over 50percent in all the regions except Niger where it was a little lower. It indicates that maize prices increased immediately after the reform with high variability.

Table 2. Regional prices of maize ₦/kg in the post-liberalization m period (1988-1992)

Regions	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
Adamawa	2.4192	1.26	6.25	1.3061	0.5399
Akwa-ibom	4.1627	1.47	11.27	2.9695	0.7134
Niger	2.1460	1.11	5.90	1.0457	0.4873
Osun	2.4333	1.41	5.57	1.2655	0.5201

Source: Computed from FOS Annual Price Data (Various Issues).

3.3 Price Fluctuation in the Post-Liberalization Period in the Long Term (1993-2000)

Table 3 reveals the movement of maize prices for the four agro-climatic zones in the post liberalization period between 1993 and 2000. The table shows sharp increase in food prices in all the regions. The variability in food prices reduced in Osun and Akwa-Ibom but increased in Niger and Adamawa. Although Niger had about 60 percent maize price variability, it had a lower mean price relative to Adamawa and Akwa Ibom.

Table 3. Regional prices of maize ₦/kg in the post-liberalization period (1993-2000)

Regions	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
Adamawa	19.3356	3.40	38.81	11.7051	0.6054
Akwa-Ibom	21.0701	8.12	46.54	9.7859	0.4644
Niger	15.0007	3.12	39.78	9.0374	0.6025
Osun	14.2306	4.85	28.07	6.5021	0.4569

Source: Computed from FOS Annual Price Data (Various Issues).

3.4 Stationarity Test for Maize Price Series

The result in Table 4 shows the stationarity test for the maize prices using ADF statistics. The results indicate that all the variables were not stationary at levels. The values of the ADF statistics were smaller in absolute term than the critical value. This showed that the null hypothesis of non-stationary could be accepted at the 5 per cent level of significance. This implies that the mean is time dependent; it has infinite variance, tendency to stray from mean

value and integrated of order one or more. When differenced once, however, the null hypothesis of non-stationary was rejected in favour of the alternative as the values of the ADF statistics were greater in absolute term than the critical value of (-2.89) at 5 per cent level of significance. The findings here corroborate earlier findings that food commodity price series are mostly integrated of order one i.e. I (1) (Alexander & Wyeth, 1994; Ogundare, 1999; Franco, 1999; Chirwa, 2001; Oladapo, 2003).

Table 4. Result of Augmented Dickey-Fuller Unit Root Tests for Maize Prices

Variables	ADF stats
L(price of maize)	-3.0516
L(Price of Sorghum)	-3.2446
L(Lagged Price of Maize)	-3.0964
L(Border parity price)	-4.5995
L(Exchange rate)	-2.9889

3.5 Results of Arch-M Model

The results of ARCH-M model for the pre and post liberalization periods are presented in Tables 5 and 6 below.

In the mean equation, in the pre liberalization period, only three variables were significant. They are the lagged price of maize, the price of sorghum and the exchange rate. In the post liberalization period, four variables were significant and they include the lagged price of maize, the price of sorghum, border parity price and the seasonal dummy. They all have positive relationship with the price of maize except the seasonal dummy.

In the variance equation, lagged price of maize, price of sorghum, time trend, border parity price and the regional and seasonal dummies affect the volatility of maize prices. Except for seasonal dummy that was not significant and the exchange rate that was significant at 10percent, other variables were significant at not more than 5percent in the post-liberalization period as in the pre-liberalization period estimation. The effect of lagged price of maize and the seasonal dummy decreased volatility in maize prices from the pre-liberalization to the post-liberalization period. Volatility in maize prices increased with changes in the price of sorghum, border parity price and time trend between the two periods. Regional differences show that volatility increased in Adamawa and Niger relative to Osun.

Table 5. Table showing ARCH-M result for the pre liberalization period

Variables	Coefficient	Standard error	Variance	Standard error
L(lag price of maize)	0.0193*	0.0117	-0.4966**	0.2422
L(Price of sorghum)	0.1919***	0.0576	-4.8765***	1.6171
L(Exchange rate)	0.3584***	0.0691	-2.0257*	1.3863
Time trend	-0.0001	0.0002	-0.0885***	-0.0885
L(Border parity price)	-0.2141	0.2698	-28.2568***	5.7760
Seasonal dummy	-0.0006	0.0055	0.0911	0.2180
Regions				
Adamawa	-0.0047	0.0080	0.7847	0.7084
Akwa-Ibom	-0.0085	0.0078	1.7267**	0.7625
Niger	-0.0052	0.0002	1.3128**	0.5826
Constant	0.0038	0.0089	52.4694***	12.2466
ARCHM				
α_1	2.6880	3.8232	0.0557	0.0636

N= 240, Wald chi2 (10) = 714.82, Log likelihood = 436.6814 Prob > chi2 = 0.0000.

Table 6. Table showing ARCH-M result for the post liberalization period

Variables	Coefficient	Standard error	Variance	Standard error
L(lag price of maize)	0.1046***	0.0332	-3.9622***	0.3606
L(Price of sorghum)	0.1756***	0.0263	-1.3963***	1.4333
L(Exchange rate)	0.0570	0.4589	0.5811	0.6274
Time trend	-0.0001	0.0001	0.4715***	0.0068
L(Border parity price)	0.1434*	0.0814	3.3712***	1.2158
Seasonal dummy	-0.0168***	0.0055	-0.5035***	0.1136
Regions				
Adamawa	-0.0049	0.0064	1.1413***	0.1327
Akwa- Ibom	-0.0059	0.0059	0.9189***	0.1642
Niger	0.0017	0.0085	1.9982***	0.1691
Constant	0.0146*	0.0078	-17.7502***	2.5150
ARCHM				
α_1	2.2212***	0.6969	0.3595***	0.0655

N= 600, Wald chi2 (10) = 37.93, Log likelihood = 785.0758 Prob > chi2 = 0.0000.

4. Discussion

As seen in Tables 1 to 3, prices were lowest in the pre liberalization period with modest increases in the period immediately after the reform. This shows that the impact of the reform increased over time. Generally, in all the four regions, prices received a significant jump in the long term. This could be attributed to pronouncement of government policy in devaluation of currency and scrapping of marketing boards that administer prices. Maize is not a major tradable commodity in Nigeria and therefore the impact of the reform is not direct. The impact is transmitted through increase in price of inputs used in maize production such as fertilizers and agrochemicals. These inputs are mostly imported and supplemented with local production. However, the National Fertilizer Company that became moribund made the acquisition of fertilizer difficult for most farmers. The increase in the cost of these inputs as a result of high exchange rate of Naira to the Dollar increased the cost of producing maize significantly thereby discouraging its use. In addition, the incessant upward review of petroleum pump prices between the mid-nineties and year 2000 increased the cost of transportation of both inputs and output of maize resulting in higher maize prices.

Although, there was general increase in food prices, the degree of variation was high in both the pre and post liberalization periods; though they differed in the different regions. The variation was lowest in the pre liberalization period relative to the post liberalization period while the highest variability was experienced in the short term period immediately after the reform. The immediate impact of the reform had wide variation in different regions. A very high coefficient of variation of 71 per cent was recorded in Akwa-Ibom but declined to 46percent in the long term. Osun recorded the highest variability in the pre reform but had the least (45 percent) in the long term which means; it had more stable prices in the long run. In other regions, the coefficient of variation was as high as 60percent. It shows that while prices became more stable in the southern agro- climatic zones in the long run, they were high in the northern savannahs.

The lagged price of maize is significant in both the pre and post liberalization periods in determining the price of maize. There was a sharp jump in the first order autocorrelation from 0.02 in the pre liberalization period to 0.10 in the post liberalization period. This implies that the long term changes in the price of maize due to free marketing are different from periods of administrative pricing. The price of its close substitute, sorghum is also significant at the 1 percent level. The effect of the price of sorghum decreased marginally between the two periods. It shows that irrespective of policy regime, the lagged price of maize and its substitute will increase the current price of maize. The real exchange rate was significant in the pre liberalization period but was not in the post liberalization period. A plausible reason may be that farmers, due to overvalued currency which made imported farm inputs cheap use more of these inputs in the pre liberalization period; but reduced the use of imported inputs after the reform. Therefore, the effect of deregulation of the exchange rate was not significant in the post liberalization period. The

border parity price was not significant in the pre- liberalization period but significant at 10 percent and positive in the post liberalization period. This implies that maize will be traded if the border price is favourable as it could be part of unrecorded cross border trade. The price of maize decreases in the rainy season which agrees with a priori expectation. Due to the influx of maize output during this season, prices fall relative to the dry season.

The effect of border parity price was highly significant in increasing the volatility of maize prices followed by the price of sorghum. This implies that it is possible that much of unrecorded informal cross border trade affects the volatility in the price of maize. This increased with the policy reform. The lagged price of maize and seasonal changes reduce volatility in the price of maize between the pre and post liberalization periods.

5. Conclusion

The empirical evidence reveals spatial and temporal differences in maize price before and after the policy reform. The mean prices and variability were generally lower in the pre- liberalization period. The results show significant increase in the mean prices after the reform which increased variability. There were significant regional differences and in the long run, the variability in the south was lower than in the north. The result revealed that exchange rate is not significant in dictating maize prices in the post liberalization period. It is believed that the high cost of imported inputs discouraged its use by farmers thus reducing the impact of the deregulation of the local currency. However, the border parity price affects the price of maize thus allowing for informal cross border trade. The lagged price of maize and the seasonal dummy reduces volatility in the price of maize between the two periods. Policy makers can stabilize maize prices by disseminating information on price movements and keeping stock of maize for the dry season. Present efforts to provide inputs at subsidized rates and effectively distribute them to farmers should be strengthened and effectively monitored to ensure access and increase output so as to increase maize stock and even seasonal supplies.

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Economic Analysis of Processing of Guava (*Psydium guajava* L.) in Uttar Pradesh State of India

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Abstract

A study was conducted to analyse the economics of guava processing with the objectives of estimating benefit cost ratio, capacity utilization, cost of processing, price spread, marketing efficiency and constraints faced by guava processors in processing of guava in Allahabad district of Uttar Pradesh (U.P.) India. The processing of fresh guava was undertaken only by 10 units (processing firms) in the study area, so all the 10 units were evaluated for the present study. The processing units included cottage scale (03), small scale (05) and large scale (02). The study revealed that returns per rupee invested in cottage scale units, was calculated as 2.74 in making jelly and 2.99 in jam; for small scale units, it was 2.28 in jelly, 2.43 in jam and 1.89 in toffee; and for large scale units, the returns per rupee invested were 2.52 in the manufacture of jelly, 2.82 in jam and 2.01in toffee. As far as capacity utilization is concerned, the large scale units utilized 92.16% of installed capacity followed by cottage scale units with 86.73% and small scale with 79.89% capacity utilization, respectively. The cost of processing per quintal of guava was found to be Rs.176.60 (cottage scale), Rs.195.58 (small scale) and Rs.222.90 (large scale). Major problems faced by processors were non-availability of skilled labours, lack of capital, setting of guava products and degree of competition, etc. It could be concluded from the study that guava processing was an economically viable entrepreneurial activity and provided ample opportunities for employment generation particularly for the local youth, and also earning much required foreign exchange. This could be possible provided the government functionaries and concerned agencies take steps towards strengthening infrastructural support for a better marketing mechanism to be in place, and introduce export promotion schemes to boost the processing industry, given its competitive advantage. Efforts need to be made to encourage local unemployed youth to opt for guava processing as their livelihood source, by providing training and financial support to them.

Keywords: Guava processing, capacity utilization, price spread, marketing efficiency, benefit cost ratio

1. Introduction

Food processing industries are of enormous significance for the development of Indian economy, because of the vital linkages and synergies that it promote between the two pillars of the economy, namely; industry and agriculture. India is one of largest producers of raw materials for food processing industry. The industry itself is under developed in India as till now only less than 2% of fruits and vegetable production is processed compared with 30% in Thailand, 70% in Brazil, 78% in Philippines and 80% in Malaysia. The total wastage in all food sectors is as high as Rs. 50, 000 crores. Even if half of the wastage could be prevented, we would have enough calories to bring the nutritional status of our poor to above subsistence level. In this scenario, food processing becomes critical. This would move up surpluses at the farm level and ensure fair prices for the producers.

It is estimated that the growth potential of this sector is enormous, as it can bring immense benefits to the economy. It also has the potential to raise the agricultural yield, augment productivity, create employment and raise the standard of living of very large number of people throughout the country, especially in the rural areas. The food processing industry has been identified as a thrust area for development.

Production of fruits and vegetables in India is about 70 million tons per year. It is estimated that nearly 35 to 40 per cent of the produce is wasted during picking, harvesting, packing, transportation, storage, marketing and consumption. Fruits and vegetables can earn 20 to 30 times foreign exchange per unit due to higher yield and better value than cereals in the international market. The development of fruits and vegetables based enterprises is gradually moving out of its rural confines into urban areas and from traditional agricultural enterprises to the corporate sector. This trend has led to the adoption of improved technology, greater commercialization and professionalism in the management of production and marketing. A number of export oriented units have potential for processing as its share in world trade foods and vegetables is lower than one per cent. The post-harvest handling, processing and management of the produce are necessary for avoiding losses. Value addition increases income and profit of the producers as well as entrepreneurs. The investment in the processing industry for producing processed agricultural products is remunerative. Therefore, keeping these facts in view, the present study; confined only to processing of Jam, Jelly and toffee, was designed with the following specific objectives:

1. To study the economics of guava processing.
2. To identify the problems faced by guava processors in processing and marketing of guava and its products, and suggest suitable remedial measures.

2. Research Methodology

The study was conducted in district Allahabad within the province of U.P., because this district has the reputation of growing best quality guava in the country. A complete list of all processing units was collected from Ministry of Food Processing Department, Lucknow. The study was conducted only for those processing units which were having FPO license. As per the FPO record there were 21 processing units in Allahabad district. Out of these 21 processing units, processing of fresh guava was undertaken only by 10 units. Therefore, all the 10 processing units were studied for the present study. The processing units were classified into three categories on the basis of the list obtained from the Ministry of Food Processing Department, Lucknow, i.e., cottage scale (03), small scale (05) and large scale (02). To get the desired information the data for the present study were collected from the selected processing units through personal interviews with the help of specially developed schedules. Simple statistical/ economic tools, as given below, were used for the estimation and interpretation of results.

2.1 Producer's Share in Consumer's Rupee

$$Ps = \frac{\text{Consumer's price - (marketing charges + middle men's margin)}}{\text{Consumer's price}} X100$$

$$Ps = \frac{\text{Net amount received by producer}}{\text{Consumer's price}} X100$$

2.2 Marketing Cost

It may be expressed as:

$$C = C_F + Cm_i + Cm_2 + Cm_3 + \ldots\ldots\ldots + C_{mn}$$

Where, C = Total marketing cost of guava;

C_F = Charges borne by the producer from the time the produce leaves the farm, till guava is sold to ultimate consumer; and Cm_i = cost incurred by the i^{th} middlemen in the process of buying and selling the product.

2.3 Marketing Margin of Middlemen

This may be expressed by two ways

a. Absolute margin of i^{th} middlemen

$$[Ami] = P_Ri - [P_{pi} + C_{mi}]$$

b. Percentage margin of i^{th} middlemen (P_{mi})

$$Pmi = \frac{PRi - (Ppi + Cmi)}{PRi} X100$$

Where, P_{Ri} = total value of receipts per unit (Sale price);

P_{pi} = purchase value of goods per unit (purchase price);

C_{mi} = costs incurred on marketing per unit.

2.4 Marketing Efficiency

The following method was used to calculate the marketing efficiency:

$$\text{Acharya's Method (MME)} = \frac{\text{Net price received by producer}}{\text{Total marketing cost} + \text{total margin of intermediaries}}$$

$$\text{MME} = \frac{-\text{FP}}{\text{MC} + \text{MM}}$$

Where, MME = Modified measure of marketing efficiency;

MC = Total marketing cost;

MM = Net marketing margin;

FP = Price received by the grower;

RP = Price paid by the consumer.

2.5 Annual Capacity Utilization

$$\text{Annual capacity utilization} = \frac{\text{Total quantity of Guava processed}}{\text{Total installed capacity}} \text{X100}$$

2.6 Benefit Cost Ratio

It is the ratio of gross income and total cost of processing.

$$\text{Benefit cost ratio} = \frac{\text{Gross income}}{\text{Total cost}}$$

2.7 Fixed and Variable Cost

The fixed and variable costs have been worked out only for three months. The processing units of the study area would process guava only for 2.5 to 3 months (November to January) during a given year. This is because the processors preferred to process only the winter season crop and during the remaining period they processed other fruits.

3. Results and Discussion

Marketing channels of processed guava products followed by processing units:

(a) Large scale

(I) Producer - Preharvest contractor - Commission agent - Processing Unit -Commission & Forwarding agent - Wholesaler - Retailer - Consumer.

(II) Producer - Commission agent - Processing unit - Commission & Forwarding agent - Wholesaler - Retailer - Consumer.

(b) Small scale

(III) Producer - Pre-harvest contractor - Commission agent - Processing unit - Commission & forwarding agent - Wholesaler - Retailer - Consumer.

(IV) Producer - Commission agent - Processing unit - Commission & forwarding agent - Wholesaler - Retailer - Consumer.

(c) Cottage scale

(V) Producer - Pre-harvest contractor - Commissions agent - Processing unit - Commission & forwarding agent - Consumers.

(VI) Producer - Commissions agent - Processing unit - Commission & forwarding agent - Consumer.

3.1 Marketing Channels of Processed Guava Products

It can be observed from the above mentioned channels of processed guava products that the processors proved to be very important intermediaries in all the channels. All the possessors of the study area generally purchased the fresh guava from wholesaler /commission agent of Mundera market (mandi). In spite of many efforts by Government Center for Fruit Preservation and Training the processing of guava was not well adopted by the processors. Only about 3 percent of total fresh guava was being processed by the guava processors in Allahabad. As far as marketing of processed products is concerned, the processors sold the processed products in local market

as well as in different distant markets. The processor moved the products through commission and forwarding agents dealing with processed products in different distant markets.

3.2 Marketing Costs Incurred in the Marketing of Raw and Processed Guava Products

3.2.1 Cost Incurred by Growers

The Table 1 indicated that the total marketing cost incurred by the producer was Rs. 148.90 per quintal in channel II, IV, and VI, which included, picking and assembling charges, grading, transportation, loading/ unloading and commission charges to commission agent, i.e., Rupees (Rs.) 13.67, 5.89, 62.31, 32.22, 5.45 and 29.36 per quintal, respectively.

Table 1. Marketing cost incurred by different intermediaries involved in the marketing of raw and processed guava products (Value in Rs. /Qtl.)

S. No.	Particulars	Channel I	Channel II	Channel III	Channel IV	Channel V	Channel VI
1	Charges paid by producer/pre-harvest contractors:						
A	Picking and assembling charges	12.34	13.67	12.34	13.67	12.34	13.67
B	Grading	5.71	5.89	5.71	5.89	5.71	5.89
C	Packing and packaging material	55.26	62.31	55.26	62.31	55.26	62.31
D	Transportation charges	31.75	32.22	31.75	32.22	31.75	32.22
E	Loading/ unloading (Palledari) charges	5.09	5.45	5.09	5.45	5.09	5.45
F	Commission charges	27.88	29.36	27.88	29.36	27.88	29.36
	Total charges	138.03	148.90	138.03	148.90	138.03	148.90
2	Charges paid by the commission agents:						
A	Market (Mandi) tax @ 2%	10.20	10.20	10.20	10.20	10.20	10.20
B	Development charges @ 0.50%	2.55	2.55	2.55	2.55	2.55	2.55
C	Miscellaneous charges	1.00	1.00	1.00	1.00	1.00	1.00
	Total charges	13.75	13.75	13.75	13.75	13.75	13.75
3	Charges paid by processors:						
A	Commission to commission and forwarding agent (15-22%)	1836.69	836.69	2142.92	2142.92	2264.76	2264.76
	Total charges	1836.69	1836.69	2142.92	2142.92	2264.76	2264.76
4	Charges paid by commission and forwarding agents:						
a	Transportation charges	128.30	128.30	55.20	55.20	34.00	34.00
b	Miscellaneous charges	12.30	12.30	10.03	10.03	6.09	6.09
	total charges	140.60	140.60	65.23	65.23	40.09	40.09
5	Charges paid by wholesalers:						
a	Risk and maintenance	131.67	131.67	131.67	131.67	-	-
b	Miscellaneous charges	85.03	85.03	85.03	85.03	-	-
	Total charges	216.70	216.70	216.70	216.70	-	-
6	Charges paid by retailer						
a	Transportation charges	45.00	45.00	45.00	45.00	-	-
b	Risk and maintenance	105.22	105.22	105.22	105.22	-	-
	Total charges	150.22	150.22	150.22	150.22	-	-

3.2.2 Cost Incurred by Pre-Harvest Contractors

It was observed from the Table 1 that the total cost incurred by the pre-harvest contractors was Rs. 138.03 in channel I, III and V which includes picking and assembling charges, grading, packing and packaging material, transportation, loading/ unloading and commission charges to commission agent (at mandi), i.e., Rs. 12.34, 5.71, 55.26, 31.75, 5.09 and 27.88 per quintal, respectively.

3.2.3 Cost Incurred by Commission Agent

The Table 1 revealed that the charges paid by the commission agent in all the channels were Rs. 13.75 per quintal of guava which includes mandi tax @ 2%, development charges @ 0.50%, and miscellaneous charges at Re. 1.00 per quintal, i.e., Rs. 10.20, 2.55 and Re. 1.00 per quintal of guava, in the three channels, respectively.

3.2.4 Charges Paid by the Processor

The marketing cost incurred by the processors varied widely among the processing units, viz., large scale, small scale and cottage scale. The marketing cost incurred by the processors was basically commission based, which they were providing to commission and forwarding agents for marketing the processed guava products. The rate of commission provided by the large scale units was 15% of gross income, small scale at 18.50% and cottage scale at 22% of the gross income, i.e., Rs. 1836.69 per quintal in channel I and II, Rs., 2142.92 per quintal in channel III and IV, and Rs. 2264.76 per quintal in channel V and IV, respectively.

3.2.5 Cost Incurred by the Commission and Forwarding Agent

It was observed from the study that the commission agent played an important role in the marketing of processed guava products as they reached every nook and corner, where the firm itself could not, as it needed a lot of time and manpower. The total cost incurred by the commission and forwarding agent was found to be Rs. 140.60 per quintal in channel I and II, Rs. 65.23 per quintal in channel III and IV and Rs. 40.09 per quintal in channel V and VI, which included transportation charges at Rs. 128.30 per quintal and miscellaneous charges at Rs. 12.30 per quintal in channel I and II. In channel III and IV, transportation charges and miscellaneous charges were Rs. 55.20 and Rs. 10.03 per quintal, respectively while as in channel IV and VI, transportation and miscellaneous charges came to Rs. 34.00 and Rs. 6.09 per quintal, respectively.

3.2.6 Cost Incurred by the Wholesalers

The total marketing cost incurred by the wholesaler was found to be Rs. 216.70 per quintal in channel I, II, III and IV which included risk and maintenance cost at Rs. 131.67 per quintal and miscellaneous charges at Rs. 85.03 per quintal. In channel V and VI the wholesalers were not involved, because the cottage scale units were selling the processed guava products through commission agents as door to door services.

3.2.7 Cost Incurred by Retailers

The total cost incurred by the retailer was found at Rs. 150.22 per quintal in channel I, II, III and IV which included transportation charges at Rs. 45.00 per quintal and risk and maintenance cost as Rs. 105.22 per quintal.

3.2.8 Capacity Utilization

It can be observed from Table 2 that average capacity of 3 months was highest in large scale (2312.82 quintals), followed by small scale (150.62 quintals) and cottage scale (51.25 quintals). As far as the capacity utilization is concerned, the large scale units utilized 92.16% of their installed capacity followed by cottage scale with 86.73% and small scale units with 79.89%, making aggregate percentage of 91.31%.

Table 2. Processing of guava bye processing units of the study area

S. No.	Category of processing unit	Average capacity of 3 months (Qtl.)	Average quantity of guava processed(Qtl.)	Capacity Utilization (%)
1.	Cottage scale	51.25	44.45	86.73
2.	Small scale	150.62	120.33	79.89
3.	Large scale	2312.82	2131.52	92.16
4.	Aggregate	838.23	765.43	91.31

* The average annual capacity of cottage scale units in the study area was 20.50 M T.

* The average annual capacity of small scale units in the study area was 60.25 M T.

* The average annual capacity of large scale unit in the study area was 925.13 M T.

3.3 Cost of Processing

3.3.1 Cottage Scale Processing Units

To work out the cost of guava processing, various fixed and variable costs were calculated for each category of units surveyed and to arrive at the cost of processing per quintal weighted average were considered.

Table 3 reveals that for the processing of guava, total fixed cost incurred by cottage scale processors accounted to only Rs. 4640.96 (i.e., 59.12 percent of the total cost). This included rental value of land, depreciation on building, depreciation on equipments, wages to permanent labours, and interest on fixed capital, i.e., 4.45, 2.48, 1.04, 39.81, and 11.33 per cent, respectively, of the total cost. Whereas the total variable cost incurred was Rs.3208.81 which included electricity charges, administrative charges, repair and maintenance, license fee to FPO, wages to casual labours, commission to commission agents (in the market), other charges (advertising and telephone), and interest on working capital, i.e., 1.83, 1.91, 1.11, 0.08, 10.19, 13.99, 8.04, and 3.72 per cent, respectively, of the total cost. Thus, the total fixed plus variable cost for cottage scale units was Rs. 7849.77. Average quantity of guava processed by cottage scale units was 44.45 quintals. Cost of processing per quintal of guava was estimated to be Rs. 176.60.

Table 3. Cost of processing of guava by cottage, small and large scale units (Value in rupees)

S. No.	Items	Cottage Unit		Small Unit		Large Unit	
		Amount	%	Amount	%	Amount	%
A.	**Fixed Cost:**						
I	Rental value of land	349.00	(4.45)	499.99	(2.12)	1687.50	(0.36)
II	Depreciation on Building @ 5%	195.05	(2.48)	900.89	(3.83)	1604.17	(0.34)
III	Depreciation on Equipments @ 5%	82.01	(1.04)	1835.48	(7.80)	10937.50	(2.30)
IV	Depreciation on owned transportation @ 5%	-	-	658.82	(2.80)	1573.76	(0.33)
V	Insurance Premium	-	-	450.00	(1.91)	5499.99	(1.16)
VI	Wages to permanent labours	3124.99	(39.81)	3525.17	(14.98)	126249.99	(26.57)
VII	Interest on fixed capital @ 10%	888.92	(11.33)	9018.50	(38.32)	112865.11	(23.75)
Total fixed cost		4640.96	(59.12)	16888.85	(71.76)	260418.02	(54.81)
B.	**Variable cost**						
I	Electricity charges	143.90	(1.83)	505.33	(2.15)	15000.00	(3.16)
II	Administrative charges	150.00	(1.91)	324.99	(1.38)	3499.99	(0.74)
III	Repair and Maintenance	87.51	(1.11)	637.56	(2.71)	23749.99	(5.00)
IV	License fee	6.44	(0.08)	18.81	(0.08)	1291.75	(0.27)
V	Wages to casual labours	800.01	(10.19)	707.50	(3.01)	87499.99	(18.41)
VI	Commission to commission agent (In Mandi)	1097.91	(13.99	2972.15	(12.62)	52648.54	(11.08)
VII	Other charges (Adv. Tel)	631.33	(8.04)	874.99	(3.72)	11499.99	(2.42)
VIII	Working cost.	2917.1	-	6041.33	-	195190.25	-
	Interest on working capital @ 10%.	291.71	(3.72)	604.13	(2.57)	19519.02	(4.11)
	Total working cost	3208.81	(40.88)	6645.46	(28.24)	214709.27	(45.19)
	Total cost (FC + VC)	7849.77	(100)	23534.31	(100)	475127.29	(100)
	Average guava processed (Qtl.)	44.45		120.33		2131.52	
	Cost of processing/q. of guava	176.60		195.58		222.90	

3.3.2 Small Scale Processing Units

Table 3 indicated that the total cost incurred by small scale processing units was Rs. 23534.31, which included fixed cost of Rs. 16888.85 and working cost of Rs. 6645.46.The fixed cost included rental value of land, depreciation on building, equipments and owned transport, insurance premium, wages to permanent labourers and interest on fixed capital, i.e., 2.12, 3.83, 7.80, 2.80, 1.91, 14.98 and 38.32 per cent, respectively. The total fixed cost was 71.76% of the total cost. The working cost included electricity charges, administrative charges, repair and maintenance, license fee to FPO, wages to casual labourers, commission to commission agent (in mandi), other charges (advertising and telephone) and interest on working capital, i.e., 2.15, 1.38, 2.71, 0.08, 3.01, 12.62, 3.72, and 2.57 per cent respectively. The total working cost was 28.24% of the total cost. The average quantity of guava processed by small scale was 120.33 quintals. Thus, the cost of processing per quintal of guava incurred by small scale processing units was Rs. 195.58.

3.3.3 Large Scale Processing Unit

Table 3 revealed that for processing of guava, total fixed cost incurred by large scale processors was estimated as Rs. 260418.02 (i.e., 54.81 per cent of the total cost), which included rented value of land, depreciation on building, equipments and owned transportation, insurance premium, wages to permanent labourers and interest on working capital, i.e., 0.36, 0.34, 2.30, 0.33, 1.16, 26.57 and 23.75per cent of the total cost, respectively. Whereas, the total variable cost incurred was Rs. 214709.27 which included electricity charges, administrative charges, license fee to FPO, wages to casual labourers, commission to commission agent (in the market). Other charges (advertising and telephone) and interest on working capital accounted for 3.16, 0.74, 5.00, 0.27, 18.41, 11.08, 2.42, and 4.11 per cent, respectively of the total cost. Thus, the total fixed and variable cost accounted for the large scale units was Rs. 475127.29. The average quantity of guava processed by large scale units was 2131.52 quintals and the cost of processing per quintal of guava was estimated as Rs. 222.90.

3.4 Costs and Returns from Processing of Jam and Jelly by Cottage Scale

3.4.1 Costs and Margins in the Processing of Jelly

The Table 4 revealed that the costs incurred by cottage scale units in the processing of one quintal of guava for making jelly were Rs.3967.83, which included cost of processing, cost of raw material, transportation charges, fuel charges, loss during processing, ingredient cost, packing and labeling, miscellaneous charges, and interest on working capital, i.e., 4.45, 12.85, 0.50, 2.15, 0.25, 31.76, 1.31, 37.38, 0.25 and 9.09 per cent, of the total cost, respectively. The highest share (37.38% of total cost) was accounted for the packing and labeling charges. From one quintal of guava, 1.13 quintals of jelly were recovered, after value addition the net margin received by the processors in selling 1.13 quintals was Rs.6908.42; per kg net margin was Rs. 61.14.

3.4.2 Costs and Margins in the Processing of Jam

It can be observed from the Table 4 that the cost incurred by cottage scale units in the processing of one quintal of guava for making Jam was Rs. 3249.53. The Jam is the by-product of Jelly, after making the jelly, remaining pulp was used for making the jam. Thus, on processing one quintal of raw guava, 1.13 quintal of jelly and 1.05 quintal of jam could be recovered. The cost which involves in making jam includes, cost of processing fuel charges, loss during processing, ingredient cost, packing and labeling charges, miscellaneous charges and interest on working capital were 5.43, 2.63, 0.22, 38.77, 1.23, 42.41, 0.22, and 9.09% of the total cost, respectively. The highest share (42.41% of total cost) was accounted for packing and labeling charges, after value addition the net margin received by the processor in selling 1.05 quintals were Rs. 6462.97 and the per kg net margin were Rs. 61.55.

3.5 Costs and Margins in the Processing of Jelly, Jam and Toffee by Small Scale Units

3.5.1 Costs and Margins in the Processing of Jelly

The Table 5 reveals that the cost incurred by small scale units in the processing of one quintal of guava for making jelly was Rs. 4106.66, which included cost of processing, cost of raw material, transportation charges, fuel charges, loss during processing, cost of ingredients (Sugar, citric acid) packing and labeling charges, miscellaneous charges and interest on working capital, i.e., 4.76, 12.42, 0.59, 2.11, 0.28, 30.68, 1.27, 38.50, 0.29 and 9.09 per cent of the total cost, respectively. On processing one quintal of raw guava 1.15 quintal of jelly were recovered. After value addition the net margin received by the processor in selling 1.15 quintal of jelly was Rs. 5237.09, whereas per kg net margin was Rs. 45.54.

Table 4. Costs and margins in processing of Jam and Jelly by cottage units

S. No.	Particulars	Jelly		Jam	
		Amount (Rs./Qtl.)	%	Amount (Rs./Qtl.)	%
2	Cost of processing	176.60	4.45	176.60	5.43
3	Cost of raw material	510.00	12.85	-	
4	Transportation charges	20.00	0.50	-	
5	Fuel charges/power input	85.40	2.15	85.40	2.63
6	Loss during processing	10.00	0.25	7.00	0.22
	Ingredient cost				
7	(a) Sugar @ 70 kg/q.	1260.00	31.76	1260.00	38.77
	(b) Citric acid @ 200, 153 gm/q.	52.00	1.31	40.00	1.23
8	Packing and labelling charges	1483.12	37.38	1378.12	42.41
9	Miscellaneous charges	10.00	0.25	7.00	0.22
10	Interest on working capital @ 10%	360.71	9.09	295.41	9.09
	Total working cost	3967.83	100	3249.53	100
11	Total quantity ready from one quintal Of guava	1.13		1.05	
12	Total returns from one quintal of processed guava	10876.25		9712.50	
13	Net margin from one quintal of processed guava (excluding marketing cost)	6908.42		6462.97	
14	Cost/kg of processed products	35.11		30.95	
15	Selling price/kg	96.25		92.50	
16	Net margins/kg (excluding marketing cost)	61.14		61.55	

Quantity of guava processed to jam & jelly - 44.45 Qtl.

Table 5. Costs and margins in the processing of jelly, Jam and toffee by small scale units

S. No.	Particulars	Jelly		Jam		Toffee	
		Amount (Rs. Qtl.)	%	Amount (Rs. Qtl.)	%	Amount (Rs. Qtl.)	%
1	Cost of processing	195.58	(4.76)	195.58	(5.79)	195.58	(2.49)
2	Cost of raw material	510.00	(12.42)	-		510.00	(6.48)
3	Transportation charges	24.22	(0.59)	-		24.22	(0.31)
4	Fuel charges/powder input	86.78	(2.11)	86.78	(2.57)	86.78	(1.10)
5	Loss during processing	11.50	(0.28)	9.50	(0.28)	11.50	(0.15)
	Ingredient cost						
6	(a) Sugar @ 70 kg/q.	1260.00	(30.68)	1260.00	(37.33)	1260.00	(16.02)
	(b) Citric acid @ 200, 190 gm/q.	52.00	(1.27)	52.00	(1.54)	49.40	(0.63)
	(c) Glucose @ 10 kg/q.	-		-		800.00	(10.17)
	(d) Skimmed milk (@ 15 kg/q.	-		-		900.00	(11.44)
	(e) Butter @ 6 kg/q.	-		-		600.00	(7.63)
7	Packing and labelling charges	1581.25	(38.50)	1457.50	(43.18)	2700.00	(34.33)
8	Miscellaneous charges	12.00	(0.29)	7.50	(0.22)	12.00	(0.15)
9	Interest on working capital @ 10%	373.33	(9.09)	306.89	(9.09)	714.95	(9.09)
10	Total working cost	4106.66	(100)	3375.75	(100)	7864.43	(100)
11	Total quantity ready from one quintal Of guava	1.15		1.06		1.35	
12	Total returns from one quintal of processed guava	9343.75		8215.00		14850.00	
13	Net margin from one quintal of processed guava (excluding marketing cost)	5237.09		4839.25		6985.57	
14	Cost/kg of processed products	35.70		31.85		58.25	
15	Selling price/kg	81.25		77.50		110.00	
16	Net margins/kg (excluding marketing cost)	45.54		45.65		51.75	

Quantity of guava processed into jelly & toffee [Jelly 87.12 Qtl.] [Toffee 33.21 Qtl.].

3.5.2 Costs and Margins in the Processing of Jam

It can be observed from the Table 5 that the cost incurred by small scale units in the processing of one quintal of raw guava for making Jam was Rs. 3375.75, which includes cost of processing, fuel charges, loss during processing, ingredient cost (sugar, citric acid), packing and labeling charges, miscellaneous charge and interest on working capital, i.e., 5.79, 2.57, 0.28, 37.33, 1.54., 43.18, 0.22, and 9.09 per cent of the total cost. On processing one quintal of raw guava by small scale units 1.15 quintal of jelly and 1.06 quintal of Jam were recovered. After value addition the net margin received by the processors in selling 1.06 quintals of jam was Rs. 4839.25 and per kg net margin was Rs. 45.65.

3.5.3 Costs and Margins in the Processing of Toffee

It can also be observed from the Table 5 that the cost incurred by the small scale units in the processing of one quintal of guava for making toffee was Rs. 7864.43, which included cost of processing, cost of raw material, transportation charges, fuel charges, loss during processing, cost of ingredients (sugar, citric acid, Glucose, skimmed milk, butter), packaging and labeling charges, miscellaneous charges and interest on working capital i.e., 2.49,6.48, 0.31, 1.10, 0.15, 16.02, 0.63, 10.17, 11.44, 7.63, 34.33, 0.15 and 0.09% of the total cost. On processing one quintal of raw guava 1.35 quintals of toffee were recovered. The net margin received by the processor in selling 1.35 quintals of toffee was Rs. 6985.57 and per kg net margin was Rs. 51.75.

3.6 Costs and Margins in the Processing of Jelly, Jam and Toffee by Large Scale Units

3.6.1 Costs and Margins in the Processing of Jelly

The Table 6 revealed that the cost incurred by large scale units in the processing of one quintal of guava for making jelly was Rs.4070.38, which includes, cost of processing, cost of raw material, transportation charges, fuel charges, loss during processing, cost of ingredients (sugar, citric acid), packing and labeling charges, miscellaneous charges and interest on working capital, i.e., 5.48, 12.53, 0.32, 2.41, 0.22, 33.17, 1.28, 35.01, 0.49 and 9.10 per cent of the total cost, respectively. On processing one quintal of raw guava by large scale 1.14 quintals of jelly were recovered, and after value addition the net margin received by the processors in selling 1.14 quintals of jelly was Rs. 6189.62 and the per kilogram net margin was Rs.54.29.

Table 6. Costs and Margins in the Processing of Jelly, Jam and Toffee by Large Scale Units

S. No.	Items	Jelly		Jam		Toffee	
		Amount (Rs./Qtl.)	%	Amount (Rs./Qtl.)	%	Amount (Rs./Qtl.)	%
1	Cost of processing	222.90	(5.48)	222.90	(6.51)	222.90	(2.67)
2	Cost of raw material	510.00	(12.53)	-		510.00	(6.11)
3	Transportation charges	13.25	(0.32)	-		13.25	(0.16)
4	Fuel charges/power input	98.20	(2.41)	98.20	(2.87)	98.20	(1.18)
5	Loss during processing	9.00	(0.22)	9.00	(0.26)	8.50	(0.10)
6	Ingredient cost						
	(a) Sugar @ 70-75 kg/q.	1350.00	(33.17)	1350.00	(39.40)	1260.00	(15.10)
	(b) Citric acid @ 200, gm/q.	52.00	(1.28)	52.00	(1.52)	52.00	(0.62)
	(c) Glucose @12kg/ q.	-		-		960.00	(11.50)
	(d) Skimmed milk @ 15 kg/qt	-		-		900.00	(10.79)
	(e) Butter @ 6 kg/q.	-		-		600.00	(7.19)
7	Packing and labelling charges	1425.00	(35.01)	1362.50	(39.77)	2940.00	(35.23)
8	Miscellaneous charges	20.00	(0.49)	20.00	(0.58)	21.00	(0.25)
9	Interest on working capital @ 10%	370.03	(9.09)	311.46	(9.09)	758.58	(9.09)
	Total working cost	4070.38	(100)	3426.06	(100)	8344.43	(100)
10	Total quantity ready from one quintal Of guava	1.14		1.09		1.40	
11	Total returns from one quintal of processed guava	10260.00		9673.75		16800.00	
12	Net margin from one quintal of processed guava (excluding marketing cost)	6189.62		6247.69		8455.57	
13	Cost/kg of processed products	35.70		31.43		59.60	
14	Selling price/kg	90.00		88.75		120.00	
15	Net margins/kg (excluding marketing cost)	54.29		57.32		60.40	

Quantity of guava processed into jelly & toffee [Jelly 1881.30 Qtl.], [Toffee 250.22 Qtl.].

3.6.2 Cost and Margins in the processing of Jam

Table 6 indicated that the cost incurred by the large scale units in the processing of one quintal of guava for making Jam was Rs. 3426.06, which included cost of processing, fuel charges, loss during processing, ingredient cost, (sugar, citric acid,) packing and labeling charges, miscellaneous charges and interest on working capital i.e., 6.51, 2.87, 0.26, 39.40, 1.52, 39.77, 0.58 and 9.09 per cent, respectively, of the total cost. On processing of one quintal of raw guava, 1.14 quintals of jelly and 1.09 quintals of jam were recovered. After value addition the net margin received by the large scale units in selling 1.09 quintals of jam was Rs. 6247.69, whereas the per kg net margin was Rs. 57.32.

3.6.3 Costs and Margins in the Processing of Toffee

It was observed from the Table 6 that the cost incurred by large scale units in the processing of one quintal of guava for making toffee was Rs. 8344.43 which included cost of processing, cost of raw material, transportation charges, fuel charges, loss during processing, cost of ingredients (sugar, citric acid, glucose, skimmed milk, butter), packing and labeling charges, miscellaneous charges and interest on working capital, i.e., 2.67, 6.11, 0.16, 1.18, 0.10, 15.10, 0.62, 11.50, 10.79, 7.19, 35.23, 0.25, and 9.09 per cent, respectively of the total cost. On processing of one quintal of raw guava 1.40 quintals were recorded by the large scale units. The net margin received by the processors in selling 1.40 quintals of toffee was Rs. 8455.57 and per kg net margin stood at Rs. 60.40.

3.6.4 Price Spread, Marketing Cost and Margins in the Marketing of Raw and Processed Guava Products

The marketing of processed products was initiated by the processors as the producers of processed products. The processors purchased the fresh guava mainly from Mundera mandi (local market in the study area) and after processing the guava fruits, they sold processed products in local market as well as in the distant markets through different middlemen. The marketing of processed product was basically commission based and the processors had to pay commission to each intermediary or they forwarded the products for some concession to make intermediaries able to earn their commissions. The main processed products of guava were jelly, jam and toffee which were manufactured by the processing units of the study area. The prices of these products hardly differed from each other, thus the analysis was performed considering the average price of these products.

In local markets the processors forwarded the guava products either through retailers or commission agents, but the cottage scale processors themselves searched out for the market of their products. They directly sold the products to consumers without the involvement of any middlemen. Commission agents were employed to capture bigger market as they sold the products by wandering door to door selling in different localities. On the other side, the retailers were able to sell the produce only to a limited number of consumers. Thus, the processors preferred to sell the produce through commission agents.

The producer's share in consumer's rupee was 0.99, 2.03, 106, 2.17, 1.71 and 3.51 per cent in channel I, II, III, V and channel VI respectively. the marketing cost incurred by the grower was 0.84, 0.89 and 1.45 in channel II, IV and VI, respectively, while in channel I, III and V the growers did not play any role in the marketing of produce as they directly sold their standing crop to pre-harvest contractors; who takes care of the crop and markets the produce. The pre-harvest contractor received a net margin of 1.10, 1.18, and 1.90% of the consumer's rupee, in channel I, III, and V, respectively. The marketing cost incurred by the pre-harvest contractors was found at 0.77, 0.83, and 1.34% of the consumer's rupee in channel I, III and V, respectively.

The Table 7 indicated that processors' share was higher as compared to that of all the intermediaries involved in the marketing of processed guava products, because the processors added value to the product. After the value addition the net amount received by the processor was found (average of Jam, Jelly and Toffee) as Rs. 5127.60 in channel I and II, i.e., 28.90% of the consumer's rupee. The net amount received by the processor in channel III and IV was found to be Rs. 4324.80 which was 25.97% of the consumer's rupee. In channels V & VI the processor's share was found as Rs. 4420.93, which was 42.94% of the consumer's rupee. The total marketing cost incurred by the processors was found at Rs. 7116.98 in channels I and II, i.e., 40.11% of the consumer's rupee. Marketing cost incurred by the processors in channel III and IV was found as Rs. 7258.53 i.e., 43.58% of the consumer's rupee. The cost incurred by the processors in channel V and VI was found as Rs. 5873.44 which was 57.05% of the consumer's rupee.

As mentioned earlier that the processors did sell their produce through commission agents. The commission agents would sell the produce on commission basis, the commission ranged from 15-22%.

Table 7. Price spread, marketing costs and marketing margins, channel wise for raw and processed guava (Quantity in quintals/value in rupees)

S.No.	Particulars	Channel I	Channel II	Channel III	Channel IV	Channel V	Channel VI
1	Producer's sale price.	175.81	510	175.81	510 (3.06)	175.81	510
I	Total cost incurred by producers.	-	148.90 (0.84)	-	148.90 (0.89)	-	148.90 (1.45)
II	Net amount received by producers.	175.81 (0.99)	361.1 (2.03)	175.81 (1.06)	361.1 (2.17)	175.81 (1.71)	361.1 (3.51)
2	Purchase price of Pre-harvest contractors.	175.81	-	175.81	-	175.81	-
I	Total Cost Incurred by Pre-harvest contractors.	138.03 (0.77)	-	138.03(0.83)	-	138.03 (1.34)	-
II	Selling price of Pre-harvest contractors.	510	-	510	-	510	-
III	Net Margin of Pre-harvest contractors.	196.16 (1.10)	-	196.16 (1.18)	-	196.16 (1.90)	-
3	Commission agent's commission from Producers/ PHCs	27.88 (0.16)	27.88 (0.16)	27.88 (0.17)	27.88 (0.17)	27.88 (0.27)	27.88 (0.27)
I	Commission agent's Commission from processors.	24.70 ((0.14)	24.70 (0.14)	24.70 (0.15)	24.70 (0.15)	24.70 (0.24)	24.70 (0.24)
II	Total Cost incurred by commission agents.	13.75 (0.08)	13.75 (0.08)	13.75 (0.08)	13.75 (0.08)	13.75 (0.13)	13.75 (0.13)
III	Net commission Received by commission agents.	38.83 (0.22)	38.83 (0.22)	38.83 (0.23)	38.83 (0.23)	38.83 (0.38)	38.83 (0.38)
4	Purchase price of Processors	510	510	510	510	510	510
I	Cost incurred by Processors (Avg.of Jam, Jelly & toffee)	7116.98 (40.11)	7116.98 (40.11)	7258.53 (43.58)	7258.53 (43.58)	5873.44 (57.05)	5873.44 (57.05)
II	Selling price of processors (Avg. of Jam, Jelly & toffee)	12244.58	12244.58	11583.33	11583.33	10294.37	10294.37
III	Net Margin Received by processors.	5127.60 (28.90)	5127.60 (28.90)	4324.80 (25.97)	4324.80 (25.97)	4420.93 (42.94)	4420.93 (42.94)
5	C& F (Commission and Forwarding agent)						
I	Commission received from processor (15-22%)	1836.69 (10.35)	1836.69 (10.35)	2142.92 (12.87)	2142.92 (12.87)	2264.76 (22)	2264.76 (22)
II	Cost incurred by Commission and Forwarding agent.	140.60 (0.79)	140.60 (0.79)	65.23 (0.39)	65.23 (0.39)	40.09 (0.39)	40.09 (0.39)
III	Net Amount received by Commission/ Forwarding agent	1696.09 (9.56)	1696.09 (9.56)	2077.69 (12.47)	2077.69 (12.47)	2224.67 (21.61)	2224.67 (21.61)
6	Purchase price of wholesalers.	12244.58	12244.58	11583.33	11583.33	-	-
I	Cost incurred by wholesalers.	216.70 (1.22)	216.70 (1.22)	216.70 (1.30)	216.70 (1.30)	-	-
II	Selling price of wholesalers.	14759.58	14759.58	13847.92	13847.92	-	-
III	Net margin received by wholesalers.	2298.30 (12.95)	2298.30 (12.95)	2047.89 (12.30)	2047.89 (12.3)	-	-
7	Purchase price of retailers.	14759.58	14759.58	13847.92	13847.92	-	-
I	Cost incurred by retailers.	150.22 (0.85)	150.22 (0.85)	150.22 (0.90)	150.22 (0.90)	-	-
II	Selling price of retailers.	17743.33	17743.33	16654.17	16654.17	-	-
III	Net margin received by retailers.	2833.53 (15.97)	2833.53 (15.97)	2656.03 (15.95)	2656.03 (15.95)	-	-
8	Purchase price of consumers.	17743.33	17743.33	16654.17	16654.17	10294.37	10294.37

*commission to C & F @22 %(cottage scale). (Figures in parentheses indicate percentage to total).

* commission to C & F @18.50 %(small scale).

* commission to C & F @15 %(large scale).

The net share received by the commission and forwarding agent was found as Rs. 1696.09 in channels I and II, i.e., 9.56% of the consumer's rupee. The net amount received by commission and forwarding agent in channel III and IV was Rs. 2077.69, i.e., 12.47% of the consumer's rupee. In channels V and VI the net share of the commission and forwarding agent was found to be Rs. 2224.67 which is 21.61% of the consumer's rupee. The cost incurred by the commission and forwarding agent was found as Rs. 140.60 in channel I and II, Rs. 65.23 in channel III and IV and Rs. 40.09 in channel V and IV, which was estimated as 0.79% in channel I and II, 0.39% in channel III , IV, V and VI of the consumer's rupee. The Table 7 also indicated that wholesaler received a net margin of Rs. 2298.30 in channels I and II, i.e., 12.95% of the consumer's rupee and Rs. 2047.89 in channels III and IV which was 12.30% of the consumer's rupee. The cost incurred by the wholesaler was found as Rs. 216.70 in channels I and II, i.e., 1.22% of the consumer's rupee and Rs. 216.70 in channel III and IV which was 1.30% of the consumer's rupee. In channels V and VI, the wholesaler did not exist.

It was also observed from the Table 5.30 that net amount received by the retailers was Rs. 2833.53 in channels I and II, which was 15.97% of the consumer's rupee and the net amount received by the retailer in channels III and IV was Rs. 2656.03, i.e., 15.95% of the consumer's rupee. The total cost incurred by the retailer was found at Rs. 150.22 in channels I, II, III and IV, which was 0.85 per cent of the consumer's rupee in channel I and II and 0.90 per cent in channels III and IV.

3.6.5 Marketing Efficiency in Different Channels of Processed Guava Products

It may be observed from the Table 8 that channel VI was found to be most efficient channel, with marketing efficiency of 0.73, followed by channel V, I, II, III and IV with marketing efficiencies of 0.71, 0.38, 0.38 0.33 and 0.33, respectively.

Table 8. Marketing efficiency of different marketing channels of processed guava products

S. No.	Particulars	Unit	Channel I	Channel II	Channel III	Channel IV	Channel V	Channel VI
1	Total marketing cost (MC)	Quintal	7621.93	7632.80	7763.48	7774.35	6011.47	6022.34
2	Total net margins of intermediaries (MM)	Quintal	5973.93	5777.77	5455.84	5259.68	196.16	-
3	Net price received by the Processor (PP)	Quintal	5127.60	5127.60	4324.80	4324.80	4420.93	4420.93
4	**Index of marketing efficiency**							
	Acharya's method (MME): 3/(1+2)	Ratio	0.38	0.38	0.33	0.33	0.71	0.73

3.6.6 Costs and Margins in the Processing of Jelly and Jam by Cottage Scale

The Table 9 indicates that the total quantity of Jam and Jelly produced by the cottage scale was 96.89 quintals i.e., Jelly 50.22 quintals and Jam 46.67 quintals. The total cost in the preparation of Jam and Jelly was Rs. 320766.07. After the sale of these two products the net receipt was Rs. 594298.93 respectively. The benefit cost ratio was found as 2.74 in case of jelly, 2.99 in case of Jam and for the firm as whole, it was 2.85.

3.6.7 Costs and Margins in the Processing of Jelly, Jam and Toffee by Small Scale Units

It is observed from the Table 10 that the total quantity of jelly, jam and toffee produced by the small scale units was found to be 237.37 quintals, i.e., in jelly- 100.19 quintals, Jam- 92.35 quintals and toffee- 44.83 quintals. The total cost in the preparation of jelly, jam and toffee was found as Rs. 912947.80. After the sale of these products the net receipt was Rs. 1109938.45. The returns per rupee of investment were found as 2.28 in jelly, 2.43 in jam and 1.89 in toffee and in aggregate it was found at 2.22.

3.6.8 Costs and Margins in the Processing of Jelly, Jam and Toffee by Large Scale Units

The Table 11 reveals that the total quantity of jam, jelly, and toffee produced by large scale units was 4545.61 quintals, i.e., 2144.68 quintals of jelly, 2050.62 quintals of jam and 350.31 quintals of toffee. The total cost in the preparation of jelly, jam and toffee was found as Rs. 16189453.86. After the sale of these products the net receipt

was Rs. 25515638.64. The returns per rupee of investment were found to be 2.52 in jelly, 2.82 in jam and 2.01 in toffee, and in aggregate it was found as 2.58.

Table 9. Total costs and margins in the processing of jelly, jam and toffee by small, large and cottage scale units

S. No.	Particulars	Jelly	Jam	Toffee	Firm as whole
A	**Small scale units**				
a.	Total quantity produced (quintals)	100.19	92.35	44.83	237.37
b.	Total cost	357678.30	294134.75	261134.75	912947.80
c.	Gross income	814043.75	715712.50	493130.00	2022886.25
d.	Net income	456365.45	421577.75	231995.25	1109938.45
e.	Benefit cost ratio	1:2.28	1:2.43	1:1.89	1:2.22
B	**Large scale units**				
a.	Total quantity produced (quintals)	2144.68	2050.62	350.31	4545.61
b.	Total cost	7656507.60	6445098.66	2087847.60	16189453.86
c.	Gross income	19302120.00	18199252.50	4203720.00	41705092.50
d.	Net income	11645612.4	11754153.84	2115872.40	25515638.64
e.	Benefit cost ratio	1:2.52	1:2.82	1:2.01	1:2.58
C	**Cottage scale units**				
a.	Total quantity produced (quintals)	50.22	46.67	-	96.89
b.	Total cost	176322.42	144443.65	-	320766.07
c.	Gross income	483367.50	431697.50	-	915065.00
d.	Net income	307045.08	287253.85	-	594298.93
e.	Benefit cost ratio	1:2.74	1:2.99	-	1:2.85

3.6.9 Constraints Encountered by Guava Processors

In the present study, it was observed that the entrepreneurs did not face any critical problem. Nevertheless, some technical constraints and the problems related to marketing of processed products were faced, which are discussed below:

1). Allahabad is known for its best guava; even then it fails to fulfil the requirements of processors. Fresh, matured, tight, seedless guava is preferred for the processing, but due to the lack of proper grading or proper knowledge about the processing of guava among the orchardists, processors were bound to purchase the mixed variety of guava that resulted in the decrease in quality of processed products.

2). The non-availability of skilled labour was found as one of the problems faced by the entrepreneurs. The labour was in abundant but they were required to be trained first before getting involved in the manufacturing of the products. All the cottage scale units of the study area reported the problem of skilled labour. Cottage scale units employed the labourers only on the basis of the demand of products in the market. If there was higher demand for processed products, surplus labour was employed. Hence, the entrepreneurs of this category faced this problem during a particular period of the season, as they did not get skilled labourers at right time. Therefore they had to train the labourers first and then to start their production. The small and large scale units generally employed the permanent labourers. But if the demand for processed guava products was higher in the markets only then they had to employ the temporary labourers.

3). Another problem reported by the processors of the study area was lack of capital inputs or financial constraints, However only cottage scale units faced this problem.

4). Setting of processed products, viz., Jelly, Jam and toffee, is a technical problem faced by cottage and small scale units. It may be either due to the lack of technical knowledge or due to some imbalanced ingredients used. This problem may cause loss to guava, because on the one hand they could not increase the price of their finished products because of competition in the markets, and on the other hand, they were bound to manufacture good quality products. However, the large scale units did not report this technical problem.

5). It is a well known fact that the processed products rarely figure in the menu of Indians. Processed products are not preferred in the regular diet. This is the main reason that only two per cent of the total fruits and vegetables are being processed in the country. The main processed products of guava are jelly and

jam which are being marketed in Allahabad as well as transported to other places. However there is lack of demand for these products as reported by the processors of the study area.

6). High degree of competition was reported by most of the entrepreneurs as they had to search for the market of their processed products. The products of cottage scale units were sold at comparatively lower rates and marketed by the processors themselves. But all the cottage scale processors had their fixed markets, thus the degree of competition was very low in this category. But in case where the products were sold through retail shops, higher degree of competition was reported. In selling the products outside Allahabad district, the small category processors had to compete with the processor of distant markets. So the processors of study area searched for those markets where the number of processors was less or where the availability of raw material was a constraint.

4. Conclusions and Policy Implications

Processing of Guava is economically feasible and hence needs to be encouraged. It provides wide scope for employment opportunities to the skilled and unskilled labourers in the rural areas, besides it has potential to earn valuable foreign exchange to the country. There is wide scope for processing industry, as production is higher than the demand particularly during the peak period. Though a variety of guava products can be prepared, but a very little portion of the surplus production of guava is utilized for processing and value addition. To avoid the glut during peak period, processing industry can prove very successful in better marketing, and boosting the interest of the guava growers towards this popular fruit for their overall prosperity. The following suggestions can prove helpful to the farming community and processing industry.

1). The Processors faced problems like unassured market for their output and high cost of fuel. In this regard the government can intervene and make arrangements for exports. Technocrats and scientists can develop energy efficient and superior technology for processing.

2). During the course of study it was found that no encouragement / importance were given by the concerned departments in the processing and marketing of Guava. Hence, the concerned development department and financial institutions must popularize the processing of Guava in the study area by providing latest technical know-how, finance and other infrastructural facilities.

3). Guava is highly nutritive and cheap; by judicious management of the guava fruits, lots of the nutritive food products can be made available to the people of the country and abroad along with wide employment and business opportunities.

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Investigation of the Antioxidative Potential of Various Solvent Fractions From Fruiting Bodies of *Schizophyllum commune* (Fr.) Mushrooms and Characterization of Phytoconstituents

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Abstract

The aim of the present study was to investigate the antioxidative potential of components extracted from the indigenous medicinal mushroom, *Schizophyllum commune,* using solvents of different polarities. The effectiveness of antioxidants in each solvent fraction was evaluated by quantitating the total phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), peroxidation inhibition in the egg yolk system, metal chelating potential, and β-carotene bleaching activity. Our findings revealved that the ethyl acetate fraction of *S. commune* had a phenolic content of 82.42±7.23 and exhibited good activities for DPPH radical scavenging (70.52±2.17%), CUPRAC (0.38±0.03), peroxidation inhibition (73.38±1.39%), metal chelating (81.29±4.19%), and β-carotene bleaching (63.25±2.5%) at concentration of 0.1 to 1.0 mg/mL. In addition, gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry were used to determine the volatile and non-volatile constituents of the potent antioxidative ethyl acetate fraction from cultivated *S. commune* fruiting bodies. This comprehensive analysis demonstrates that the ethyl acetate fraction of *S. commune* fruiting bodies is a viable source of natural antioxidants that can be utilized for functional foods and nutraceutical applications.

Keywords: volatile, ethyl acetate, oxidative stress, free radical, total phenolic content

1. Introduction

Numerous physiological processes in living organisms produce oxygen-centered free radicals and other reactive oxygen species (ROS) as by-products. The most common free radical species are superoxide radicals, peroxide radicals, reactive nitrogen radicals and nitric oxide (Halliwell, 1996). The uncontrolled production of free radicals and ROS can result in cell death and tissue injury (Cheung et al., 2005). Oxidative damages caused by free radicals contributes to a wide variety of pathological conditions, including atherosclerosis, respiratory disorders, neurodegenerative diseases, inflammatory diseases, cancer, hypertension and ageing (Anderson, 1999). Almost all organisms have evolved antioxidant defenses and repair systems to protect them against oxidative damage. For example, mammalian cells possess intracellular antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, which protect against the formation of free radicals (Ferreira et al., 2006). However, these systems are insufficient to completely prevent oxidative damage. Thus, exogenous dietary antioxidants, which can scavenge free radicals and oxidants, have been suggested as beneficial protective agents for the human body. Therefore, the consumption of dietary antioxidants might prevent diseases.

Mushrooms are functional foods that can be a great source of nutrition and physiologically beneficial, non-toxic medicine (Wasser & Weis, 1999). Mushrooms can contain a wide variety of free radical scavenging molecules, including polysaccharides, polyphenols, and dietary antioxidants (vitamin C, E, and carotenoids) that have been of particular interest in the fight against degenerative diseases. So far, phenolic compounds are considered to be one of the most important non-essential dietary components for inhibiting free radicals in the prevention of vascular diseases, some forms of cancer, and destructive oxidative stress (damaging DNA, protein and

membranes) (Rabajoli et al., 1996). Some researchers have even indicated that phenolic substances, such as flavonoids and phenolic acids, are considerably more potent antioxidants than vitamin C and E (Cao et al., 1997).

Schizophyllum commune (Fr.) is an-edible mushroom that is widely distributed, growing on every continent except Antarctica (Yim et al., 2009). For this reason, this mushroom has been the object of numerous studies related to, sexuality, genetics, and morphogenesis. In addition, Oso (1981) reported that *S. commune* has significant medical properties. Infact, studies have indicated that schizophyllan, a polysaccharide isolated from *S. commune,* is a promising therapeutic agent, which displays antitumor, anticancer, and immunomodulating activities. Indeed, recent scientific reports identifying biologically active compounds within various mushrooms have validated their medicinal efficacy (Lindequist et al., 2008). In spite of the potential medical benefits related to *S. commune,* its phenolic content, antioxidative activity, and chemical composition have not been well estabilished. Therefore, in this study, we aimed to investigate the antioxidative activities and myco-chemical characterization of solvent fractions obtained from *S. commune* fruiting bodies.

2. Method

2.1 Cultivation of S. Commune Fruiting Bodies

S. commune was cultivated in plastic bags using sawdust as a substrate. The harvested mushroom fruiting bodies were rinsed first with tap water and then with de-ionized water. The mushrooms were dried 50-60°C and then ground into a fine powder using a blender. The antioxidative compounds present in the *S. commune* fruiting bodies were then extracted with various solvents.

2.2 Preparation of Solvent Extract and Fractions

The preparation of mushroom extract and solvent fractions was performed according to the method of Duan et al. (2006) with some modification. *Schizophyllum commune* powder (500 g) was extracted with 2.5 L of methanol: dichloromethane (2:1) in an air tight, clean flat bottomed container for 3 days at room temperature with occasional stirring and shaking. The extract was then filtered through Whatman No 1 filter paper. The extraction was repeated twice. The organic solution from each extraction was combined and evaporated under vacuum until dry, yielding crude extract, which was then dissolved in 90% aqueous methanol. The solution was partitioned with hexane (3 x 100 mL), and the aqueous methanol was evaporated under reduced pressure to give a semisolid, which was then dissolved in 100 mL of distilled water and further partitioned successively with dichloromethane (DCM; 3 x 100 mL) and ethyl acetate (EA; 3 x 100 mL), respectively (Figure 1).

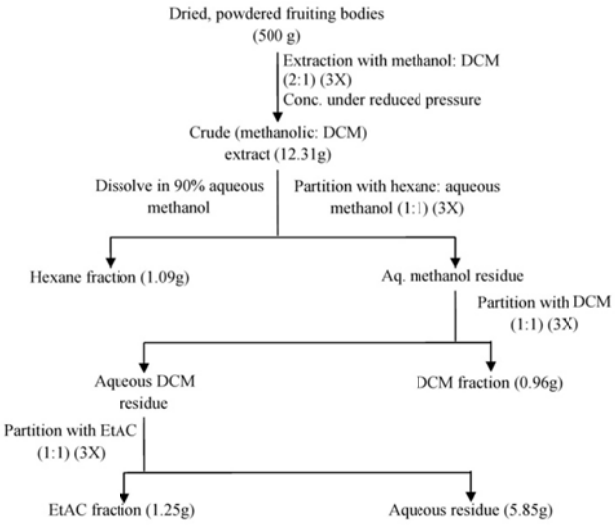

Figure 1. Extraction and fractionation of fruiting bodies of *S. commune* mushroom

2.3 Sources of the Chemicals

The chemicals used in the present study were of analytical reagent grade and were purchased from Sigma-Aldrich.

2.4 Investigation of Antioxidant Activity of S. commune Mushrooms

The antioxidant potential of S. commune solvent fractions was investigated using the following standard assays and compared to quercetin, a natural antioxidant compound.

2.4.1 Total Phenolic Content (TPC)

TPC of *S. commune* solvent fractions were quantified using Folin-Ciocalteu reagent based on a modified version of the Slinkard and Singleton method (1977). Briefly, 250 µL of 10 % Folin-Ciocalteu reagent, was added to 250 µL of each solvent fraction and shaken. After 2 minutes, 500 µL of 10 % sodium carbonate solution was added. The mixture was incubated in the dark for 1h before absorbance was read at 750 nm. TPC was calculated on the basis of the calibration curve of gallic acid (2-10 µg/mL) and expressed as gallic acid equivalents (GAEs), in mg/g of extract.

2.4.2 Scavenging Effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radicals

The DPPH radical scavenging assay was done following the method of Noorlidah et al. (2012). Aliquot (0.1 mL) of each solvent fraction with different concentration (0.1 and 100 mg/mL) was mixed with 3.9 mL of 0.06 mM DPPH dissolved in methanol. The solution was shaken vigrorously and the absorbance was measured at 515 nm, using methanol as blank. The decrease in absorbance was monitored at 0 min, 1 min, 2 min, and every 15 mins until the reaction reached its plateau. The percentage of free radical scavenging activity, was calculated using the following equation:

$$\textit{Radical scavenging activity (\%)} = A_0 A_s / A_0 \text{ x } 100$$

where A_0 is the absorbance of 0.06 mM methanolic DPPH only, and A_s is the absorbance of the reaction mixture. IC_{50} value (half maximal inhibitory concentration) of the most potent solvent extract was calculated from the graph of radical scavenging activity against extract concentration.

2.4.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

The CUPRAC assay was performed according to the method of Ozturk et al. (2007) with some modifications. The test mixture contained 1 mL of each of the following: 10 mM of copper (II), 7.5 mM neocuproine, and 1 M ammonium acetate buffer (pH 7.0). Briefly, 1 mL of solvent fraction (0.1-100 mg/mL) was added to each reaction tubes to achieve a final volume of 4 mL. The tubes were then incubated for 30 min at room temperature before absorbance at 450 nm was recorded against a blank.

2.4.4 Inhibition of Lipid Peroxidation of Buffered Egg Yolk

Inhibition of lipid peroxidation was determined according to the method of Daker et al. (2008). Briefly, the reaction mixture contained 1 mL of fowl egg yolk emulsified with 0.1 M phosphate buffer (pH 7.4) to obtain a final concentration of 25 g/L and 100 µL of 1000 µM $FeSO_4$. Concentration of 0.1-100 mg/mL of each solvent fraction was added, shaken and incubated at 37°C for 1h before being treated with 0.5 mL of freshly prepared 15% trichloroacetic acid (TCA) and 1.0 mL of 1% thiobarbituric acid (TBA). The reaction tubes were further incubated in a boiling water bath for 10 min. The tubes were then cooled to room temperature and, centrifuged at 3,500 g for 10 min to remove the precipitated protein. Finally, the formation of thiobarbituric acid reactive substances (TBARS) in 100 µL of supernatant was determined by reading the absorbance at 532 nm. Buffered egg with Fe^{2+} only was used as control in this assay. The percentage inhibition (IC_{50}) was calculated using the following equation:

$$\textit{Inhibition (\%)} = A_0 A_s / A_0 \text{ x } 100$$

where A_0 is the absorbance of the control, and A_s is the absorbance of the sample. IC_{50}value (half maximal inhibitory concentration) of the most potent solvent extract was calculated from the graph of inhibition of lipid peroxidation against extract concentration.

2.4.5 Metal Chelating Activity

The ability of the fraction to chelate iron (II) was estimated according to the method of Dastmalchi et al. (2008). An aliquot of each sample (200 µL) was mixed with 100 µL of $FeCl_2 \cdot 2H_2O$ (2.0 mmol/L) and 900 µL of MeOH. After 5 min, the reaction was initiated by addition of 400 mL of ferrozine (5.0 mmol/L) and further incubated for 10 min. Then absorbance was read at 562 nm. The chelating activity (%) was calculated using the following equation:

$$\textit{Chelating activity (\%)} = [Abs_{control} - (Abs_{sample} / Abs_{control}] \text{ X } 100.$$

2.4.6 β-carotene Bleaching Assay

β-carotene bleaching activity of the various mushroom solvent fractions (0.1 to 100 mg/mL) was evaluated according to the method of Noorlidah et al. (2012) with some modifications. Briefly, 2 mg of β-carotene was dissolved in 10 mL of chloroform. Then, linoleic acid (40 µL) and Tween 80 emulsifier (400 µL) were added to this mixture and subsequently chloroform was evaporated under vacuum prior to the addition of 100 mL of distilled water. Then, 4.8 mL of this mixture was mixed with various concentrations of each solvent fractions and the absorbance was measured at 470 nm. The percentage inhibition was calculated using the following formula:

$$Inhibition\ (\%) = A_0 A_s / A_0 \text{ x } 100$$

where A_0 is the absorbance of the control, and A_s is the absorbance of the sample.

2.5 Mycochemical Characterization of S. commune

2.5.1 Liquid Chromatography –Tandem Mass Spectrometry (LC-MS/MS) Analysis of the Ethyl Acetate Fraction of *S. commune*

The LC-MS/MS analyses were carried out using AB Sciex 3200 Q trap with Perkin Elmer UHPLC FX15. The chromatograph was coupled to an electrospray ionization (ESI) mass spectrometer through a turbo V spray source. The analytical condition employed a phenomenex aqua column (50 mm x 2.1 mm, particle size 3 µm), mobile phase nitrogen, an, optimum flow rate of 0.4 mL/min, and an injection volume of 10 µL. The diode array detector recorded the spectra from 100 to 1200 nm. The MS analyzer was operated in the ESI negative mode.

2.5.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of the Ethyl Acetate Fraction of *S. commune*

GC/MS analyses were performed on a gas chromatograph directly coupled to a mass spectrometer system. HP-5ms silica capillary column (30 m x 250 µm, 0.25 µm film) was used under the following conditions: oven temperature program from 70°C (2 min) to 300°C (6 min) with the final temperature maintained for 29 min; injector temperature of 250°C; He carrier gas, flow rate of 1 mL/min; injection volume of 1.5 µL; splitless injection technique; ionization energy of 70 eV, electronic ionization (EI) mode; ion source temperature of 200 °C ; scan mass range of 50-550 m/z; and interface line temperature of 300°C . Peak identification was based on comparison with mass spectra from the National Institute of Standards and Technology (NIST 08 and NIST 08s) library and published data.

2.6 Statistical Analysis

All experiments were performed in triplicate and data were recorded as means ± standard deviations. Statistical analyses were carried out using one-way analysis of variance (ANOVA). Differences among means were analysed by least significant difference at 95% ($P<0.01$).

3. Results and Discussion

3.1 TPC

As shown in Figure 2a, analysis of the distribution of phenolic compounds in *S. commune* solvent fractions revealed that the dichloromethane fraction contained the highest phenolic content (86.51±6.70 mg GAE/g), followed by the ethyl acetate (82.42±7.23 mg GAE/g), aqeous residue (59.39±8.13 mg GAE/g), crude (41.51±1.14 mg GAE/g), and hexane (37.12±4.77 mg GAE/g) fractions. In regard to the relationship between TPC and the antioxidant activity, it has been reported by several researchers that phenolic compounds in mushrooms significantly contribute to their antioxidant properties. In this study, there was positive correlation between TPC and metal chelating ability ($r^2=0.765$), which was highly significant ($p<0.001$). In addition, there was significant correlation between TPC and antioxidant capacity ($r^2=0.415$, $p<0.001$), as shown by the β-carotene bleaching assay. Indeed, antioxidant activity increased proportionally to the phenolic content, and a linear relationship between DPPH-radical scavenging activity and TPC was established ($r^2= 0.084$, $p<0.001$). Additionally, TPC was significantly correlated with antioxidant activity as measured by both the CUPRAC ($r^2=0.015$, $p<0.001$) and lipid peroxidation ($r^2= 0.015$, $p<0.001$) assays.

3.2 Scavenging Activity Against DPPH Radical.

The DPPH radical assay is most commonly used for investigating the antioxidant activities of natural compounds. In the DPPH assay, solvent fractions or extracts are tested for their ability to reduce the stable radical DPPH$^{\bullet}$ to the yellow-colored diphenylpicrylhydrazine. Specifically, the method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant, which results in the formation of a non-radical form of DPPH (DPPH-H) (Shon et al., 2003). As seen in Figure 2b, the ethyl acetate fraction of *S. commune* showed the highest activity in this assay (70.52±2.17 mg/mL; IC_{50}-70 µg/mL), which was higher than

the positive control (quercetin) at all concentrations tested (0.1-1.0 mg / mL). Among the different solvent fractions isolated from the crude extract, the dichloro methane, ethyl acetate, water residue, and hexane fractions, revealed no or only moderate activities in this assay. Thus, the ethyl acetate fraction showed the most potent activity, indicating that the compounds with the strongest DPPH radical scavenging activity in *S. commune* were of medium polarity. Notably, the observed activity of this fraction was significantly higher than all of the soluble fractions, the water residue, and the positive control, (quercetin at the same concentration).

3.3 Reducing Power Assay (CUPRAC)

The reducing power of antioxidant compounds can be indicated by their ability to reduce oxidized intermediates produced during the lipid peroxidation process by donating electrons. However, while the ferric-reducing antioxidant power (FRAP) assay is the most commonly used method for determining reducing potential of antioxidants, it has two major flaws: (1) FRAP assay is conducted under acidic condition (pH 3.6) to maintain iron solubility; (2) FRAP assay does not measure thiol antioxidants, such as glutathione. Thus, FRAP may not give values that are to physiological conditions. In the present study, we used the CUPRAC assay, which is based on the reduction of Cu (II) to Cu (I) by antioxidants. The reducing potentials for the different fractions of *S. commune* as determined by the CUPRAC assay, are presented in Figure 2c. The results indicated that the ethyl acetate fraction had the highest reducing power when compared with the positive control (quercetin). Notably, studies have indicated that the *in vitro* physiological procedure yields a higher reducing power than the chemical procedure (Perez-Jimenez J & Saura-Calixto, 2005). In addition, there was a moderate correlation between reducing power of ethyl acetate fraction of *S. commune* and TPC (r^2=0.015, p<0.001). Thus, ethyl acetate fraction exhibited the highest activity (0.38±0.03), followed by the dichloromethane, crude, hexane, and water residue fractions.

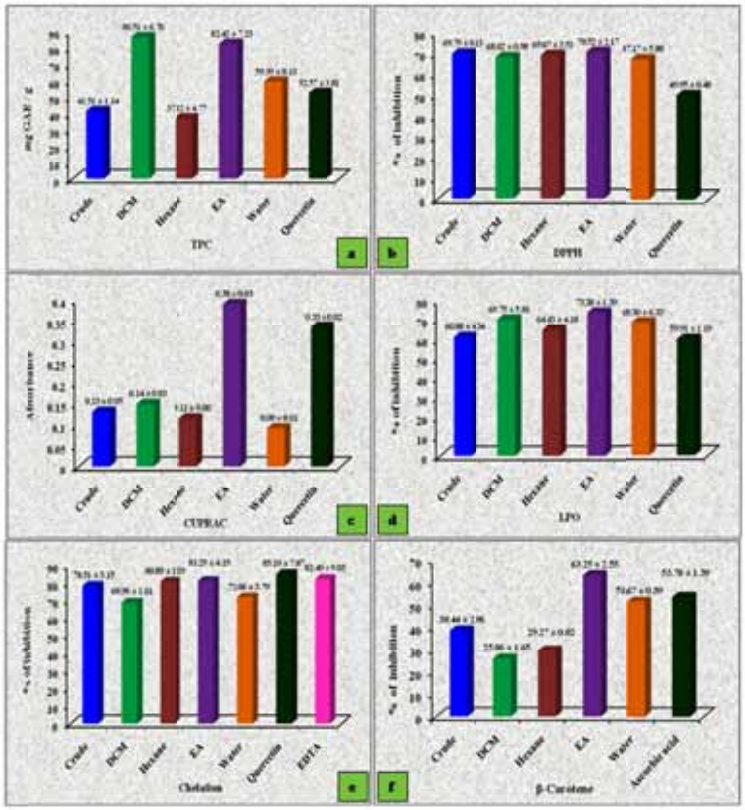

Figure 2. Total phenolic content (TPC) analysis (a) DPPH radical scavenging activity (b), antioxidant activity by CUPRAC (c), lipid peroxidation (LPO) activity (d), metal chelating ability (e), and β-carotene bleaching assay (f) are shown for the various solvent fractions from *S. commune* mushrooms at 1mg /mL

3.4 Lipid Peroxidation Assay

The unsaturated sites in fatty acids are easily attacked by free radicals, promoting the formation and regulation of

lipid radicals. In this process, double bonds of lipids undergo rearrangements that result in destruction of lipids, producing breakdown products, such as malondialdehyde. Thus the estimation of malondialdehyde in a mushroom fraction helps to evaluate its protection level against lipid peroxidation, (i.e., antioxidants present in sample protect against the hazardous effects of free radicals on unsaturated fatty acids, minimizing the production of malondialdehyde) (Michielin et al., 2011). In our study, lipid peroxidation induced by Fe^{2+} was estimated by the presence of TBARS. The ability of the various *S. commune* solvent fractions to inhibit peroxidation of phospholipids in egg yolk is shown in Figure 2d. These data demonstrate that the, ethyl acetate fraction exhibited the highest inhibition of lipid peroxidation (73.38±1.39%; IC_{50}–9.0 µg/mL) followed by dichloromethane (69.75±5.81%) water residue (68.30±6.33%) hexane (64.43±4.18%) and crude extract (60.88±4.34%) respectively. Moreover, quercetin showed a lower inhibition potential compared to the ethyl acetate fraction (59.91±1.19%).

The process of lipid peroxidation has been suggested to proceed *via* a free radical chain reaction (Halliwell, 1989), which has been associated with cell membrane damage. This membranouse damage has been suggested to contribute to various diseases, including diabetes. Incubation of egg yolk homogenates in the presence of $FeSO_4$ causes a significant increase in lipid peroxidation. It is possible that the high level of inhibition on lipid peroxidation displayed by the ethyl acetate fraction is related to the presence of phenolic compounds, which have been correlated with antioxidant activity (Gulcin et al., 2002).

3.5 Metal Chelating Activity Assay

Transition metals, such as iron, can stimulate lipid peroxidation by generating hydroxyl radicals through the Fenton reaction, accelerating lipid peroxidation into peroxyl and alkyl radicals. Therefore, these metals can drive the lipid peroxidation reaction. Chelating agents can inhibit radical generation by stabilizing transition metals, consequently reducing free radical damage. In addition, phenolic compounds have the potential to bind to metal ions due to their chemical structures, and have been shown to exhibit antioxidant activity through the chelation of metal ions (Zhao et al., 2008). For the metal chelating assay, chelating agents disturb, ferrozine complex formation resulting in a decrease in the red color. As shown in Figure 2e, the iron chelating ability of the ethyl acetate fraction (81.29±4.19%; IC_{50}–49 µg/mL) was the most active, but was lower than that of ethyl acetate fraction and the controls, quercetin (85.19±7.87%) and EDTA (82.49±9.03%). Moreover, the chelating abilities of the other fractions, including hexane (80.89±1.19%), crude (78.51±3.15%), water residue (72.06±3.79%) and dichloromethane (68.96±1.61%). These data suggest, that the extracts can protect against oxidative damage by sequestering iron (II) ions, which may otherwise catalyze Fenton-type reactions or participate in metal catalyzed hydroperoxide decomposition reactions (Dorman et al., 2003). On the other hand, the iron (II) chelating properties of the fractions be attributed to the presence of endogenous chelating agents, mainly phenolics. In fact, certain phenolic compounds have functional groups that are oriented in a manner that, can allow chelation of metal ions (Thompson et al., 1976). Chelating agents may serve as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of metal ions. Thus we propose that the low moderate ferrous ion chelating effects of the ethyl acetate fraction could protect against oxidative damage.

3.6 β- carotene-linoleic Acid Bleaching Assay

While iron (III) reduction and synthetic free radical scavenging models are valuable tools for analyzing the potential antioxidant activity of natural compounds, these systems do not use a food or biologically relevant oxidizable substrate; therefore, no direct information regarding protective action of the extracts can be determined (Dorman et al., 2003). For this reason, it was considered important to assess the inhibitory effect of *S. commune* extracts on the oxidation of β-carotene. In the β-carotene bleaching assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals, which attack the chromophore of β-carotene. This, results in a bleaching of the reaction emulsion. Thus, an extract that is capable of retarding/inhibiting the oxidation of β-carotene can be described as a free radical scavenger and primary antioxidant (Liyana-Pathirana & Shahidi, 2006).

As can be seen in Figure 2f, all of the extracts were capable of inhibiting the bleaching of β-carotene by scavenging linoleate-derived free radicals. The ethyl acetate fraction was more effective than (the ascorbic acid control) (63.25±2.5%). It has been suggested that the polarity of an extract might impact its activity in water (i.e., non-polar compounds can be are more effective antioxidants than polar compounds due to a ‘concentrating effect’ within the lipid phase). However, in this work we have used different solvent fractions and found an appreciable inhibition of β-carotene bleaching using the ethyl acetate fraction of *S. commune*. This phenomenon has been described by Koleva and his co-author, who reported inhibition of bleaching of β-carotene by a polar extract of *Sideritis* species (Koleva et al., 2002). According to the β-carotene bleaching data, the ethyl fraction was capable

of scavenging free radicals in a complex heterogeneous medium.

3.7 Identification of Chemical Constituents in Ethyl Acetate Fraction by LC/MS Analysis

The LC/MS chromatogram of the ethyl acetate fraction of *S. commune* at 240 nm is displayed in Figure 3. In addition, Table 1 shows the retention time, mode (+/–), λ max, molecular weight, and the respective phytoconstituents identified. In the LC/MS analysis of the following were observed as negative molecular ions at $[MS\text{-}H]^+$: hydroxybenzoic acid at an m/z of 138.12, protocatechuic acid at at an m/z of 154.12, Vitamin E at an m/z of 430.71, and mevastatin at an m/z 390.51. Also, phenolic acids were observed as negative molecular ions at $[MS\text{-}H]^+$ at m/z of 293.0, 297.0, 323.0, 327.0, 367.0, 379.0, 383.0 and 409.0. Notably, these compounds idendified in the ethyl acetate fraction of *S. commune* have been reported to have several biological activities. For example protocatechuic acid has antioxidant and anticancer activities (Tanaka & Tanaka, 2011), vitamin E has been reported to possess antioxidant activity (Rezk et al., 1983), and mevastatin is an HMG-CoA reductase inhibitor (Hanjani et al., 2001).

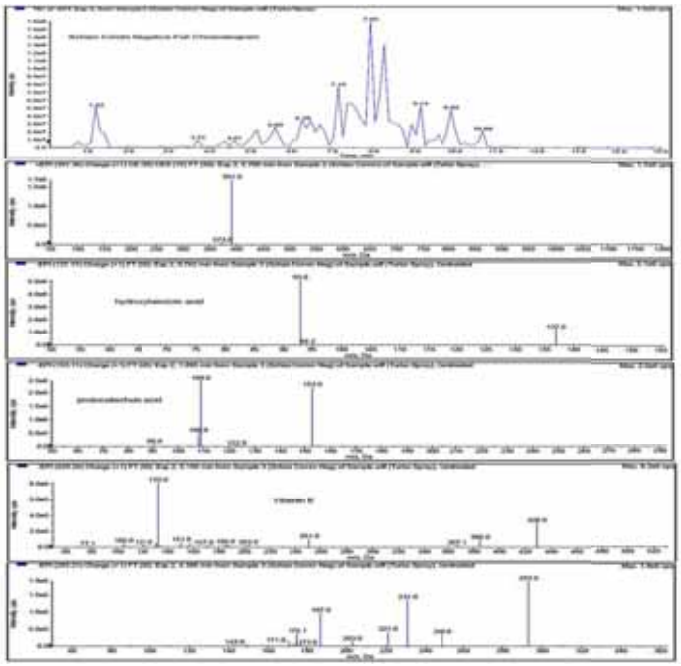

Figure 3. LCMS/MS chromatogram of the ethyl acetate fraction of *S. commune* mushroom

Table 1. Identification of non-volatile chemical constituents in ethyl acetate fraction of *S. commune* by LCMS/MS

S. No	Rt (min)	Mode (+/–)	Compounds	Molecular formula	Molecular weight
1	0.762	–	Hydroxybenzoic acid	$C_7H_6O_3$	138.12
2	1.095	–	Protocatechuic acid	$C_7H_6O_4$	154.12
3	5.156	–	Vitamin E	$C_{29}H_{50}O_2$	430.71
4	5.708	–	Mevastatin	$C_{23}H_{34}O_5$	390.50

3.8 Identification of Chemical Constituents in Ethyl Acetate Fraction by GC/MS Analysis

The GC-MS chromatogram of the ethyl acetate fraction is displayed in Figure 4. As shown in Table 2, the 44 chemical constituents from GC/MS analysis were characterized based on peak area percentage and relative retention time (Rt), which were obtained from already published data (Tatsis et al., 2007). The major components present in the ethyl acetate fraction of S. commune were niacinamide (19.88%), 4-pyridinecarboxamide (19.88%), 9-octadecanamide (18.85%), methyl-9-methyltetradecanoate (18.83%), dodecanamide (17.26%), hexadecanamide (17.26%), linoleic acid ethyl ester (17.10%), cycloeicosane (16.23%), hexadecanoic acid, (methyl ester 14.85%), 1-octadecane (14.23%), and pentadecanoic acid, (methyl ester 13.83%). Many of these compounds identified by GC/MS in the ethyl acetate fraction of S. commune have been suggested to have

biological activities. In fact, niacinamide, was reported to be an effective dietary precursor for NAD^+ and to inhibit poly ADP-ribose polymerase-1 (PARP-1) activity (Surjana et al., 2010), In addition, ergosterol not only reduces inflammation related pain and decreases incidence of cardiovascular diseases, but also has antioxidant, antimicrobial, anticomplementary and antitumor activities (Yuan et al., 2006). Also, linoleic acid ethyl ester has been reported to possess antioxidant activity (Fagali & Catala, 2008). Therefore, the presence of these phytochemicals makes S. commune a potential source of bioactive compounds.

Table 2 Identification of volatile chemical constituents in ethyl acetate fraction of *S. commune* by GC/MS

S.No	Name of Compounds	Retention time	Peak Area (%)	Molecular formula	Molecular weight
1	Phenol	3.705	3.57	C_6H_5OH	94.11
2	Benzo [1,2,5] thiadiazole, 4-bromo-7-(4-methylpiperazine)	5.347	0.66	$C_{11}H_{13}BrN_4O_2S_2$	377.28
3	1,2- Benzenediol	6.314	1.03	$C_6H_6O_2$	110.11
4	dl-Mevalonic acid lactone	6.629	0.29	$C_6H_{10}O_3$	130.14
5	Indole	7.664	0.43	C_8H_7N	117.11
6	N-Acetylindole	7.664	0.43	$C_{10}H_9NO$	159.06
7	5H-1-Pyrindine	7.664	0.43	C_8H_9N	119.16
8	Benzaldehyde, 3-hydroxy	8.506	0.37	$C_7H_6O_2$	122.12
9	L-Pipecolinic acid	8.672	0.60	$C_6H_{11}NO_2$	129.20
10	Formyl glutamine	8.672	0.60	$C_6H_{10}N_2O_4$	174.15
11	4-Methylproline	8.672	0.60	$C_6H_{11}NO_2$	129.15
12	Niacinamide	9.152	19.88	$C_6H_6N_2O$	122.12
13	4- Pyridinecarboxamide	9.152	19.88	$C_6H_6N_2O$	122.14
14	Benzeneethanol, 4-hydroxy	9.433	12.25	$C_8H_{10}O_2$	138.16
15	Phenol, 2,6-bis (1,1-dimethylethyl)	10.360	0.45	$C_{14}H_{22}O$	206.32
16	Ethyl citrate	12.099	0.64	$C_{12}H_{20}O_7$	276.28
17	n-Hexadecanoic acid	15.206	1.83	$C_{16}H_{32}O_2$	256.42
18	Tridecanoic acid	15.203	1.83	$C_{13}H_{26}O_2$	214.34
19	Chlorpyrifos	15.629	0.17	$C_9H_{11}Cl_3NO_3PS$	350.59
20	9,12-octadecadienoic acid	16.499	0.40	$C_{18}H_{32}O_2$	280.44
21	Decanedioic acid, bis (2-ethylhexyl) ester	22.210	2.00	$C_{26}H_{50}O_4$	426.67
22	Ergosterol	25.591	14.12	$C_{28}H_{44}O$	396.65
23	Benzene, 1,2,3-trimethyl	3.739	7.46	C_9H_{12}	120.19
24	Benzene, 1,2-dimethyl	4.471	1.56	C_8H_{10}	106.17
25	Benzene, 2-ethyl-1,4-dimethyl	4.826	1.17	$C_{10}H_{14}$	134.21
26	Benzene, 1,2,4,5-tetramethyl	5.290	1.49	$C_{10}H_{14}$	134.22
27	1,3,8-p-Menthatrine	5.747	0.46	$C_{10}H_{14}$	134.21
28	Azulene	6.234	0.88	$C_{10}H_8$	128.17
29	Naphthalene	6.234	0.88	$C_{10}H_8$	128.17
30	Undecane	7.601	1.33	$C_{11}H_{24}$	156.31
31	Octane, 2-methyl	7.601	1.33	C_9H_{20}	128.25
32	Tridecane	7.601	1.33	$C_{13}H_{28}$	184.36
33	Niacinamide	8.923	3.91	$C_6H_6N_2O$	122.12
34	Phenol, 2-ethyl	9.324	1.09	$C_8H_{10}O$	122.16
35	Pentadecanoic acid, methyl ester	13.833	1.08	$C_{16}H_{32}O_2$	256.42
36	Methyl 9-methyltetradecanoate	18.833	1.08	$C_{16}H_{32}O_2$	256.42
37	1-Octadecane	14.239	0.49	$C_{18}H_{36}$	252.48
38	Hexadecanoic acid, methyl ester	14.857	3.29	$C_{17}H_{34}O_2$	270.45
39	Cycloeicosane	16.236	0.40	$C_{20}H_{40}$	280.53
40	Linoleic acid ethyl ester	17.106	2.91	$C_{20}H_{36}O_2$	308.49
41	Dodecanamide	17.266	5.31	$C_{12}H_{25}NO$	199.33
42	Hexadecanamide	17.266	5.31	$C_{16}H_{33}NO$	255.44
43	9-Octadecanamide	18.857	23.70	$C_{18}H_{35}NO$	281.47
44	Benzene, 1,2,3-trimethyl	3.739	7.46	C_9H_{12}	120.19

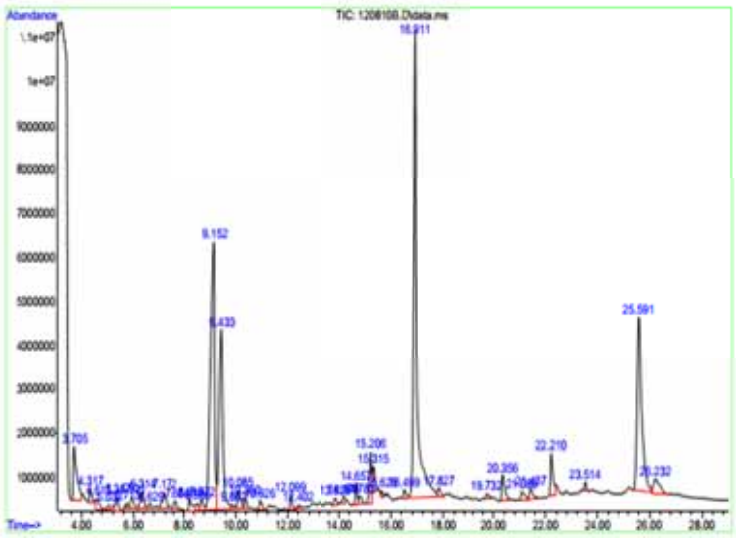

Figure 4. GC/MS chromatogram of the ethyl acetate fraction of *S. commune* mushroom

Here, we have demonstrated that the ethyl acetate fraction of S. commune has antioxidant compounds that can protect against oxidative damage in our bodies and prevent chronic diseases. The impact of these compounds on human health and in the control of free radical mediated diseases will need to be determined through future in vivo studies. These natural constituents could be exploited as cost effective food/feed additives for human and animal health.

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Population Structure, Linkage Disequilibrium and Selective Loci in Natural Populations of *Prunus davidiana*

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Abstract

Prunus davidiana (Carrie're) Franch is a very important resource for the restoration in dry and arid areas, genetic improvement of peach, and extraction of health-promoting components. To effectively use the resource, we must have a measure of genetic diversity of *P. davidiana* and its population structure. LD (Linkage disequilibrium) provides information for association mapping underlying the phenotypic variation observed. Selective loci reveal adaptive evolution processes resulting from natural selection. A set of 190 genotypes from seven natural populations（SXTB, SIYQ, SXFX, NXXJ, SIJC, GSHT, GSHS) of *P. davidiana* collected from the range of *P. davidiana* in China was fingerprinted with 23 SSR markers, and analyzed with spatial structure, pairwise Fst (differentiation coefficient), PCA (principal coordinate analysis), estimation of groups of populations with STRUCTURE software, selective loci obtained from lnRH tested by standardization distribution and Grubbs. Our results demonstrate that population structure of four groups existed among populations through complementary analyses of the above mentioned methods; significant LD numbers from 22 to 129 between loci within unstructured populations were detected; there were five selective loci in all populations and two common selective loci for local natural selection between populations. We should conserve four populations among seven populations; these selective loci may provide information for disclosing adaption evolution and candidate genes according to selective loci and alleles; LDs inform how to use them for association analysis.

Keywords: *a* species of the *subgenus Amygdalus*, microsatellite, population relation, LD, positive selection

1. Introduction

Prunus davidiana (Carr.) Franch., a wild species and perennial woody plant, is native to China (Yu, 1979; Wang, 1988) and is found in wild areas of China. Wild *P. davidiana* was recorded more than 3000 years ago (Wang & Zhuang, 2001), and Meyer (1915) found wild trees in Shaanxi and Gansu provinces in China which are considered as the center of origin of *P. davidiana*. *P. davidiana* has tolerance to cold temperatures, drought, high soil of pH and resistance to peach aphid (Wang & Zhuang, 2001; Bassi & Monet, 2008). *P. davidiana* is often used as rootstock (Yu, 1979) for fruit production of peach, nectarine and plum in Northwestern and Northern China. Furthermore, it can be crossed with peach and nectarine to improve resistance to plum pox, powdery mildew, leaf curl and etc. (Moing et al., 2003), to breed rootstocks adaptable to marginal soils and more resistant to replant problems (Edin & Garcin, 1994; Pisani & Roselli, 1983; Roselli et al., 1985) or to select for self-incompatibility genes (Foulongne et al., 2003). *P. davidiana* can also be used as landscape trees because of their early flowering and attractive bark, and strength of limbs wood (Bassi & Monet, 2008). *P. davidiana* is widely used as an alternative prior species for ecological restoration in dry and arid areas of northwestern China (Wang & Zhuang, 2001). Seeds of *P. davidiana* have 45% oil that is used in Chinese medicine (Wang & Zhuang, 2001). Stems of *P. davidiana* contain health-promoting components that increase the inhibitory activity on total reactive oxygen species (ROS) and hydroxyl radical (*OH) (Choi, 1991; Jung et al., 2003). Propagation of *P. davidiana* is typically from seeds of isolated natural populations in wild places, so natural populations of *P. davidiana* keep relative stability and integrity of gene pool for genetic analyses. From above mentioned aspects, it is necessary to study on

genetic characterization of natural populations of *P. davidiana*, which provides some information for conservation and utilities.

SSR (Simple sequence repeat or Microsatellite) technology is usually preferred among molecular biology methods due to SSR markers displaying co-dominant inheritance, hypervariability and having high cross-species transferability (Tauraz, 1989; Sosinski et al., 2000; Wünsch, 2009). More than 300 SSR markers have been isolated and characterized in the subgenus *Amygdalus* of *Prunus* (Sook et al., 2008). The markers provide a very reliable and convenient tool for the analyzing genetic diversity of *P. davidiana*. Genetic diversity studies have been performed in peach and other species of the *subgenus Amygdalus* (Aranzana et al., 2002, 2003,2010; Bouhadida et al., 2007; Cheng, 2007a,b; Cheng & Huang, 2009; Cipriani et al., 1999; Dirlewanger et al., 2002; Shiran, 2007; Sosinski et al., 2000; Testolin et al., 2000) however, there are no studies on the population structure, LD and selection of natural populations of *P. davidiana*.

Measuring the population structure of *P. davidiana* using neutral markers is an important first step in association genetic studies in order to avoid false associations between phenotypes and genotypes that may arise from nonselective demographic factors (Krutovsky et al., 2009), and it is more efficient for management and utilization of germplasm (Cho et al., 2008). Softwares or methods such as Cluster analyses using UPGMA (the unweighted pair group method with arithmetic mean method), NJ (Neighbor Joining method) and MP (Maximum parsimony method) (Nei and Kumar, 2000) to identify groups and subgroups according to similarity or distance; STRUCTURE software (Pritchard et al., 2000) for deciding K groups by genetic background analysis; PCA (principal coordinate analysis) implemented by GenALEx6.2 software (Peakall & Smouse, 2006) for characterizing population structure by means of principal coordinates. Some papers have been published about both wild populations (Belaj et al., 2007; Besnard et al., 2007) and cultivars (Inghelandt et al., 2010; Li et al., 2010) in plants and used for guidance of conservation and LD (linage disequilibrium) analyses.

Generally, genetic mapping comes from two basic methods, one is traditional QTL (Quantitative trait loci), and the other is advanced LD (Linage disequilibrium loci). QTL mapping requires segregating populations derived from biparental crosses and has resolution limited (Ecke et al., 2009). LD mapping has higher number of recombination events and a higher resolution in polymorphic populations (Ewens & Spielman, 2001; Jannink et al., 2001). Testing of LD can be calculated with Pairwise LD, Multi-locus LD, Haplotype-specific LD, Model-based LD and recombination (Mueller, 2004). LD has been utilized for genetic mapping of traist or disease loci in humans and model organisms (Mueller, 2004). LD has also successfully been used in plants, and significant LD between loci has been detected and the extent and decay of LD have been observed to vary between expressed species populations and subpopulations(Agrama & Eizenga, 2007; Berloo et al., 2008; Rossi et al., 2009; Comadran et al., 2010; Inghelandt et al., 2010; Brazauskas et al., 2011; Myles et al., 2011). These results provided preconditions for selecting populations or subpopulations for LD mapping, and for evaluating number of markers for use in LD mapping.

Commonly used measures for inferring the present selection are increased linkage disequilibrium between loci; reduced polymorphism; or a skewed allele frequency spectrum at individual loci (Schlotterer et al., 2002). Selection can be detected by methods like lnRV, lnRH, Fay and Wu's H test, the E test, and the joint DH test, the MFDM test (Schlotterer, 2002; Schotterer & Dieringer, 2005; Fay & Wu, 2000; Akey et al., 2002; Sabeti et al., 2002; Zeng et al., 2006; Innan & Kim, 2008; Li, 2010) as well as different models (Excoffier et al., 2009). Selective loci as signature of selection (Kane & Rieseberg, 2007; Chapman et al., 2008); identification of candidate loci (Schlotterer et al., 2002); environmental adaption (Kane & Rieseberg, 2007; Coyer et al., 2011); local Selective Sweeps (Schlotterer, 2002).

There are no published studies on population structure, LD and selection loci of *P. davidiana*. In this study, we investigated seven populations with 23 SSRs that cover the peach genome and appear not tightly linked markers (Aranzana et al., 2003). In this study, we aim to 1) analyze population structure and measure genetic variation among populations and use the information to guide the conservation and use of the germplasm; 2) determine if LD exists between loci among populations and use the information to choose the appropriate strategy for genetic association mapping; 3) search for loci showing evidence of selection in the whole population or between populations to detect genes associated with adaption evolution.

2. Materials and Methods

2.1 Plant Materials

We selected accessions of seven natural populations of *P. davidiana* from the center of origin Shannxi and Gansu provinces and surrounding areas Shanxi and Ningxia provinces in China. Young leaves from more than 30 accessions from each population were collected. The distance between any two accessions collected in a

population were separated by no less than 50 meters. The seven populations sampled in Taibai, Shaanxi; Yangquan, Shanxi; Fuxian, Shaanxi; Xiji, Ningxia; Jiaocheng, Shanxi; Huating, Gansu and Heshui, Gansu are abbreviated as SXTB, SIYQ, SXFX, NXXJ, SIJC, GSHT, GSHS, respectively. Samples were stored in sealed bags containing silica material for DNA extraction, and their geographical information was recorded (Table 1; Figure 1).

Figure 1. Seven populations (NXXJ, GSHS, GSHT, SITB, SXFX, SIYQ and SIJC) of *P. davidiana* collected from the areas

Table 1. Geographic information of collected populations

Name of abbreviation	Place of collection	Latitude	Longitude
SXTB	Taibai county, Shaanxi province	34.098	107.310
SIYQ	Yangquan county, Shanxi province	37.782	113.427
SXFX	Fu county, Shaanxi province	35.973	109.059
NXXJ	Xiji county, Nixia municipality	36.018	106.219
SIJC	Jiacheng county, Shanxi	37.655	111.735
GSHT	Huating county, Gansu province	35.234	106.696
GSHS	Heshui county, Gansu province	36.097	108.524

2.2 Molecular Analysis

Genomic DNA was extracted using a modified CTAB extraction protocol (Doyle & Doyle, 1987). In order to fit the samples into 96 well plates, the number of DNA samples from each population 26 to 28 to conform to a total of 192 samples in two plates. PCR amplifications were run on different two platforms. For fragment separation on 3% high resolution MetaPhor® (Cambrex Charles City Inc, IA) agarose – 1X TBE gels, the amplifications were performed in a total volume of 20 µl with final concentrations of 50 ng of DNA, 0.2 µM of both primers, 200 µM of each dNTP (New England Biolabs, Ipswich, MA) and 0.5 U of New England Biolabs' *Taq* DNA polymerase in 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl. DNAs of all accessions was analyzed using 23 published SSR markers which were previously mapped in peach (Table 2). To determine the allelic size amplified by the markers, we selected DNAs of a few accessions from each population amplified by 23 SSRs for fragments analysis ABI 3130 (Applied Biosystems, Foster City, CA). PCR conditions were the same as above with the exception of 0.02 µM of M-13–tagged forward primer, 0.2 µM of reverse primer and 0.2 µM of M-13-tagged dye (6'-FAM, VIC, NED or PET) (ABI). Thermo Scientific MBS Satellite Thermal Cyclers (Thermo Fisher Scientific, Waltham, MA) were used. The PCR program was performed for 3 min of initial denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature (T_a) and 1 min at 72°C, then a final extension step of 5 min at 72°C for each set of primer combinations.

Table 2. Twenty-three SSR markers used for amplification of individuals of seven populations in *P. davidiana*

Marker	Linkage group	Forward sequence	Reverse sequence	Reference
BPPCT 006	G8	GCTTGTGGCATGGAAGC	CCCTGTTTCTCATAGAACTCACAT	Dirlewanger et al., 2003
BPPCT 007	G3	TCATTGCTCGTCATCAGC	CAGATTTCTGAAGTTAGCGGTA	
BPPCT 008	G6	ATGGTGTGTATGGACATGATGA	CCTCAACCTAAGACACCTTCACT	
BPPCT 015	G4	ATGGAAGGGAAGAGAAATCG	GTCATCTCAGTCAACTTTTCCG	
BPPCT 017	G5	TTAAGAGTTTGTGATGGGAACC	AAGCATAATTTAGCATAACCAAGC	
BPPCT 020	G1	CGTGGATGGTCAAGATGC	ATTGACGTGGACTTACAGGTG	
BPPCT 025	G6	TCCTGCGTAGAAGAAGGTAGC	CGACATAAAGTCCAAATGGC	
BPPCT 028	G1	TCAAGTTAGCTGAGGATCGC	GAGCTTGCCTATGAGAAGACC	
CPPCT 002	G3	GGAGCTGCAATATTGCTG	GTTAGGGAAGCATCTCAC	Aranzana et al., 2002
CPPCT 006	G8	AATTAACTCCAACAGCTCCA	ATGGTTGCTTAATTCAATGG	
CPPCT 016	G1	AATTCCCTATGGAAATTAGA	CGCATATTATAGGTAGGAAA	
CPPCT 017	G7	TGACATGCATGCACTAAACAA	TGCAAATGCAATTTCATAAAGG	
CPPCT 022	G7	CAATTAGCTAGAGAGAATTATTG	GACAAGAAGCAAGTAGTTTG	
CPPCT 033	G7	TCAGCAAACTAGAAACAAACC	TTGCAATCTGGTTGATGTT	
UDP96-001	G6	AGTTTGATTTTCTGATGCATCC	TGCCATAAGGACCGGTATGT	Cipriani et al., 1999
UDP96-003	G4	TTGCTCAAAAGTGTCGTTGC	ACACGTAGTGCAACACTGGC	Testolin et al., 2000
UDP96-005	G1	GTAACGCTCGCTACCACAAA	CCTGCATATCACCACCCAG	
UDP96-013	G2	ATTCTTCACTACACGTGCACG	CCCCAGACATACTGTGGCTT	
UDP97-403	G3	CTGGCTTACAACTCGCAAGC	CGTCGACCAACTGAGACTCA	
UDP98-024	G4	CCTTGATGCATAATCAAACAGC	GGACACACTGGCATGTGAAG	
UDP98-025	G2	GGGAGGTTACTATGCCATGAAG	CGCAGACATGTAGTAGGACCTC	
UDP98-409	G8	GCTGATGGGTTTTATGGTTTTC	CGGACTCTTATCCTCTATCAACA	
UDP98-412	G6	AGGGAAAGTTTCTGCTGCAC	GCTGAAGACGACGATGATGA	

When performing PCR for multifluorophore fragment analysis, the conditions above mentioned were followed except for primer pairs with T_a significantly lower than 58ºC (T_a for M-13 forward primer). In such cases, 4 additional cycles were performed at the annealing temperature of the SSR marker followed by 35 cycles at the annealing temperature of the M-13, as described above. PCR amplicons, using 3% MetaPhor® - 1X TBE agarose gels along with New England Biolabs' low molecular weight DNA marker, were visualized with ethidium bromide under UV light, and after pooling the four amplicons together (4 different fluorophores). The samples were cleaned with ExoSAP-IT (USA Scientific or USB) according to manufacturer protocols and run on an ABI 3130 with GeneScan™ 600 LIZ® (Applied Biosystems) internal size standard. PCR products were analyzed by GeneScan with the ABI 3130 and read by Gene Mapper V.4.0 (Applied Biosystem) for multifluorophore fragments.

2.3 Data Analysis

DNAs of accessions from populations were amplified, and their bands with 23 SSR markers, which corresponded to exact sizes detected by Gene Mapper V.4.0, were recorded in Excel.

Genetic variation of SSR markers, including indexes of identifying genotype number, gene diversity, PIC (Polymorphic information content), f (Inbreeding coefficient), was performed using Powermarker software（Liu & Muse, 2005）; Genetic characteristics of including average number of alleles per locus, expected heterozygosity and Theta(H) under the infinite model of populations, were analyzed with Arlequin ver 3.5.1.2 software（Excoffier & Lischer, 2010）.

Population structure was performed with four complementary analyses on genotypic data. First, spatial structure was detected with GenALE x 6.2 software (Peakall & Smouse, 2006) based on genetic distances among populations; second, PCA was implemented with GenALEx6.2 software. Based on the distribution of all accessions along the first three axes, we could detect whether there was any grouping of individuals from populations; third, we used natural populations as a priori groups to test with Wright's Fst index (Weir & Cockerham, 1984) if there was differentiation between populations. The empirical distribution of no differentiation was obtained with Arlequin ver 3.5.1.2 using 10000 permutations; fourth, STRUCTURE 2.3 software, based on the Bayesian model of clustering method (Pritchard et al., 2000), was implemented. We used admixture model assumption to identify K groups of individuals. The assumed K groups varying from 2 to 10 were calculated with thirty replicate runs per K value, a burn in period length of 100000 and a post burn in simulation length of 200000. We decided final K groups through LnP (D) values according to the method recommended by Evanno et al. (2005). Individuals can be allocated into groups with different membership coefficients corresponding to the sum of all being equal to 1.

LD analysis of unstructured populations detected by STRUCTURE was performed using Arlequin ver 3.5.1.2 software under unknown phase between alleles from two heterozygous loci. When allele frequencies for LD were used, those below 5 percent were removed. The number of permutations for LD was 10000, without breaking genotypes to prevent any disequilibrium within loci (Hardy-Weinberg) to affect the significance of disequilibrium between loci. LD between a pair of loci was tested for genotypic data using a likelihood-ratio test, whose empirical distribution was obtained by a permutation procedure (Slatkin & Excoffier, 1996).

Selective loci were detected with Arlequin ver 3.5.1.2 from the alleles at 23 loci of all accessions according to demand of format in finite model with settings of 30000 permutations. A plot of Fst values against Het/(1-Fst) with permutations and observations was generated. If a locus located out of the plot area, the outlier locus is the most possible selected locus. Schotterer and Dieringer (2005) developed quantitative model-free statistics to identify loci that exhibited the largest reduction in microsatellite diversity ln RH which was more powerful than ln RV (Schlotterer, 2002). Ln RH can be obtained with the expected heterozygosity of compared populations, based on a stepwise mutation mode (Ohta & Kimura, 1973). Ln RH should be approximated by a Gaussian distribution under neutrality. Selective loci as outlier loci were checked between populations by using method of ln RH (Schotterer & Daniel, 2005). For monomorphic loci in a population, we used the method of Kauer et al. (2003) to adjust one additional allele different from the others for avoiding division by zero in the calculation of the ratio between populations. If a locus falls beyond the predetermined confidence bounds (i.e. 95% of a standard normal distribution), it indicates a significant reduction in genetic diversity (Harr et al., 2002). Grubbs' test (Motulsky, 2003), also known as the maximum normed residual test, is a statistical test used to detect outliers in a univariate data set assumed to come from a normally distributed population. We also used Grubbs' test to detect outlier values which should be considered as selective loci. Diagrams of allelic frequencies of selective loci between populations were produced in Excel.

3. Results

3.1 Genetic Diversity of SSR Markers and Populations

The 23 loci amplified by the SSR markers revealed a total of 148 alleles. The number of genotypes identified ranged from 3 with CPPCT002 to 37 with UDP98-412, and the mean of 14.2 for all the markers for 190 accessions. Gene diversity and PIC had similar orders except for a few slight differences, displaying that their values of CPPCT002 and UDP96-005 were the highest and lowest, respectively. Inbreeding coefficient screened by the markers disclosed extreme homozygosity of loci except BPPCT006 (Table 3). For analyses of populations, the mean numbers of alleles per locus ranged from 4.261 in SIJC to 2.826 in SXTB with the mean of 3.7 among seven populations. Expected heterozygosity and Theta (H) under the infinite model of the populations were the same orders as the mean numbers of alleles per locus except SIJC (Table 4).

Table 3. Genetic variation of 23 SSR markers in 190 individuals of seven populations

Marker	Genotype No.	Gene Diversity	PIC	f
BPPCT006	5.0	0.2961	0.2849	-0.1172
BPPCT007	16.0	0.7811	0.7463	0.5840
BPPCT008	22.0	0.7528	0.7219	0.4218
BPPCT015	18.0	0.7816	0.7508	0.7117
BPPCT017	9.0	0.6790	0.6146	0.8149
BPPCT020	5.0	0.4806	0.4054	0.3015
BPPCT025	9.0	0.6961	0.6343	0.5180
BPPCT028	8.0	0.1937	0.1846	0.6754
CPPCT002	3.0	0.0612	0.0593	0.8287
CPPCT006	10.0	0.7189	0.6696	0.9199
CPPCT016	8.0	0.4626	0.4188	0.5127
CPPCT017	3.0	0.3354	0.2792	0.8282
CPPCT022	10.0	0.6989	0.6436	0.6101
CPPCT033	8.0	0.3649	0.3211	0.6841
UDP96-001	13.0	0.3884	0.3680	0.4736
UDP96-003	12.0	0.6944	0.6415	0.7435
UDP96-005	36.0	0.8680	0.8539	0.4926
UDP96-013	10.0	0.2976	0.2825	0.2244
UDP98-024	32.0	0.8048	0.7812	0.7135
UDP98-025	14.0	0.6895	0.6544	0.8405
UDP97-403	21.0	0.7923	0.7638	0.8215
UDP98-409	18.0	0.7134	0.6718	0.6254
UDP98-412	37.0	0.8552	0.8388	0.3377
Mean	14.2	0.5829	0.5474	0.6059

Note: PIC short for polymorphic information content; f for inbreeding coefficient.

Table 4. Average number of alleles per locus, expected heterozygosity and Theta(H) under the infinite model of populations

Population	Number of alleles per locus		Expected heterozygosity		Theta(H)
	Mean	s.d.	Mean	s.d.	Mean
SXTB	2.826	1.497	0.31804	0.26825	0.46636
SIYQ	3.652	1.748	0.45334	0.27104	0.82929
SXFX	4	1.907	0.52194	0.23561	1.09177
NXXJ	3.957	2.325	0.49197	0.29691	0.96837
SIJC	4.261	2.137	0.49912	0.25348	0.99651
GSHT	4.174	1.969	0.53811	0.21719	1.16504
GSHS	3.174	1.557	0.44173	0.26043	0.79124
Mean	3.72	1.877	0.46632	0.25756	0.90123

Note: Mean as average value of the item, s.d as standard deviation.

Expected heterozygosity calculated through $\hat{H} = \frac{n}{n-1}(1 - \sum_{i=1}^{k} p_i^2)$

3.2 Population Structure

According to data of latitude and longitude and amplified bands of accessions of populations, geographic distances and genetic distances between populations were obtained with GenALEx6.2 software. When we analyzed correlation coefficients based on genetic distance and geographic distance between populations, all distance classes displayed no significance at 5% level, which meant there was no spatial structure of populations, that is, the end points were not located beyond the upper or lower red dots lines (Figure 2; Table 5).

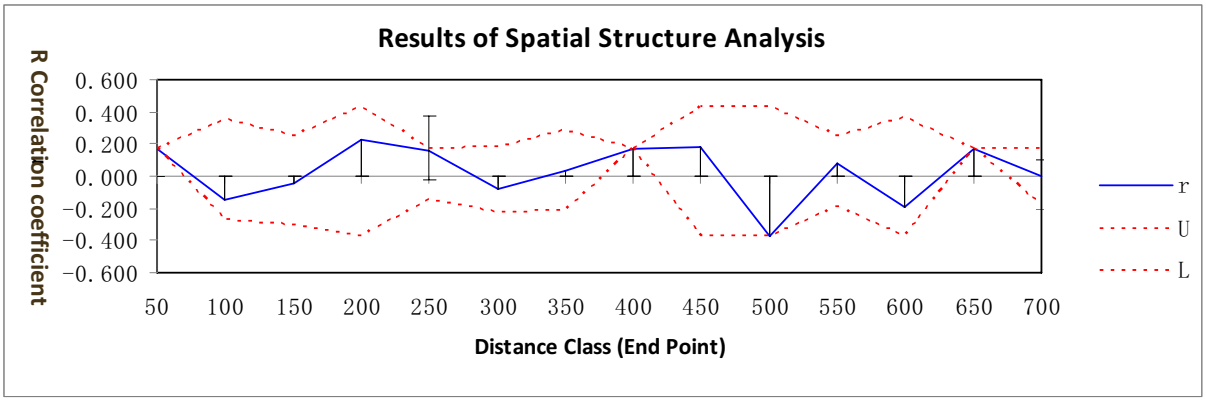

Figure 2. Spatial structure analyses of seven populations

Note: U (Ur error) and L (Lr error) error bars bound the 95% confidence interval about r as determined by bootstrap resampling.

Table 5. Correlation of genetic distance and geographic distance among populations

Distance Class (End Point)	50	100	150	200	250	300	350	400	450	500	550	600	650	700
r	0.167	-0.143	-0.040	0.223	0.153	-0.082	0.035	0.167	0.178	-0.377	0.078	-0.196	0.167	-0.004
U	0.167	0.347	0.251	0.435	0.172	0.179	0.282	0.167	0.435	0.435	0.250	0.361	0.167	0.171
L	0.167	-0.277	-0.311	-0.377	-0.145	-0.225	-0.217	0.167	-0.377	-0.377	-0.188	-0.377	0.167	-0.183
P(r-rand >= r-data)	1.000	0.860	0.640	0.260	0.070	0.690	0.470	1.000	0.290	1.000	0.290	0.870	1.000	0.500
P(r-rand <= r-data)	1.000	0.170	0.370	0.860	0.940	0.330	0.550	1.000	0.780	0.070	0.720	0.230	1.000	0.520
Correction	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167
r uc	0.000	-0.310	-0.207	0.056	-0.013	-0.248	-0.132	0.000	0.011	-0.544	-0.089	-0.363	0.000	-0.170
U uc	0.000	0.180	0.085	0.268	0.006	0.012	0.115	0.000	0.268	0.268	0.084	0.194	0.000	0.004
L uc	0.000	-0.444	-0.477	-0.544	-0.312	-0.392	-0.383	0.000	-0.544	-0.544	-0.354	-0.544	0.000	-0.350
Mean Bootstrap r	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.008
Ur error	-0.167	0.143	0.040	-0.223	0.220	0.082	-0.035	-0.167	-0.178	0.377	-0.078	0.196	-0.167	0.104
Lr error	0.167	-0.143	-0.040	0.223	0.181	-0.082	0.035	0.167	0.178	-0.377	0.078	-0.196	0.167	0.196
Ur	0.000	0.000	0.000	0.000	0.374	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100
Lr	0.000	0.000	0.000	0.000	-0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.199
Intercept	76.871													

Note: r, U and L values are adjusted by the correction factor. Uncorrected values are shown as r uc, U uc, L uc. Bootstrap mean, Ur, Lr are also adjusted by the correction factor.

Upper (Ur error) and lower (Lr error) error bars bound the 95% confidence interval about r as determined by bootstrap resampling.

Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of No spatial structure for the combined data set as determined by permutation.

Fst was calculated for all pairs of populations, and all pairwise differences between populations appeared significant at the 5% level (Table 6). It meant there was genetic differentiation between the populations. When we used all accessions from the populations for PCA, the first three axes explained 24.81%, 23.08% and 16.39% of total variation, respectively. Accessions from SXTB or SIYQ almost clustered together according to their originations, whereas accessions from the other five populations distributed in overlapping (Figure 3).

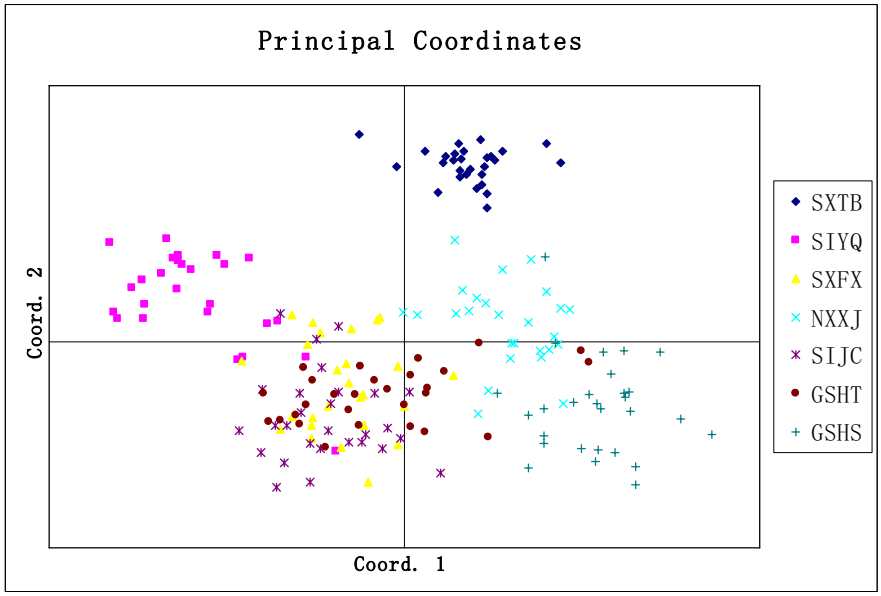

Figure 3. Principal coordinates analysis of 190 individuals from seven populations

We used STRUCTURE2.3 software in admixture model to analyze genetic structure of populations. When performed with assumed K = 2 to 10, there were no distinct groups to be decided because of the values of LnP(D) slightly increasing with values of K, so we used the method suggested by Evanno et al. (2005) to calculate K groups through values of LnP(D), and the highest peak of the curve line was found at K = 4 (groups) (Figure 4). Accessions' membership probabilities of seven populations allocated in four groups were more than 0.83 just except 0.77 from GSHT. Accessions from populations were distributed among assumed four groups similar to the groups with PCA analysis (Figure 3).

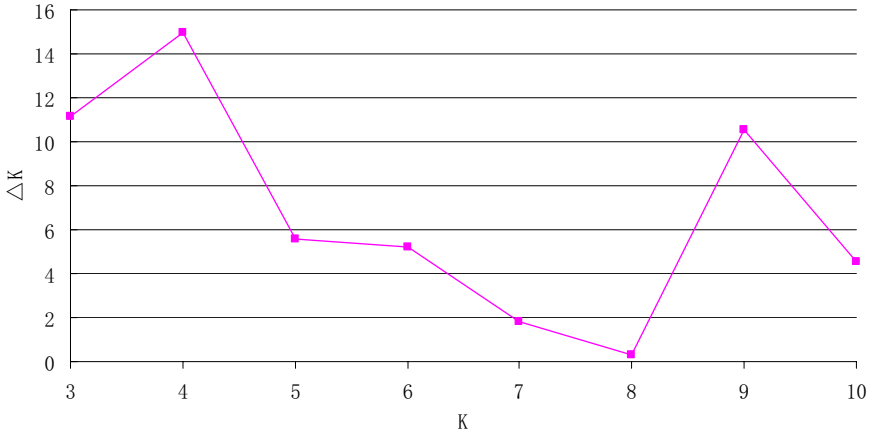

Figure 4. Detecting the number of cluster of 190 individuals from seven populations. ΔK calculated as ΔK = m|L''(K)|/s[L(K)]. The modal value of this distribution is the true K(*) or the uppermost level of structure, here four clusters (Evanno et al., 2005)

3.3 LD

Because population structure can cause spurious LD, we combined the four methods discussed above to identify unstructured populations (almost the same color in Figure 5) including SXTB; SIYQ;SXFX,SIJC and GSHT; NXXJ and GSHS for further studying, meanwhile, the whole population including seven populations was used as control to compare with unstructured populations. We performed the aforementioned populations to investigate significance of genotypic disequilibrium between all loci after removing low frequent alleles (only considering MAF>=0.05). There were some significant LDs between loci of 23 SSRs, displaying that 199 and172 in the whole seven populations; 22 and 8 in SXTB; 37 and 20 in SIYQ; 129 and 95 in SXFX, SIJC and GSHT; 72 and 50 in NXXJ and GSHS at 5% and 1% significant level were found (Figure 6, 7, 8, 9, 10).

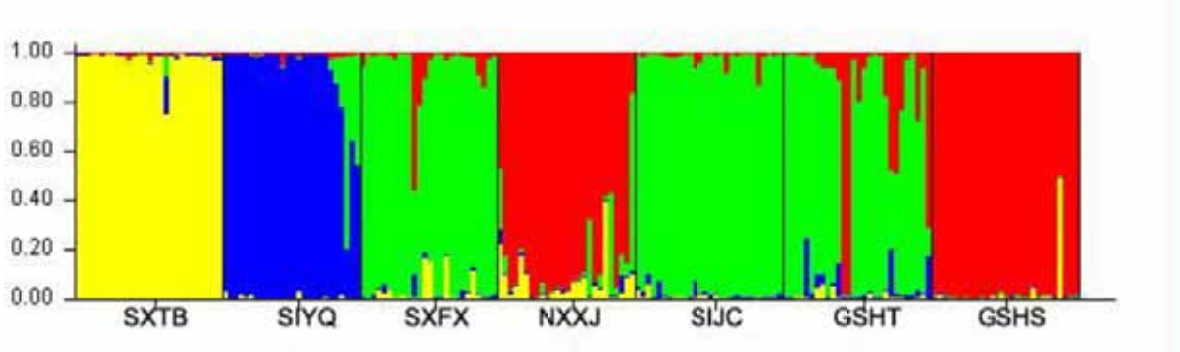

Figure 5. Population structure of 190 individuals from seven populations using 23 loci (k=4)

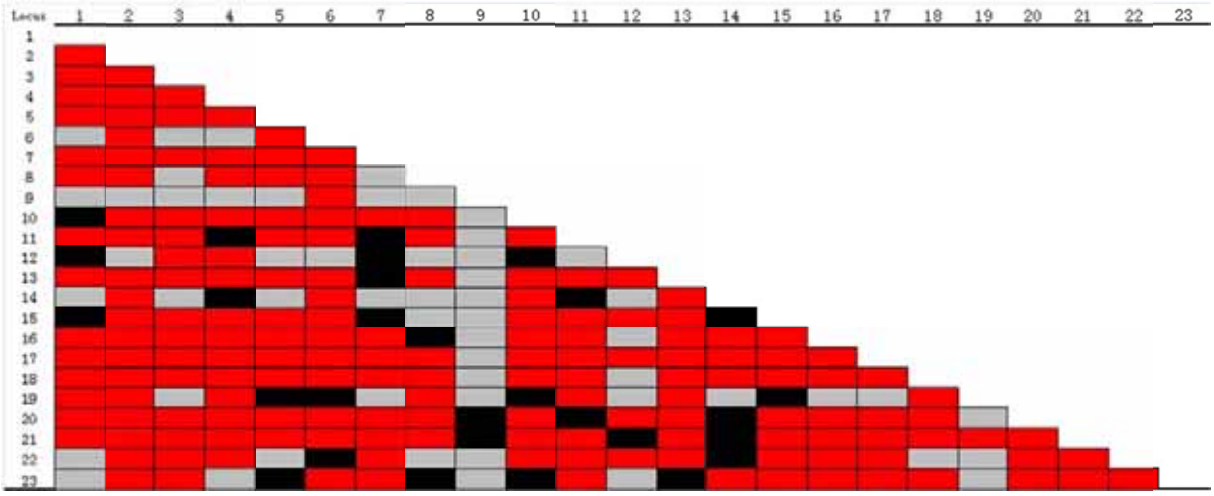

Figure 6. Test of linkage disequilibrium between all pairs of loci in seven populations

Note: Red and black filled cells mark indicates significant linkage disequilibrium of pairwise loci at the 1% or 5% significance level. The numbers 1, 2, ……, 23 represent the SSR loci amplified in Table 2 in order. Below is as the same.

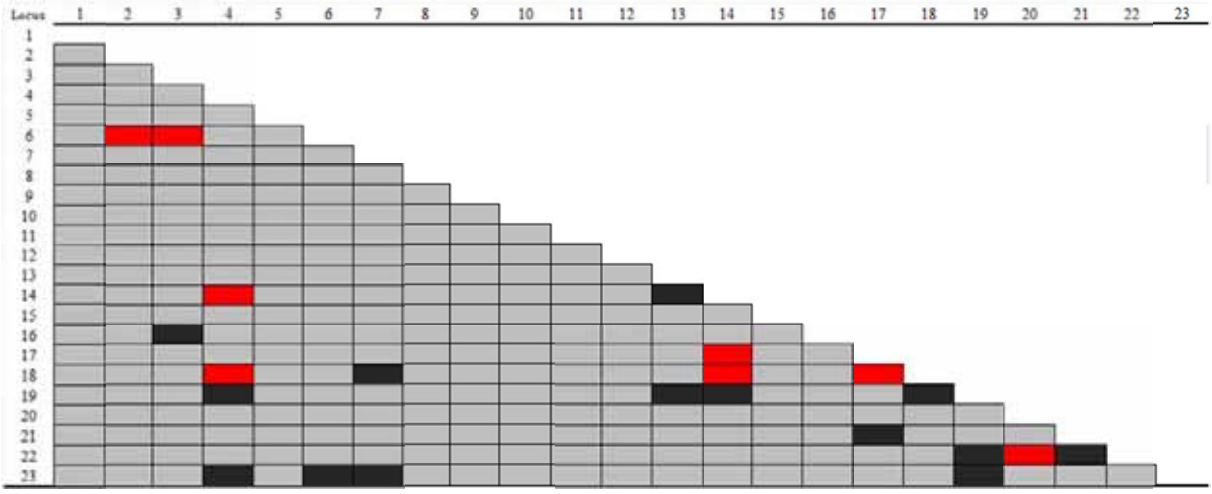

Figure 7. Test of linkage disequilibrium between all pairs of loci in the SXTB population

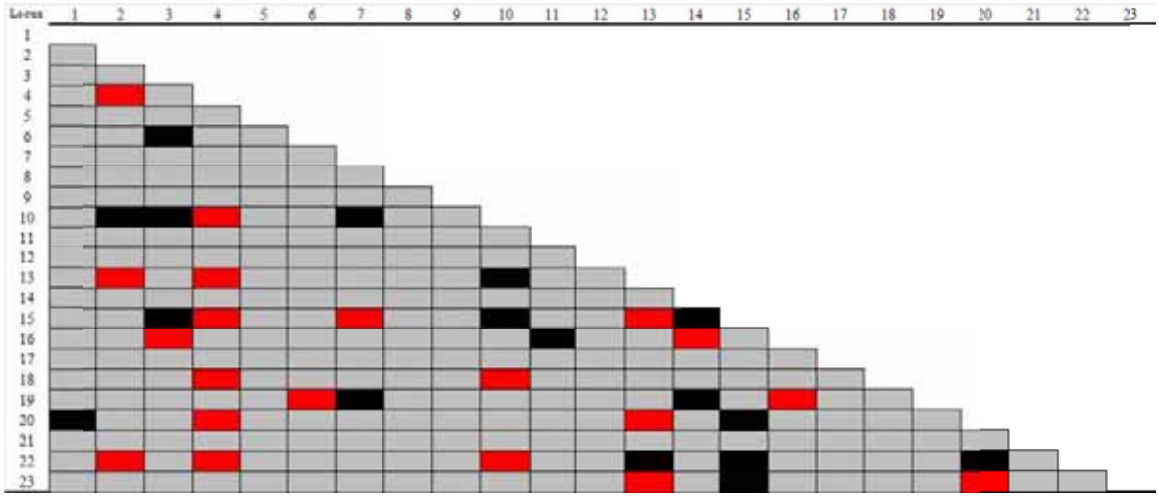

Figure 8. Test of linkage disequilibrium between all pairs of loci in the SIYQ population

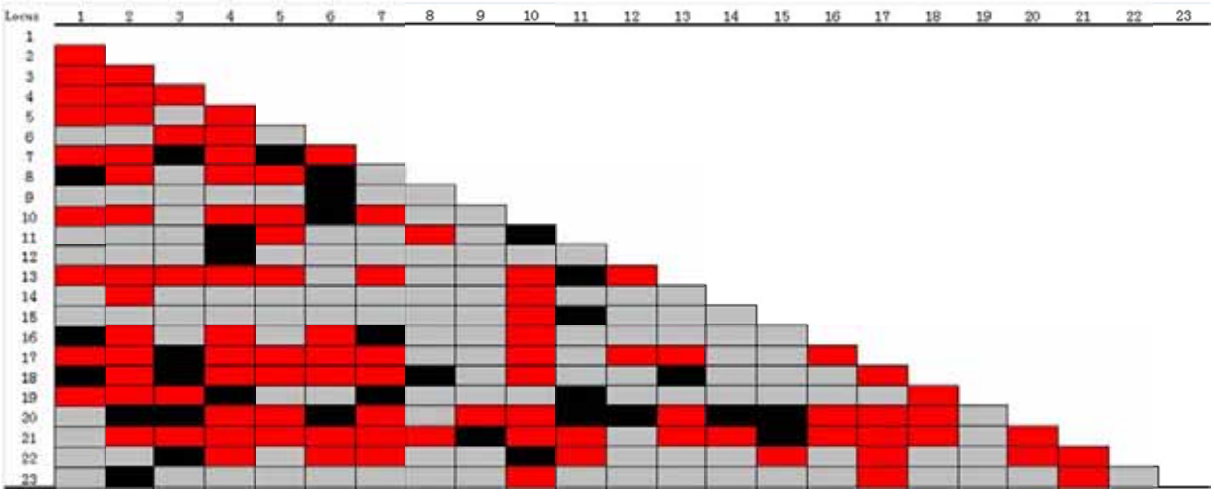

Figure 9. Test of linkage disequilibrium between all pairs of loci in the SXFX, SIJC and GSHS populations

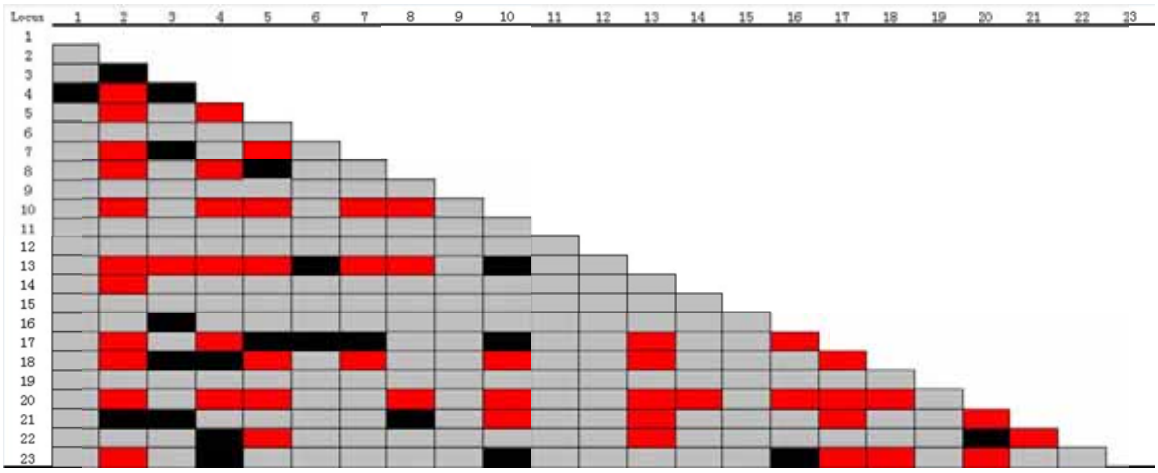

Figure 10. Test of linkage disequilibrium between all pairs of loci in the NXXJ, GSHS populations

3.4 Selective Loci

Wild populations under different environments experienced natural selection, so selective loci maybe appeared in whole populations or between populations. We used Arlequin ver 3.5.1 in finite model to detect selective loci (Table 7). There were five loci with significance at 5% level among populations, of which two with 1% level were apparently located outside of scattered dots plot of 30000 of coalescent simulations performed for all populations (Figure 11). When data of lnRH values were tested using beyond 95% confidence scale, 18 outlier loci or selective loci were found between populations (Electronic supplementary material S1), while data of lnRH values were tested by Grubbs, there were only three outlier loci between populations (Electronic supplementary material S2). Although the two testing methods detected different number of selective loci, there still were two similar loci, BPPCT 025 (between SXFX and SIJC) and CPPCT022 (between SIYQ and NXXJ). Allele frequencies in loci BPPCT 025 or CPPCT022 between populations apparently were different from each other, and variation of allele number became narrow (Figure 12a, b).

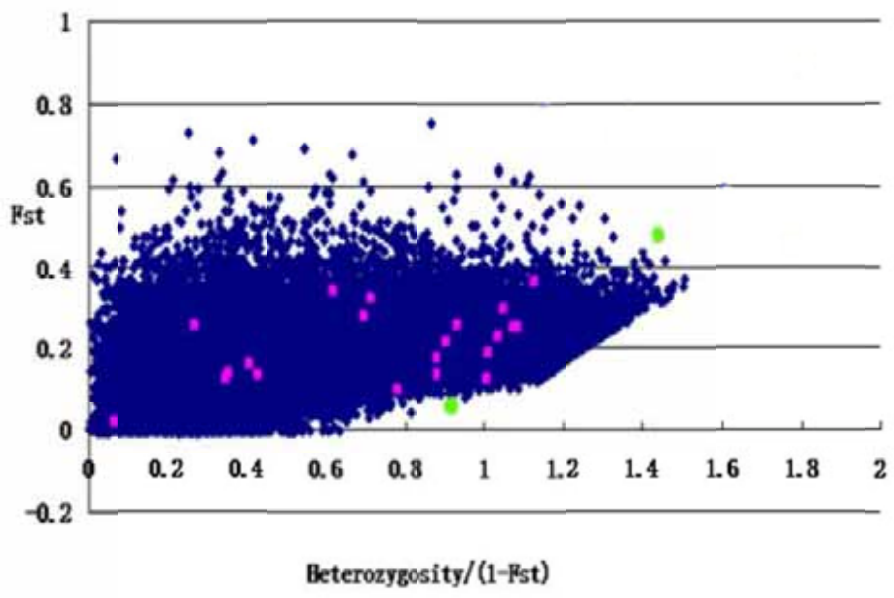

Figure 11. Joint distribution of Fst against heterozygosity. Pink diamonds correspond to observed microsallite loci, whereas blue dots are simulated loci under finite model with 30000 permutations. Significant loci ($P < 0.01$) are shown as large green dots

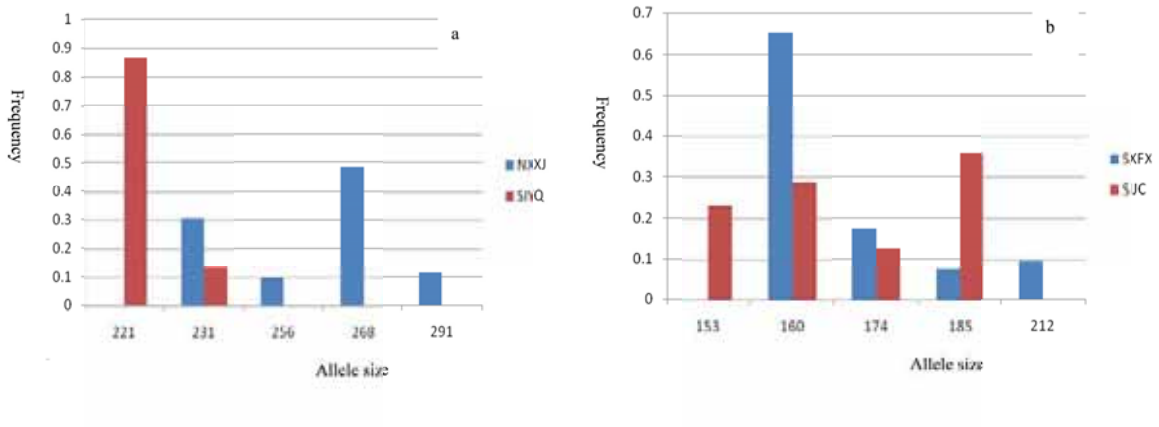

Figure 12. Frequency distribution of allele sizes with significance(P<0.01) under mutual comparisons of all populations. a) Frequency distribution of alleles amplified by BPPCT 025 between NXXJ and SIYQ populations. a) Frequency distribution of alleles amplified by CPPCT 022 between SXFX and SIJC populations

4. Discussion

Information of genetic diversity from both markers and populations in this study appeared very affluent. Inbreeding coefficients with positive values inferred to self-compatible type as main reproduction, justifying that isolated populations has very few chance to exchange genes from outside populations, and conforming to population differentiation among populations (Table 6).

Table 6. Fst values between populations calculated with distance method of pairwise difference

	SXTB	SIYQ	SXFX	NXXJ	SIJC	GSHT	GSHS
SXTB		*	*	*	*	*	*
SIYQ	0.323		*	*	*	*	*
SXFX	0.301	0.212		*	*	*	*
NXXJ	0.207	0.248	0.163		*	*	*
SIJC	0.317	0.239	0.179	0.190		*	*
GSHT	0.277	0.204	0.174	0.170	0.147		*
GSHS	0.320	0.334	0.236	0.141	0.243	0.173	

Note: Fst Values listed below diagonal and "*" with significance at 5% level shown above diagonal.

Seven populations were analyzed with population spatial structure, displaying that no correlation between genetic distance and geographic distance (Figure 2). There is not any spatial structure, at least two reasons are assumed as follows: first, the populations separates with long distance, and pollination is very difficult for individuals from different population; second, fruits of *P. davidiana* cannot be eaten because of no flesh, so fruits and seeds seldom have been brought to other places for growing as natural populations. The fact conformed to inbreeding coefficients with most positive values detected by the markers. Just as through analysis of spatial genetic structure of the Laperrine's olive which was very isolated endangered populations (Besnard et al., 2007), evident genetic particularities have to be urgently considered for their endemism. STRUCTURE and PCA analysis can identify population structure and be effectively applied for deciding groups. Population structure of wild olives and commercial maize (Belaj et al., 2007; Inghelandt et al., 2010) built four gene pools for gemplasm, respectively. By using successful methods, Population structure indicated there were four groups in this study (Figure 3, 4, 5). Fst between all studied populations were significant (Table 6). All information from above analyses for population structure of *P. davidiana* infers that we try to conserve individuals from four groups of these wild populations.

LDs in the whole population and unstructured populations expressed great differences. What reason is for highest value in the whole population is that there is population structure existed in it, which causes spurious LD. For example, Wang et al. (2008) found that 63.89% LDs of loci pairs at a 1% level were in the entire sample, but a range of 18.75–40.28% was in the subgroups. We selected unstructured populations for further analyzing of LD, Many LDs of loci pairs in unstructured populations were detected, which explained that natural populations might have experienced genetic bottleneck from their progenitor and natural selection for a long time, and self-compatible individuals generated genetic drift because some deadly genes became homogeneous. LDs screened the populations creates precondition for association mapping and marker assisted selection (MAS). In this study, mean value of 25.7% of loci pairs (SXTB; SIYQ; SXFX SIJC and GSHT; NXXJ and GSHS were 8.6%, 14.6%, 51.0% and 28.5% at 5% significant level, respectively) in *P. davidiana* (Figure 7, 8, 9, 10) was higher than that of 15.1% of the three subpopulations of cultivars in related *P. persica* (melting peaches, nectarines and non-melting peaches were 13.9%,13.4% and 18%, respectively) (Aranzana et al., 2010). The two species belonged to the same genus had more difference of LD, while the latter maybe came from more recombination due to cultivars bred from crossing. Other studies (Barnaud et al., 2009; Rossi et al. 2009) also justified that domestication bottlenecks and vegetative propagation are the primary factors responsible for this difference between cultivated and wild grapevine. Differences of LDs among unstructured populations may be explained that they had different number of accessions, membership of accessions and differential selection for adaptation to complicated environments or for special traits in these populations, but the information still can help us to select ideal populations for association mapping.

Table 7. Selective loci detected in finite model for all individuals from seven populations

Locus	Obs.Het. BP	Het./(1-Fst)	Obs. Fst	Fst P-value
BPPCT 006	0.304	0.354	0.140	0.304
BPPCT 007	0.805	1.074	0.250	0.298
BPPCT 008	0.765	0.883	0.134	0.066
BPPCT 015	0.801	1.035	0.226	0.436
BPPCT 017	0.697	0.934	0.254	0.318
BPPCT 020	0.501	0.695	0.279	0.248
BPPCT 025	0.702	0.780	0.100	0.018*
BPPCT 028	0.199	0.268	0.256	0.243
CPPCT 002	0.061	0.062	0.020	0.065
CPPCT 006	0.741	1.050	0.295	0.142
CPPCT 016	0.483	0.713	0.322	0.137
CPPCT 017	0.340	0.406	0.163	0.368
CPPCT 022	0.748	1.438	0.480	0.000**
CPPCT 033	0.369	0.425	0.133	0.225
UDP96-001	0.408	0.618	0.341	0.123
UDP96-003	0.710	0.907	0.217	0.494
UDP96-005	0.880	1.006	0.125	0.018*
UDP96-013	0.303	0.346	0.125	0.246
UDP97-403	0.820	1.011	0.189	0.319
UDP98-024	0.719	1.127	0.362	0.032*
UDP98-025	0.813	1.085	0.251	0.289
UDP 98-409	0.727	0.882	0.176	0.265
UDP98-412	0.861	0.921	0.065	0.000**

Note : '*'and '**' represent significant level at 1% and 5%, respectively

To adapt to various environments, natural populations through selection have caused variation of alleles. Generally, as long as favorable genes were fixed for positive selection, usually as expression of outlier loci, variation of gene frequencies became low. We used Arlequin ver 3.5.1.2 in finite model to detect outlier loci for individuals from all populations. Five loci were significant for selection in all populations, inferring that genes experienced coinciding evolution with history of demography (Table 7). Two of these loci with 1% significant level, located beyond 30000 permutations plot (Figure 11), demonstrated two positive selective types, one with low Fst at the bottom of the plot was balancing selection; the other with high Fst at the upper of the plot and high heterozygosity was directional selection (Excoffier et al., 2009). On the other hand, we detected selective loci appeared between populations. Selective loci using lnRH tested by both standard distribution scale and Grubbs appeared different number (Electronic supplementary material S1, Electronic supplementary material S2). From statistics, Grubbs test, used for detecting outliers, is more strictly than test of standard deviation. Both methods detected common selective loci: BPPCT 025 loci between SXFX and SIJC, CPPCT022 loci between SIYQ and NXXJ. Allele frequencies displayed apparently differences between populations (Figure 12a,b) as local selective sweep found in human populations (Kayser et al., 2003; Schlotterer, 2002). Some genes of the two loci are very possible responsibility for disclosing adaption evolution and digging out candidate genes.

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Determination of Races and Biovars of *Ralstonia solanacearum* Causing Bacterial Wilt Disease of Potato

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Abstract

A survey was carried out in some selected potato growing districts of Bangladesh during December to February 2011 to know the status of bacterial wilt of potato caused by *Ralstonia solanacearum* in terms of its incidence and severity. The results showed that the highest wilt incidence was recorded in Munshigonj (22.65%), followed by Nilphamari (19.98%) and the lowest incidence was recorded in Jamalpur (9.07%). The highest bacterial wilt severity was recorded in Munshigonj (3.80), while the lowest wilt severity was recorded in Jamalpur (2.90). A total of 44 isolates (*R. solanacearum*) were obtained from the wilted potato plant samples i.e. 20 from Munshigonj, 17 from Nilphamari and 7 from Jamalpur and the isolates were divided into three groups. The production of pink or light red colour with whitish margin on TZC medium by the bacterial isolates indicated all groups of *R. solanacearum* isolates were virulent. The results of pathogenicity test revealed that all groups of *R. solanacearum* isolates were able to cause wilt symptoms in potato plants and brown rot symptoms in potato tuber. On the other hand, all biochemical tests were used for the identification of *R. solanacearum* isolates. The biovar test using the oxidization of disaccharides (sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) by *R. solanacearum* isolates confirmed that all groups of *R. solanacearum* isolates belong to biovar III. The race identification of *R. solanacearum* isolates by pathogenicity test on brinjal, tomato, tobacco and chilli indicating a narrow host range (only in potato) and were categorized in race 3. Therefore, the *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh were belonging to Biovar III and Race 3.

Keywords: biovar, race, *Ralstonia solanacearum*, wilt, potato

1. Introduction

Potato (*Solanum tuberosum* L.) is a herbaceous tuber crop belonging to the family Solanaceae. It is one of the three leading staple food crops next to rice and wheat and is of course the most important vegetable grown in Bangladesh and also in the world (Ahmed & Talukder, 1978). It is cultivated and recognized as popular vegetable throughout the entire tropical and subtropical region of the world (Hayward, 1991). Potato is locally known as “Alu”. The crop extends substantial amount of high quality protein and essential vitamins, minerals and trace elements to the human diet (Horton, 1987). It produces more carbohydrates per unit amount than either rice or wheat. In Bangladesh, potato is a crop of great economic significance.

The major potato growing areas of Bangladesh are Munshigonj, Jamalpur, Nilphamari, Jessore, Bogra, Chandpur and Panchagorh. The total acreage of potato is 977 thousand hectares with total annual production of 5268 million tones (BBS, 2009). It contributes alone as much as 54% of the total annual vegetable production of Bangladesh (BBS, 2009). However, the production of potato is quite low as compared to the major potato growing countries of the world. The reasons behind the low yields of potato are different bacterial, fungal, viral and nematodes diseases and poor management practices. Among these, diseases are the most predominant limiting factors the potato bacterial wilt was the most important problem in Bangladesh. A total of fifteen diseases have been recorded in Bangladesh of which early and late blight, wilts (Bacterial, fungal and

nematodes), scab, stem rot, stem canker/scurf, potato leaf roller, potato mosaic virus, dry rot and soft rot are the major diseases of potato in Bangladesh (Christ, 1998).

Bacterial wilt caused by *Ralstonia solanacearum* (Smith, 1986), formerly called *Pseudomonas solanacearum* (Yabuuchi et al., 1976), a soil-borne gram-negative bacterium is a recognized parasite in over 200 families of plants, including potato, brinjal and tomato as well as many native plant species. The bacterium normally invades plant roots from the soil through wounds or natural openings, colonizes the intercellular space of the root cortex and vascular parenchyma, and eventually enters the xylem vessel and spreads up into the stem and leaves. Affected plants suffer chlorosis, stunting, wilting, and usually die rapidly. Losses caused by the disease are known to be enormous but cannot be accurately estimated because of abandonment of wilt-susceptible crops in many parts of the world.

The species *R. solanacearum* is a complex taxonomic unit with broad physiological and genetic diversity. *R. solanacearum* was classified into five races on the basis of different host range (Buddenhagen et al., 1962; He et al., 1983; Pegg & Moffet, 1971), and six biovars according to the ability to oxidize three hexose alcohols and three disaccharides (Hayward, 1964, 1991; Hayward & Hartman, 1994; He et al., 1983). Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not based on gene-for-gene interactions i.e., different cultivars carrying different R gene(s). Instead, these are determined based on the pathogenicity of each isolate in different kinds of host plants. Although the biovar and race systems are widely accepted for the classification of *R. solanacearum,* there is no definite correlation between biovar and race. Each race transects the biovars and each biovar contains various races. The only positive correlation between the biovar and race systems exists for biovar 2 and race 3 (Patrice, 2008).

The main control strategy for bacterial wilt has been the use of resistant varieties. However, the stability of bacterial wilt resistance in brinjal, potato and tomato, is highly affected by pathogen density, pathogen strains and several soil factors. The other control methods such as cultural, chemical and biological were not found effective against bacterial wilt disease of potato due to wide host range and genetic diversity of its pathogen, *R. solanacearum*. Information on its pathogen population especially biovars and races are essential to formulate a pathogen-targeted and geographically-targeted integrated management strategy against the disease. Therefore, the present study was undertaken to determine the biovars and races of *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh at least to step forward for designing an effective management approach.

2. Materials and Methods

2.1 Surveying and Sampling

A survey was carried out to know the status of bacterial wilt of potato in Bangladesh in terms of its incidence and severity in some selected districts viz. Jamalpur, Munshigonj, Panchagarh, Bogra, Jessore, Chandpur and Nilphamari during December to February, 2011. At least three locations in each district and five farmers' fields from each location were surveyed to record the bacterial wilt incidence and severity. For a quick field diagnosis, the streaming of milky white masses of bacterial cells (ooze) confirmed the disease is bacterial wilt caused by *R. solanacearum* and to distinguish bacterial wilt from vascular wilts caused by fungal pathogen and nematode. At least 10 samples of the diseased plants were collected from each of the surveyed district and were brought to the laboratory for the isolation of different group of isolates of *R. solanacearum*.

2.2 Assessment of Disease Incidence and Severity

The status of bacterial wilt of potato was surveyed in terms of its incidence and severity. Data on wilt incidence were recorded in at least three locations from five farmer's fields for each district. Then the per cent wilt incidence was calculated by the following formula:

$$\%\ \text{Wilt incidence} = \frac{\text{Number of wilted plants in each field}}{\text{Total number of plants in each field}} \text{X } 100$$

Five plants were randomly selected from each farmer field from each location to calculate the wilt severity in each district. The severity of bacterial wilt was recorded based on the severity scale as described previously by Horita and Tsuchiya (2001). Briefly, 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = Plant dies.

2.3 Isolation, Identification, Purification and Preservation of R. solanacearum

The wilted plant samples collected from the fields were washed under running tap water to remove sand and soil. The stem of the infected potato plants were surface sterilized with 70% alcohol and cut into two halves and the cut ends were dipped into water in test tubes. After waiting around half an hour, bacterial ooze was seen to come

out into the water from the infected stem. Then a loop full of water was streaked on the Nutrient Agar (NA) plate. The plates were then incubated at 28°C for at least 24 hours to grow the bacterium in the medium. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC) plate (Kelman, 1954). The isolates of *R. solanacearum* were then preserved in 10% skim milk and kept at -20°C refrigerator for subsequent studies. The collected isolates from different potato growing districts were classified into three groups based on the obtained districts. To confirm the isolates of *R. solanacearum*, the pathogenicity test was performed on one month old potato seedlings by soil inoculation method with approximately 10^8 CFU/ml. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the centre was selected for each group of isolates and multiplied in a TTC (without adding Triphenyl Tetrazolium Chloride) for pathogencity test. At 30-40 days age of tobacco plants, bacterial suspension (approximately 10^8 CFU/ml) of each isolate representing a group was injected into the intracellular space of the leaf with a hypodermal syringe. Hypersensitive reaction was observed daily and continued until five days after infiltration.

2.4 Biochemical Characterization of R. solanacearum

2.4.1 Identification of Virulent and Avirulent Isolates

The virulent (colonies with pink or light red colour or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) strains of *R. solanacearum* were identified in TTC medium containing 0.005% TTC (Kelman, 1954).

2.4.2 Biochemical Tests for the Identification of *R. solanacearum*

Several biochemical tests viz. Gram staining reaction, Potassium hydroxide solubility test, Kovac's oxidase test, Levan test and Sugar fermentation test were performed for confirmation of *R. solanacearum* isolates as described previously by Rahman et al. (2010) and Hossain (2006). Single isolate of *R. solanacearum* from each group was randomly selected for biochemical tests.

2.4.3 Determination of Biovars

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides (sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) as described previously by Hayward (1964) and He et al. (1983). The biovars were determined in the mineral medium ($NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0 g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 200µl of the melted medium was dispensed into the wells of microtitre plate. Inoculums for each group of isolate was prepared by several loop full of bacteria from 24-48 hours old cultures to distilled water to make suspension containing about 10^8 CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28°C. The plates were then examined after 3 days of inoculation for changing pH which was indicated by the change of colour (Schaad, 1988).

2.5 Races Identification

The races of *Ralstonia solanacearum* were identified by pathogenicity test on wide host range (Denny & Hayword, 2001). Seedlings of brinjal, tomato, tobacco and chilli were raised in tray. One month (30 days) old seedlings (brinjal, tomato & chilli) were inoculated by soil inoculation method. The incubated plants were then kept in the net house until symptoms development.

3. Results and Discussion

3.1 Incidence and Severity of Bacterial Wilt

A total of seven selected potato growing districts viz. Jamalpur, Munshigonj, Panchagarh, Bogra, Jessore, Chandpur and Nilphamari were surveyed to know the status of bacterial wilt of potato in terms of its incidence and severity. However, bacterial wilt infection was noticed in three districts namely Munshigonj, Nilphamari and Jamalpur. A significant variation was observed in terms of bacterial wilt incidence among the selected growing areas surveyed (Table 1). The survey results showed that the highest bacterial wilt incidence was recorded in Munshigonj (22.65%), followed by Nilphamari (19.98%) while the lowest bacterial wilt incidence (9.07%) was recorded in Jamalpur (Table 1). On the contrary, the highest bacterial wilt severity was recorded in Munshigonj (3.80), while the lowest bacterial wilt severity was recorded in Jamalpur (2.90) during the time of survey (Table 1). Moreover, these variations of wilt incidence and severity may be attributed due to the diversity of *R. solanacearum* isolates and also due to the variations in soil factors prevailing in different locations surveyed. Differences of wilt incidence and severity were also reported in eggplant due to the great diversity of host plants

affected by this pathogen, phenotype and genotype of *Ralstonia solanacearum,* its wide geographical distribution, and the range of environmental conditions conducive to bacterial wilt (Rahman et al., 2010).

Table 1. Incidence and severity of bacterial wilt at selected potato growing areas in Bangladesh

Areas Surveyed	Number of isolates	Group	Wilt incidence (%)	Wilt severity (1-5 scale) *
Munshigonj	20	I	22.65a	3.80
Nilphamari	17	II	19.98b	3.00
Jamalpur	7	III	9.07c	2.90
LSD			1.371	
Level of significance			**	

** Significance at 1% level of probability; *Severity data recorded at the time of survey.

3.2 Isolation and Identification of the R. solanacearum Isolates

A total of 44 *R. solanacearum* isolates were obtained from the wilted potato plant samples i.e. 20 from Munshigonj (Group I), 17 from Nilphamari (Group II) and 7 from Jamalpur (Group III) (Table 1). Although equal numbers of samples were collected from each of the surveyed area, the number of isolates varied because of failure of isolation of the bacterium from all the infected plants. All of the *R. solanacearum* isolates collected from wilted potato plants produced cream colour or off-white colour colonies on NA media after 24 hours of incubation at 28°C.

3.3 Pathogenicity and Hypersensitive Response (HR) Test

The results of pathogenicity test revealed that all the isolate groups of *R. solanacearum* were able to produce wilt symptom in potato plants incubated by soil inoculation method (Table 2). The isolates of *R. solanacearum* were also tested for inducing brown rot symptom on the potato tubers. The results revealed that all isolates groups of *R. solanacearum* were able to produce brown rot symptoms on tubers. On the contrary, the *R. solanacearum* isolate obtained from wilted brinjal plants was not capable to produce any brown rot symptom in the potato tuber. The isolates of *R. solanacearum* collected from the wilted potato plant were tested for hypersensitive reaction in tobacco. The result showed that none of the isolates was able to cause the death of local cell of tissue between veins of tobacco leaves.

Table 2. Pathogenicity, hypersensitive response (HR) and biochemical tests of different isolate groups of *R. solanacearum*

Isolate Name	Number of isolates	Pathogenicity test and colour test on TTC media	HR Test	Gram staining reaction	KOH solubility test	Kovac's oxidase test	Levan test	Sugar fermentation test				Inference
								Dextrose	Sucrose	Manitol	Lactose	
Group I	20	+	-	+	+	+	+	+	+	+	+	*R. solanacearum*
Group II	17	+	-	+	+	+	+	+	+	+	+	*R. solanacearum*
Group III	7	+	-	+	+	+	+	+	+	+	+	*R. solanacearum*

+ Positive reaction.

- Negative reaction.

GroupI: Munshigonj, Group II: Nilphamari and Group III: Jamalpur.

3.4 Biochemical Tests

3.4.1 Gram's Stain

The Gram's staining reaction was performed using crystal violate. The microscopic results showed that all of the isolates of *R. solanacearum* did not retain violet colour i.e. the isolates retained counter stain (pink colour). Therefore, all isolates of *R. solanacearum* representing each group are gram negative and straight or curved rod shaped which is the characteristic feature of any plant pathogenic bacteria (Table 2).

3.4.2 Potassium Hydroxide Solubility Test

The gram negative test of *R. solanacearum* was also confirmed by Potassium hydroxide solubility test. The result revealed that a elastic thread or viscous thread was observed when loop raised from the bacterial solution by toothpick, a few centimeters from glass slides in case of all group indicating that all groups of *R. solanacearum* isolates are gram negative (Table 2).

3.4.3 Kovac's Oxidase Test

Kovac's oxidase test was also carried out to know the oxidation ability of *R. solanacearum* isolates. the result showed that all groups of *R. solanacearum* isolates were able to develop deep blue colour with oxidase reagent within few seconds which indicated that the tested group of *R. solanacearum* isolates were Gram negative (Table 2).

3.4.4 Levan Test

Levan is an intracellular bacterial polysaccharide (beta-2, 6-1 linked D-fructan), whose potential and actual uses are similar to those of dextral (Avigad, 1968). In this study, induction of the Levan was performed in NA medium containing 5% sucrose, Levan sucrase (E.C.2.4.1.10), which catalyzes the synthesis of Levan form sucrose, is produced by a number of bacteria including *R. solanacearum*. The result showed that all group of *R. solanacearum* isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 2).

3.4.5 Sugar Fermentation Test

The *R. solanacearum* is able to oxidize the sugars which are indicated by colour change (reddish to yellow). The results of sugar fermentation test clearly showed that all groups of *R. solanacearum* isolates obtained from the wilted potato plants samples were able to oxidize the four (4) basic sugars (Dextrose, sucrose, manitol and lactose) by producing acid and gas. The acid production in sugar fermentation test by bacterial isolates were indicated by the colour change from reddish to yellow, gas production was noted by the appearance of gas bubbles in the inverted Dhuram's tubes and the oxidation of sugar manitol by the bacterial isolates indicated by the production of yellow to red colour (Table 2). The isolates were also characterized by the hypersensitive response (HR) test in tobacco and also by different biochemical tests. These results were supported by the findings of Dhital et al. (2001) who observed that *R. solanacearum* was able to produce wilt symptoms in potato, HR in tobacco leaves and was found positive by a series of biochemical tests. Also, the Gram staining and KOH solubility test indicated that the isolates of *R. solanacearum* are gram negative. Suslow et al. (1982) reported that the KOH technique is far easier and faster to distinguish gram negative and gram positive bacteria than the traditional Gram-straining test in which dyes are employed. Like other gram negative bacteria, the isolates of *R. solanacearum* were able to develop blue colour in Kovac's test by oxidizing the sugars and were able to ferment four basic sugars (Dextrose, sucrose, manitol and lactose) to produce acid and gas. The isolates of *R. solanacearum* were also able to produce round or circular domed shaped colonies in sucrose medium. When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose. During this fermentation process, the bacteria utilize sucrose for maintenance and their growth.

3.5 Identification of Virulent/Avirulent Strains of R. solanacearum

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman Tetrazolium Chloride (TZC) agar test. In this test, virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation. Result of this test showed that all groups of *R. solanacearum* isolates collected from different growing areas produced pink or light red colour colonies or colonies with characteristic red centre and whitish margin on TZC medium (Table 2). This indicates that all *R. solanacearum* isolates were virulent. Kelman (1954) reported that avirulent colony types of *R. solanacearum* could be easily differentiated by the pigmentation from the wild virulent types. *R. solanacearum* developed two types of colonies on tetrazolium chloride (TZC) medium on which virulent colonies appear as white with pink centres and non-virulent colonies appear as small off-white colonies. On this medium, typical bacterial colonies appear fluidal, irregular in shape, and white with pink centres after 2 to 5 days incubation at 28ºC as reported by Champoiseau (2008). *R. solanacearum* produced fluidal colonies with pink or light red colour on TZC media after 24 hours of inoculation as reported previously by Rahman et al. (2010).

3.6 Biovar Differentiation

The result of the biovar test showed that all *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5 days. The oxidation reaction was indicating by the change of colour. The results revealed the change of colour blue to yellow indicating the oxidization of sugars by bacterial isolates. Therefore, all groups of *R. solanacearum* isolates belong to biovar III as shown in Table 3. On the other hand, all the control plates of different sugar and sugar alcohol remain unchanged. The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported previously by Hayward (1964), He et al. (1983), Kumar et al. (1993). Also, they observed that biovar III oxidizes both disaccharides and hexose alcohols, biovar II oxidizes only disaccharides whereas Biovar I oxidizes hexose alcohols only, and biovar IV oxidizes only alcohols.

Table 3. Differentiation of *Ralstonia solanacearum* into biovars and races

Isolate group	Utilization of carbohydrates						Biovars	Races
	Dextrose	Maltose	Lactose	Sorbitol	Manitol	Dulsitol		
1	+	+	+	+	+	+	III	3
2	+	+	+	+	+	+	III	3
3	+	+	+	+	+	+	III	3

+ Positive reaction, - Negative reaction.

Isolate Group 1: Munshigonj, Isolate Group 2: Nilphamari and Isolate Group 3: Jamalpur.

3.7 Identification of Races

There is no biochemical test for race identification of bacterial wilt pathogen *R. solanacearum.* The races of *R. solanacearum* were identifying by pathogenicity tests in wide host range such as brinjal, tomato and chilli. The result of the pathogenicity test showed that none of the group of *R. solanacearum* isolates tested in the study was not able to cause wilt symptom in inoculated brinjal, tomato and chilli plants indicating a narrow host range but the isolates produced wilt symptom in potato seedlings (Table 2). Therefore, all groups of *R. solanacearum* isolates causing bacterial wilt of potato collected from three selected growing areas belong to race 3. On the other hand, isolate obtained from wilted brinjal plant inducing wilt symptom in tomato, chilli and brinjal which is belonging to race 1. Denny and Hayward (2001) identified race of *R. solanacearum* by host range. The findings of the present study are also supported by Buddenhagen et al. (1962) who classified *R. solanacearum* into three races who found only one race. Race 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds. In addition to race 2 that causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp*.,* while race 3 affects potato and tomato but it is weakly virulent on other solanaceous crops. Aragaki and Quinon (1965) reported that race 4 infected ginger in the Philippines. He et al. (1983) reported race 5 from mulberry in China. Five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions (French, 1986). Patrice (2008) reported that *R. solanacearum* was initially subdivided into races and biovars based on variability in host range. He added that five races have been identified within the species. Strains of *R. solanacearum* have also been divided into five host-specific races by Pradhanang et al. (2000). However, the results of this study primarily indicated that bacterial wilt pathogen of potato; *R. solanacearum* is belonging to race 3 although no wilt symptom was observed in tomato.

4. Conclusion

The incidence of bacterial wilt varied in the major potato growing areas may be due to the species complex of the pathogen, *R. solanacearum* and also for various soil factors. Biovar III and Race 3 of *R. solanacearum* was only prevalent in all growing areas of potato in Bangladesh. The findings of the present study will be useful for designing the study of the population structures of *R. solanacearum* using the molecular approaches with special emphasis on its integrated management.

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Anti-Microbial Properties of Secondary Metabolites of Marine *Gordonia tearrae* Extract

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Abstract

The objective of this study is to screen four species of marine actinomycetes: *Gordonia* (SPTG111), *Rhodococcus* sp (BP33), *Gordonia* sp (BP5), *Brevibacterium antarcterium* (SPTG45). These bacteria were grown in seven different media, namely Braine Heart Infusion broth (BHIB), Nutrient broth, Marine broth, Trypticasein Soy Broth, Tryptic Soy Broth, Luria Bertani broth, and Lauryl Sulfate Broth. Brine heart infusion broth (BHIB) was used to growth bacteria for the antibacterial activity test. Crude extract of *Gordonia tearrae* was tested for antimicrobial activity against *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (*MRSA*), *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli, Salmonella typhi*, *Pseudomonas aeruginosa* and *Candida albicans* using agar diffusion method. The minimal inhibitory concentration (MIC) of *G. tearrae* crude extract was determined by the microbroth dilution method. Ethyl acetate extract of this isolate showed significant antibacterial activity against Gram negative bacteria *E. coli* (20 mm), *Pseudomonas aeruginosa* (9 mm), *Salmonella typhi* (12 mm) and Gram positive bacteria *E. faecalis* (7 mm), *Bacillus cereus* (21 mm), and *MRSA* (30 mm), and yeast *C. albicans* (21 mm). The MIC value of antimicrobial activity ranged from 25 to 6.3 µg/ml.

Keywords: *Gordonia tearrae*, BHIB, yield of extraction, antibacterial, antifungal, MIC

1. Introduction

Infectious disease mortality rates are increasing in developed countries (Pinner et al., 1996). Jones et al. (2008) reported the emergence of 335 infectious diseases between 1940 and 2004 in the global human population. These negative health trends call for a renewed interest in infectious disease as well as effective strategies for treatment and prevention. Natural products are one of the most important sources of antibiotics (Bull & Stach, 2007). With respect to the development of new antimicrobials, the marine environment holds great promise for the discovery of novel bioactive compounds. Marine sponges are among the most ancient multicellular animals. These sessile, filter feeding animals are a rich source of novel biologically active metabolites and offer great potential for drug discovery and, in the long term, for treatment of cancer and infectious diseases (Blunt et al., 2007). Members of the phylum Actinobacteria and specifically the order Actinomycetales have been identified as abundant members of sponge-associated microbial communities (Hentschel et al., 2002; Zhang et al., 2006). Their existence in the marine environment has been further shown in marine sediments as well as in the deepest ocean trenches (Bredholdt et al., 2007; Maldonado et al., 2005). Actinomycetes are of considerable interest owing to their ability to produce new chemical entities with diverse pharmacological activities. Marine actinomycetes in particular have yielded numerous novel secondary metabolites (Lam, 2006). Several antibiotics have already been derived from marine actinomycetes (Baltz, 2008) and at present, two thirds of natural antibiotics are obtained from actinomycetes. They also serve as alternative sources of biologically active substances (Behal, 2003) especially in combating infectious diseases which today are leading health problems with high morbidity and mortality in the developing countries (Black et al., 1982). Several reports are available on antibacterial and antifungal activity of marine actinomycetes (Suthindhiran & Kannabiran, 2009; Bredholt et al., 2008) but relatively few studies are available for *Gordonia* species. *Gordonia tearrae* is one of 27 *Gordonia* species of marine actinomycetes (Arenskotter et al., 2004). Previously known as "*Gordona tearrae*" and "*Rhodococcus tearrae*", it is a nocardioform actinomyces isolated from soil and water (Tsukamura, 1971; Stackebrandt, 1997). In this study,

we report the antimicrobial activities of methanol crude extract of *Gordonia tearrae* (SPTG 11-1) which isolated from pulau tinggi sponge.

2. Materials and Methods

2.1 Actinomycetes Bacteria Collection

Four actinomycetes includes *Gordonia* (SPTG 11-1), *Brevibacterium antarcterium.* (SPTG 45), were isolated from (Pulau Tinggi sponge), and *Gordonia* sp (BP5), *Rhodococcus* sp (BP 33), were isolated from (Langkwi Kedai Sediment) in Malaysia. They were collected from National University of Malaysia microbe culture unit, and cultured in starch casein agar, marine agar, Actinomycetes isolation agar. These Incubated at 30°C, 1-2 weeks.

2.2 Microbial Pathogens

Bacterial and fungal pathogens tested include. *Escherichia coli* (ATCC 10536)*, Salmonella typhi* (ATCC51312)*, Pseudomonas aeruginosa* (ATCC 27853)*, Enterococcus faecalis* (ATCC14506), *Bacillus cereus* (ATCC11774)*, MRSA* (ATCC11632) and *Candida albicans* (ATCC10231). This strain was maintained on nutrient agar slants (NA; Difco, USA) at 4°C. Test cultures were obtained from the National University of Malaysia microbe culture collection unit.

2.3 Physiological and Morphological Characterization of the Gordonia

Physiological and morphological characterization of *Gordonia* SPTG111 isolate was determined as described by the Bergey's Manual of determinative bacteriology. Following the observation of the colony morphology of *Gordonia* on BHIA, Gram staining of overnight pure cultures was subsequently done. The presence of respiratory enzymes, Oxidase and Catalase were investigated using standard methods as described as Kovacs (1956) and Vera and Power (1980). Motility test was conducted using SIM medium. Different salt concentrations of different media were used for salt tolerance assay.

2.4 Molecular Taxonomy, Sequencing and Phylogenetic Analysis

The genomic DNA of *Gordonia* SPTG111 was isolated by CTAB/NaCl method. The isolate was grown in 50 ml of BHI broth using the universal primers (published forward primer (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (5' GGT TAC CTT GTT ACG ACT 3') for the amplification of DNA. These sequences were acquired from FirstBase Laboratory, Sdn. Bhd., Malaysia. A final PCR reaction volume of 50 µl containing 1 µl supernatant containing DNA, 1 x GoTaq Flexi Buffer (Promega, USA), 2 mM $MgCl_2$ (Fermentas, USA), 1 µM of each forward and reverse primer, 200 µM each dNTP and 2.5 U Taq polymerase. Amplification was carried out in a Mini-cycler (MJ Research, USA). The PCR protocol used was 95°C for 2 min, 51°C for 30 s, 72°C for 45 s, 22 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 45 s. Amplification was followed by a final extension at 72°C for 2 min. After the PCR reaction was completed, 10 µl of obtained PCR product was electrophoresed on a 1% 1× Tris-acetate-EDTA agarose gel containing 1:10,000 gel red. A 1kb DNA ladder (Promega, USA) was also included in the run. PCR products were purified using QIAquick Purification Kit (QIAGEN, Germany) according to manufacturer's instructions. Sequencing was carried out by automatic DNA sequencing machine (ABI Prism 377) at FirstBase Laboratory, Sdn Bhd, Malaysia. Phylogenetic tree was generated by means of neighbour relationships using MEGA software version 4.0.2.

2.5 Scanning Electron Microscopy (SEM)

BHI agar containing isolates that have been incubated for 5 days were cut into small (1 cm^3) pieces using a sterile spatula. For the purpose of fixing, these pieces were immersed in glutaraldehyde 2.5% (w/v) and kept at 4°C for 24 hours. The samples were subsequently washed three times with phosphate pH 7 buffer solution for 10 minutes. Then the samples were dehydrated by an ethanol series beginning with 30% and 50% for 15 minutes and 70% for 24 hours at 4°C. Extended hydration process was achieved using ethanol 80, 85, 90 and 95% for 15 minutes. Specimens in 100% ethanol were critical-point dried in a CO_2 atmosphere, gold-coated for 5 minutes and observed under scanning electron microscope (SEM Philips XL30 model).

2.6 Evaluation of Different Media Broth for Bacterial Growth

A modified method of (Augustine et al., 2005) was used. The bacterial isolates were inoculated into seven different media, Brain Heart Infusion Broth (BHIB), Nutrient Broth (NB), Marine Broth (MB), Trypticasein Soy Broth (TrySB), Tryptic Soy Broth (TSB), Luria Bertani Broth (LBB), and Lauryl Sulfate Broth (LSB). These were then incubated for 5-7 days at 30°C and a shaking speed of 200rpm. To extract bioactive metabolites compounds, the bacterial cultures obtained were thereafter centrifuged for 15 min at 10,000 rpm. Cell-free supernatants obtained were extracted and mixed with an equal volume of ethyl acetate and kept in rotary shaker

overnight (200 rpm). The solvent layers were collected and then evaporated in a rotary evaporator to obtain crude extracts filtered using 0.45 µm membrane filter, for use in screening for antimicrobial activity.

2.7 Antimicrobial Activity Assay of Gordonia Tearrae Strain

The crude extract of *Gordonia* SPTG111 obtained was dissolved in methanol used for testing against bacterial and fungal pathogens by disc diffusion method as described by Berghe and Vlietinck (1991). This crude was pipetted on the sterile paper disk (AA Whatman, Healthcare, UK, Limited) and left to air dry room temperature around 6 hours. Using sterile swab to streak the pathogens bacteria were following a 0.5 McFarland turbidity standard followed by placing the disc containing 30 µg crude on the agar .The plates were incubated at 37℃ for 24 h during which antimicrobial activity was evidenced by the presence of a clear zone of inhibition surrounding the disk. Each test was repeated three times and the antimicrobial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared to chloromphnicol 30 µg as a control.

2.8 Minimum Inhibition Concentration (MIC)

The MIC of the crude extract of *Gordonia tearrae* was determined using microbroth dilution method (Carbonnelle et al., 1987) in Mueller Hinton broth. The MIC was considered as the lowest concentration of the crude extract which inhibited visible growth of the microbe in Mueller Hinton or Sabouraud broth supplemented with 10% glucose and 0.5% phenol red. For susceptibility testing, in a first step Mueller Hinton broth (50 µl) was distributed from the first to the twelfth test tubes. Dry extracts was dissolved in DMSO (100 µl) and subsequently in Mueller Hinton broth, to reach a final concentration of 1000 µg/ml. These solution (50 µl) was added to the first well of each microtiter line. The dilutions was done by transferring the solution (50 µl) from the first to the eleventh tube. A volume of 50 µl was finally discarded from the eleventh tube. The twelfth tube served as growth control where no sample (extract, reference antibiotics) was added. A microbial suspension (50µl, 10^5 colony forming units), obtained from an overnight growth at 37 °C was added to each well. The final concentration of the extracts adopted to evaluate the antimicrobial activity ranged from 200 to 0.8 µg/ml. Tests were incubated aerobically at 37°C for 24 h before being read. The MIC was considered as the lowest concentration of the sample that prevented visible growth or changed in color from red to yellow due to the formation of acidic metabolites corresponding to microbial growth.

2.9 Statistical Analysis

The data were presented as mean ±S.D. Statistical computations were calculated using ANOVA one way for windows software. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Physiological and Morphological Characterization of the Gordonia Tearrae:

This strain of Gordonia tearrae was observed to be deeply orange pigmented colony,and irregular and rough , aerobic, gram-positive, catalase- urease- oxidase positive, non motile, nocardioform (mixture of rod-shaped or coccoid elements) confiemd by the SEM image (Figure 1) that do not generate spores.

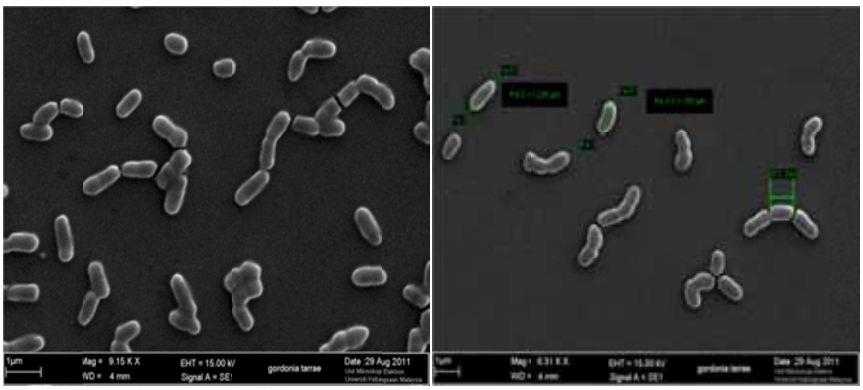

Figure 1. Scanning electron microscopy of *Gordonia tearrae*. Bar indicates 1 µm

3.2 Molecular Taxonomy, Sequencing and Phylogenetic Analysis

The almost complete 16S rRNA gene sequencing and NCBI blast search retrieved from GenBank showed a similarity of 100% alignment with the genus *Gordonia and Gordonia tearrae* being the closest fit (Figure 2). The size of PCR products obtained was ~1.5 kb, detected using 1% agarose gel electrophoresis.

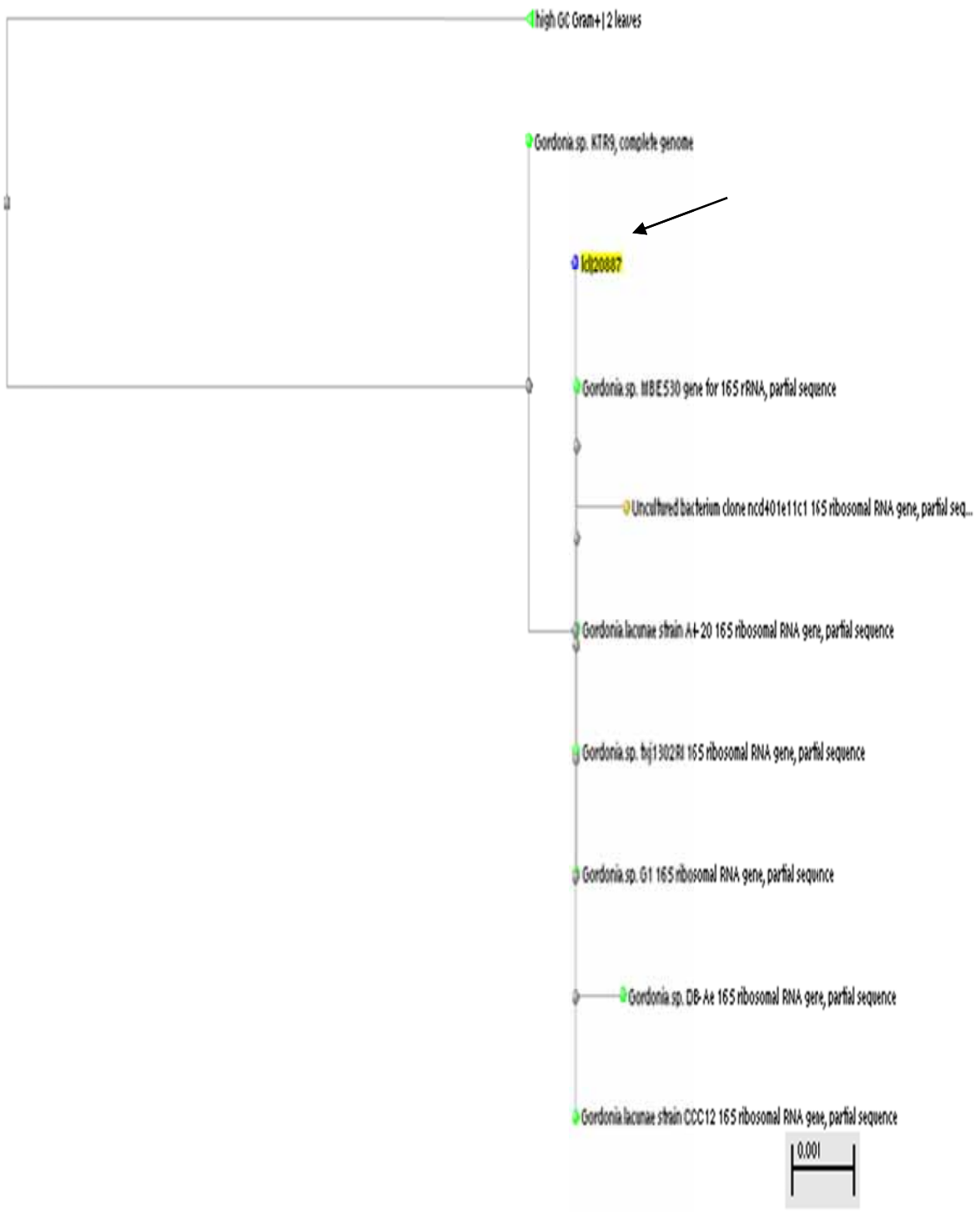

Figure 2. Phylogenetic relationship of *Gordonia* to related bacteria based on neighbour-joining tree analysis of 16S r RNA gene sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

3.3 Evaluation of Different Media Broth for Bacterial Growth

Gordonia tearrae showed maximum yield of extraction in BHIB compared with Trypticasein SB, L.BB, TSB, MB, NB and LSB respectively. The significantly high level of secondary metabolites compounds ($p < 0.005$, n=3) (Figure 3).

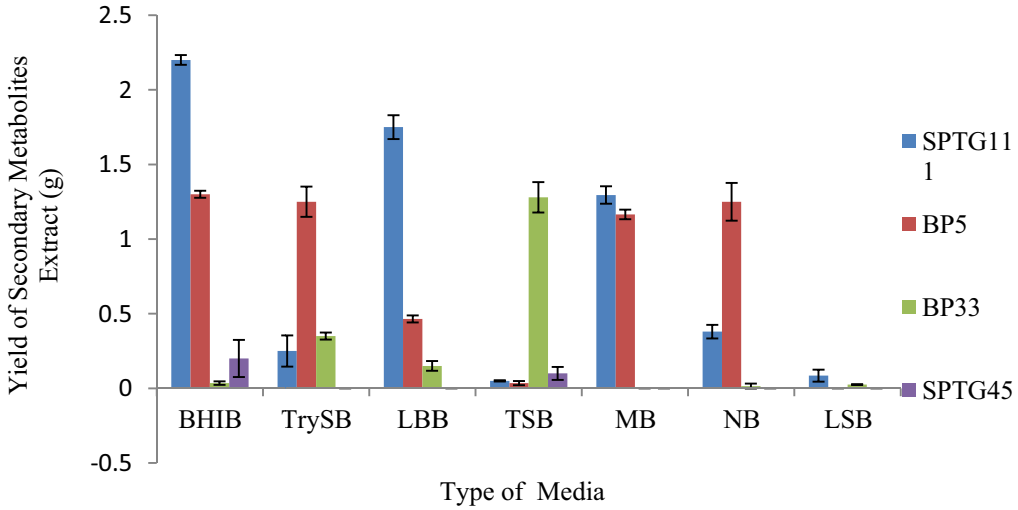

Figure 3. The yield of secondary metabolites of *Gordonia tearrae* SPTG111

3.4 Antimicrobial Activity Assay

The 30 µg/disc concentration of ethyl acetate crude extract of *Gordonia tearrae* principally extracted by BHIB, showed higher antimicrobial activities (Figure 4) with inhibition zones ranging from 7-30 mm as compared with BP3, BP5 and SPTG45 which were extracted with same media (range 1-25 mm) (Table 1). Of all the tested bacteria used in this study, antimicrobial effect was highest among *S. aureus* for *Gordonia tearrae* isolate in addition to BP33 and BP5. Notable also was the fact that extract from isolate SPTG45 was not active against most of the tested isolates on all four growth media. However, there were exemptions for *S.typhi* and *P. aeruginosa* where only small zones of inhibition were noticeable using BHI broth (Table 1). Only extract from *G. tearrae* was effective against MRSA in levels comparable to the control drug, Chloramphenicol.

Figure 4. No 2 is antimicrobial activity of *Gordonia tearrae* crude extracted by BHI broth aginest *C.albicanse* and *MRS*

Table 1. Antimicrobial activity of crude extracts of four marine actinomycetes (mm) extracted with different four selected media

Media	BHIB				LBB				MB				TSB				
Tested isolate	*SPTG 111*	*BP 33*	*BP 5*	*SPTG 45*	*SPTG 111*	*BP 33*	*BP 5*	*SPTG 45*	*SPTG 111*	*BP 33*	*BP 5*	*SPTG 45*	*SPTG 111*	*BP 33*	*BP 5*	*SPTG 45*	CH
Escherichia. coli	20	5	15	/	15	/	12	/	17	4	/	/	16	3	7	/	15
Enterobacter.feacalis	7	/	10	/	11	/	14	/	0	/	/	/	5	/	8	/	12
Bacillus. cereus	21	/	12	/	20	/	12	/	15	/	/	/	19	2	2	/	30
MRSA	30	/	/	/	1	/	/	/	10	/	/	/	4	/	/	/	30
Salmonella. typhi	12	2	5	3	6	/	6	/	6	2	1	/	1	/	1	/	17
Pseudomonas .aeruginosa	9	1	7	7	3.5	/	2	/	4	1	/	/	1.5	2	/	/	12
Candida. albicans	25	5	10	/	5	/	10	/	0	3	/	/	19	5	8	/	20

- (/) the sample was not active.

- (CH) Chloramphenicol (30 µg /disc).

- Brain Heart Infusion Broth-BHIB, Luria Bertani Broth- LBB, Marine Broth- MB, Trypticasien soya broth-TSB.

3.5 Minimum Inhibition Concentration (MIC)

The MIC values of the *Gordonia tearrae* extract obtained by microbroth dilution method are presented in Table 2. These values ranged from 3.12 -12.5 µg/ml, most of them were less than those obtained from the reference drug Chloramphenicol. This indicated *G.tearrae* crude extract have strong activity on pathogen bacteria.

Table 2. Minimum inhibitory concentration (MIC) of *Gordonia tearrae* crude extract

Tested microbe	Extracts MIC	CH*
MRSA	6.3	25
Bacillus cereus	3.12	25
Enterococcus faecalis	25	25
Escherichia coli	6.3	25
Salmonella typhyi	12.5	50
Pseudomonas earoginosa	25	50
Candida albicans.	6.3	25

(CH)*Chloramphenicol 30µg.

4. Discussion

Marine actinomycetes are of a great importance due to their astounding ability to produce numerous biologically active compounds including antibiotics, anticancer and antiphrastic agents. Marine actinomycetes thus present an arsenal of current and potential drugs particularly in the global battle against the rising menace of antibiotic resistance n both clinical and environmental settings (Williams, 2009). The *Gordonia tearrae* (SPTG 111) strain used in this study was obtained from the UKM culture collection unit, where it was deposited and labelled as previously isolated from marine sponge at Pulau Tinggi. The sequencing of PCR amplicons of the 16S rRNA gene showed 100% sequence identity to *Gordonia* sp. Isolate Bg17 (HF548413.1), a bacteria isolate from Rubinskoe water reservoir, Russia; *Gordonia* sp. G1 16S ribosomal RNA gene, partial sequence FJ939311.1 isolated in a study of degradation of crude oil and utilization of hydrocarbon compounds by bacterial isolates from used engine oil-contaminated soil; *Gordonia tearrae* strain MRbS27 (FJ959396.1), an isolate from a study on the diversity of bio-compound producing heterotrophic, rhizosphere bacteria from Stachytarpheta crassifolia,

a Brazilian semi-arid plant and *Gordonia tearrae* strain K22-39 (EU333873.1) isolated from a study on culturable bacteria from a cold desert of the Indian Himalayas. The selected strain when cultured in Brain Heart Infusion Broth (BHIB), showed higher activity against tested microbial pathogens. Our observation of BHIB supporting the highest level of accumulation of crude secondary metabolites is in concert with the findings of Marinho et al. (2009) who reported that BHI supported growth of a bacteriocin producer strain of *P. putida*. In this study, we used the zones of inhibition of the lawn of bacterial cells around extracts filled disc as an indication of antibacterial activity. This zone of inhibition ranged from 5.5 mm for extracts at 30 µg/disc concentration to 6.3 for 25 µg/ml of crude extract. The crude extract of ethyl acetate of *Gordonia tearrae* showed an activity that was significantly different from that of chloramphinicol, which in this case was used as reference drug. This observation was recorded when crude extract was tested against *Staphylococcus aureus, Candida albicans, Bacillus cereus*, and *E. Coli.* Notably, the observed MIC values for the crude extract washigher than that of chlramphenicol (Table 2).

5. Conclusion

In conclusion, secondary metabolites produced by marine actinomycetes usually have distinct chemical structures, which may form the basis for the synthesis of novel drugs. Much undoubtedly, these targets may pave the way for more highly effective antibiotics. This study investigated the antibacterial effect of ethyl acetate extract of *Gordonia tearrae* (marine actinomycetes) against pathogenic bacteria. Brain heart infusion broth media was able to stimulate the highest level of production of metabolic compounds as compared to other growth media. The marine actinomycetes described here show promising activity in the area of antimicrobial activity. The crude extract of *Gordonia tearrae* was active (MIC ≤ 3.12 µg/ml) against at least one of the tested microorganisms *Bacillus cereus.* Further work is currently going on to elucidate possible toxicity studies associated with the extracts.

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The Research of Cold Stress on Three Revetment Plants

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Abstract

In order to select proper revetment plant to apply in the rock slope protection, we used *Cynodon dactylon* as control to investigate the effect of cold stress on two wild revetment plants (*Pogonatherum paniceum* and *Eriophorum comosum*) in current study. With the dropping of temperature (0°C, -5°C, -10°C, -15°C, -20°C), the content of soluble sugar, soluble protein, and free proline in these three plants rose first, and then decreased. Meanwhile, the chlorophyll content declined, but the electrolyte osmotic rate increased. Under the five different temperatures, the content of soluble sugar and soluble protein were highest ($P < 0.05$) in *E. comosum*, the lowest ($P < 0.05$) in *C. dactylon*, and *P. paniceum* in the middle. The free proline contents in *E. comosum* was similar to *P. paniceum* at -15°C, and -20°C, but both of them were significant greater than that in *C. dactylon* ($P < 0.05$). Additionally, *C. dactylon* had the highest electrolyte osmotic rate, and *E. comosum* was the lowest. Meanwhile, the semilethal low temperature was the lowest (-13.6°C) in *E. comosum* as compared to *C. dactlylon* (-6.86°C) and *P. paniceum* (-9.37°C). Both the contents of Chlorophyll *a* and Chlorophyll *b* were highest in *P. paniceum*, lowest in *C. dactylon*, and those in *E. comosum* were medium. These suggested that *E. comosum* may have the strongest cold resistance capacity, and that in *C. dactylon* was the weakest. The results showed that *E. comosum* was the best option to the rock slope protection due to their strongest anti-cold ability among these three grasses.

Keywords: revetment plant, cold stress, cold resistance, osmotic adjustment materials, electrolyte osmotic rate, Chlorophyll, semilethal low temperature

1. Introduction

The development in socio-economic cannot achieve without infrastructure development, especially in the construction of road and railway. However, which also brought inevitable destruction on the ecological balance, leading to bareness in surface, decreasing the soil anti-erosion capacity, and aggravating the soil erosion (Wang & Peng, 1997). Thus, it is very necessary to carry out vegetation restoration in bare rock slope. The key in which is not the slope protection technique and soil matrixes, but the selection of proper revetment plant (Zhen & Fang, 2009).

However, research in eco-engineering for the rock slope protection is still at the starting stage in China (Xiong et al., 2011). At present, single pasture or lawn grass is selected for rock slope protection, but which is often followed by inadaptability. When the temperature is lower, the water stress occurs, and the survival rate decreases (He et al., 2012). Nevertheless, wild lithophytes can effectively solve this problem due to their advantageous traits (Liu & Han, 2007). It has been reported that lithophytes can directly grow in steep rock and cemented masonry stone, and they have greater resistance to drought, cold, disease and barren, and fecundity than other grasses. Therefore, the selection of proper lithophytes has important significance in the rock slope protection (Li et al., 2004b). According to the survey by Li et al. (2006), *Pogonatherum paniceum* and *Eriophorum comosum* can be used as revetment plants because of the characteristics in strong deep roots, and well tolerant to barrenness, drought and water erosion. They can grow in a crack between rocks, and form symbiotic relationships with lower organisms, such as algae, lichen, moss, etc (Li & Luo, 2006).

At present, systemic researches have been performed in seed certification, drought adaptability, microbial ecological distribution in rhizosphere soil, plant tissue culture, and genetic diversity of *Pogonatherum paniceum* and *Eriophorum comosum* (Li et al., 2004a; Wang et al., 2005; Zhuang et al., 2010). However, the study on anti-cold ability of these two grasses has not been reported yet. In the current research, we used *Pogonatherum*

paniceum and *Eriophorum comosum* as experimental materials, and Cynodon dactylon as control to study the cold adaptability of these two plants. Results of this study could provide scientific theoretical basis for selection of revetment plant.

2. Materials and Methods

2.1 Site for Experiment

The experiment was carried out at the teaching practice base of the college of Forestry, Sichuan Agricultural University, Ya'an, Sichuan province, China. Average altitude, mean annual precipitation, annual temperature and relative air humidity in this area is 620 m, 1800 mm, 16.2 ºC, and 79%, respectively. The annual cumulative temperature (≥10 ºC) is 5231 ºC, annual sunshine is 1039.6 h, and frost-free period is 298 d.

2.2 Experimental Design

Wild *Pogonatherum paniceum*, *Eriophorum comosum* and *Cynodon dactylon* were collected from the rock slope at the town of Duoying, Ya'an, and the slope gradient was steeper than 85%. Pot experiment was used in this study. The experimental soil was purple sandy shale soil, and which was the heavy clay soil with a pH value of 6.2.

The collected seedlings were planted on 10th May 2012. Six seedlings were planted in each pot with 20 duplicates in each grass. Fresh leaves were collected from three grasses at 7 am on 15th November 2012. The collected leaves were stored in ice bags, and taken back to the lab rapidly. Leaves were cleaned with deionized water, and dried by the filter paper. Then, five different temperatures include 0°C, -5°C, -10°C, -15°C, and -20°C were employed to treat leaves, and 10 duplicates in each temperature treatment.

2.3 Indexes Measuring

The membrane permeability was measured by conductimeter (DDS-11A). The soluble protein content was tested with staining of coomassie brilliant blue R 250, and followed the described by Zhang (Zhang, 2006). The soluble sugar content was measured by sulfuric anthrone reaction based on the method of Li (Li, 2000). The Chlorophyll content was determined according to Wintermans and De Motes (Wintermans & De Mots, 1965). The free proline was measured directly using the ninhydrin colorimetric method of Troll and Lindsley (Troll & Lindsley, 1955)

2.4 Statistical Analysis

One-way ANOVA was used to compare the effect of treatments and, Tukey's test was used for the multiple comparison. Statistics analysis was performed using SPSS 17.0 software, and the P-value for significance was set at $P < 0.05$.

3. Results

3.1 Effect of Low Temperature Stress on Membrane Permeability of Leaf Cells

As shown in the Figure 1, the electrolyte osmotic rate of three grasses went up with the decrease of temperature. The electrolyte osmotic rate of *C. dactylon* increased quickly from 0 to -15°C, and slower growth was found after -15°C. However, the rising trend was slowly before -10°C and then increased sharply in *E. comosum* and *P. paniceum*. Additionally, the electrolyte osmotic rate was lowest in *E. comosum*, greatest in *C. dactylon*, and *P. paniceum* was medium at all temperatures.

The semilethal low temperatures of three plants were calculated based on the fitted regression equation of the electrolyte osmotic rate (Table 1). The semilethal low temperature was lowest in *E. comosum* (-13.6°C), highest in *C. dactylon* (-6.86°C), and that in *P. paniceum* (-9.37°C) in the middle, which were consistent with the electrolyte osmotic rate of three plants.

Table 1. The semilethal temperature of three plants

Grass	Logistic equation	LT_{50}/°C	R_2
E. comosum	$Y = 67.513/(1+e^{2.461-0.198x})$	-13.6	0.9965
P. paniceum	$Y = 92.765/(1+e^{1.482-0.154x})$	-9.37	0.9876
C. dactylon	$Y = 80.516/(1+e^{1.978-0.366x})$	-6.86	0.9915

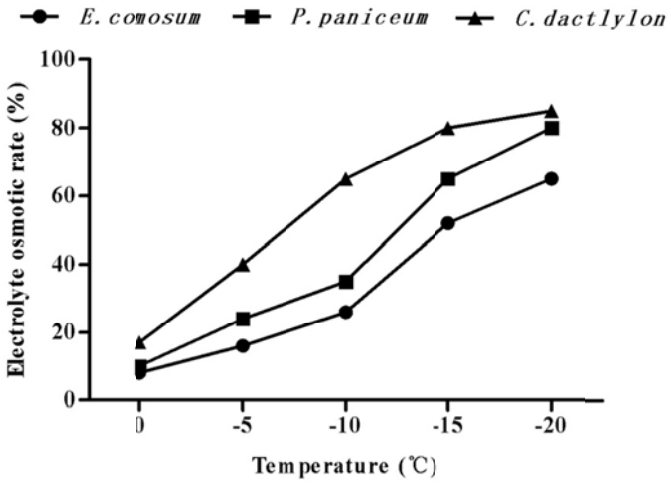

Figure 1. Variation of electrolyte osmotic rate in leaf of three plants under low temperature stress

3.2 Effect of Low Temperature Stress on Osmotic Adjustment Materials of Leaf

In our study, the variation trends of soluble sugar were similar in the three plants (Figure 2). The soluble sugar content increased from 0 to -15°C and then decreased, and the decline in *C. dactylon* was the fastest. In addition, *E. comosum* had the highest soluble sugar content ($P < 0.05$) at the five temperatures, and those in *C. dactylon* were lowest ($P < 0.05$) in all points.

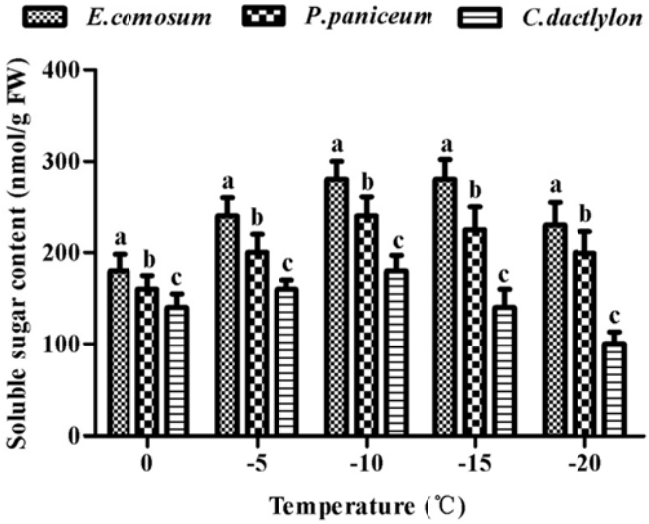

Figure 2. Variation of soluble sugar content in leaf of three plants under low temperature stress

Different lowercase above the columns indicate significant differences ($P < 0.05$).

The soluble protein content in *C. dactylon* and *P. paniceum* rose from 0 to -10°C, and followed by slow declines (Figure 3). However, the soluble protein content in *E. comosum* slowed down rapidly after reaching the peck at -15°C. It was also can be seen in the Figure 3, *C. dactylon* had the lowest ($P < 0.05$) soluble protein content under the different temperatures, the highest was found in *E. comosum* ($P < 0.05$).

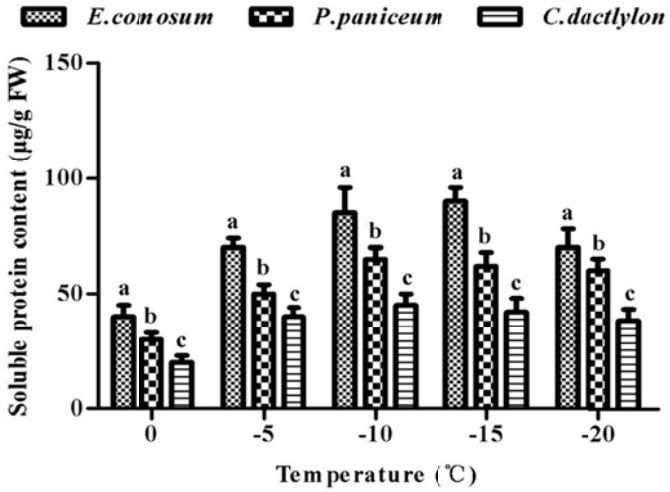

Figure 3. Variation of soluble protein content in leaf of three plants under low temperature stress

Different lowercase above the columns indicate significant differences ($P < 0.05$).

It can be seen from Figure 4 that the change trends in three plants were different. Specifically, the free proline content in *E. comosum* went up from 0 to -10°C, and declined sharply from -10°C to -15°C, then decreased gradually. The free proline content in *P. paniceum* increased slowly as the temperature decrease, and got to the top at -15°C, then slowed down gradually. However, the free proline content in *C. dactylon* rose from 0 to -5°C, and followed by a decrease trend. Meanwhile, the free proline content of *E. comosum* was highest at all temperatures ($P < 0.05$), and lowest in *C. dactylon* ($P < 0.05$) under the temperatures of 0°C, -5°C and -10°C. But the free proline content in *E. comosum* was similar to that in *P. paniceum*, and significant than that in *C. dactylon* ($P < 0.05$) under the temperature of -15°C and -20°C.

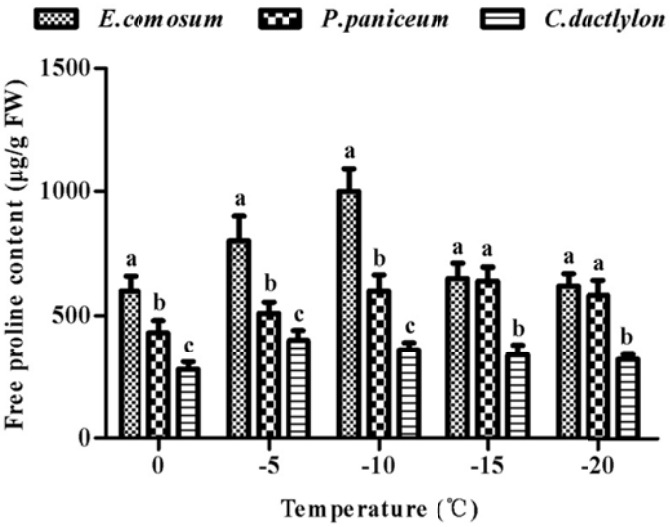

Figure 4. Variation of free proline content in leaf of three plants under low temperature stress

Different lowercase above the columns indicate significant differences ($P < 0.05$).

3.3 Effect of Low Temperature Stress on Chlorophyll Content of Leaf

The effect of low temperature stress on chlorophyll *a* and chlorophyll *b* content of leaf was shown in Figure 5(A) and 5(B), respectively. The content of chlorophyll *a* and chlorophyll *b* in three plants descended with decreasing disposed temperature. The decrease trend of chlorophyll *a* content was slower that of chlorophyll *b*. Both

chlorophyll *a* and chlorophyll *b* were lowest in *C. dactlylon* among experimental grasses at five treated temperatures, the highest chlorophyll a content was found in *P. paniceum*, and that in *E. comosum* was medium.

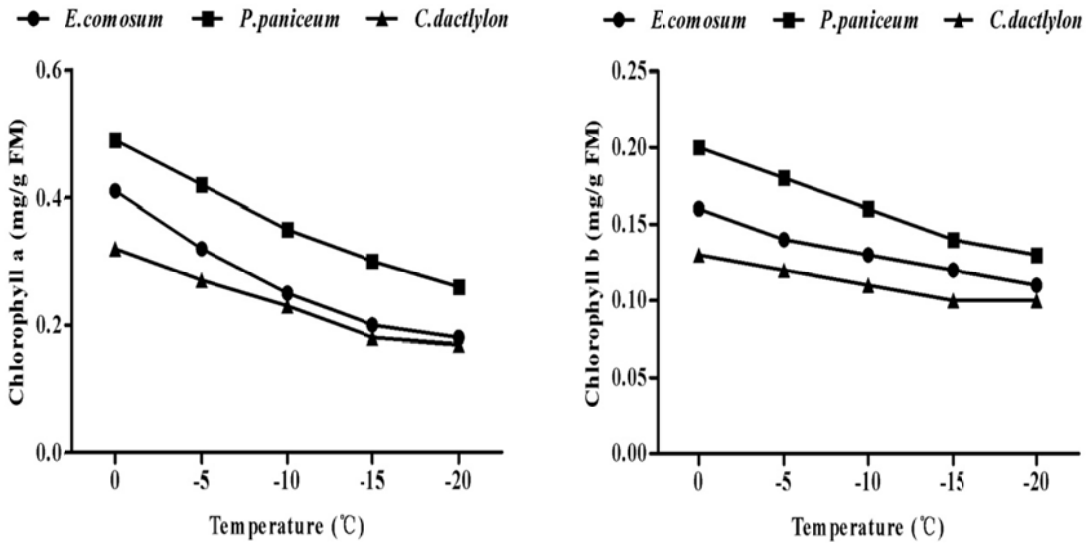

Figure 5. Variation of chlorophyll content in leaf of three plants under low temperature stress

4. Discussion

In general condition, the production and cleanup of free radical exists in dynamic equilibrium in plants. When the plant is under the environment stress, the balance of free radical will be destroyed. In this case, the accumulation of free radical can cause harm to cells, and the plasmalemma system will be the first by this impact (Iriti & Faoro, 2009). Under the low temperature stress, both the structure and functions of plasmalemma are damaged, which cause the increase in cell membrane permeation and electrical conductivity, and can reflect the damaged condition of plant (Wang et al., 2011). The electrical conductivity in plant with strong anti-cold ability is lower than that in plant with weak cold-resistance at the same temperature (Sun et al., 2009). Chen et al (1999) demonstrated that plant will suffer irreversible damage when a wide-range increase is occurred in the electrical conductivity between two temperatures (Chen, 1999). In our study, the electrolyte osmotic rate of three grasses went up with the temperature decreasing. This showed that the damage degree of cell membrane rose as temperature dropped. In addition, both the electrolyte osmotic rate at temperatures and the semilethal low temperature were lowest in *E. comosum*. This illustrated that *E. comosum* have strongest anti-cold ability among these three plants. Tian et al. (2011) investigated the effects of chilling stress on relative electric conductivity in different cultivars of *Anthunium andraenum*. Their results concluded that the relative electric conductivity was lowest in Alabamb which had the strongest chilling resistance (Tian et al., 2011).

The content of soluble sugar, soluble protein and free proline are most important osmotic adjustment materials (Babu et al., 1999). As an osmoregulatory factor, the soluble sugar can regulate osmotic potential, and assist in enhancing stress resistance (Irigoyen et al., 2006). The accumulation of soluble sugar can improve the osmotic pressure and water-retaining capacity of plant, thus boosting the cold resistance (Ma et al., 2010). The soluble protein has stronger hydrophilia, and can improve the water-possessing ability and protoplasm elasticity in cell as well. Thus, the increase of soluble protein can also strengthen the stress resistance (Zhu et al., 2011). In this experiment, both the soluble sugar and soluble protein content were highest in *E. comosum*, and lowest in *C. dactlylon* at all temperatures. These also suggested that the cold resistance in *E. comosum* were highest among the three grasses. Moreover, the soluble sugar and soluble protein content went up from 0 to -15°C in all measured grasses. These may be explained that plants accumulated soluble sugar and soluble protein to fight with cold stress from 0 to -15°C. But as temperature continued to drop, cells need them to maintain the energy supply, or cells suffered irreversible damage, thus they decreased after -15°C. Xu et al. (2011) studied the changes of osmotic regulatory metabolites in eight bamboo species under low temperature stress. The results showed the contents of soluble protein and soluble sugar changed significantly under low temperature, and which form the cold-resistance varieties were higher than that of the others (Xu & Dai, 2011).

It has been demonstrated that the free proline content is closely related with cold resistance in plant, the accumulation of which is considered an adaptation to the cold stress (Dörffling et al., 2008). The increase of proline can decrease the water potential and raise the water-retaining capacity under the low temperature. Thus, its accumulation can as a measure of anti-cold ability (Ashraf & Foolad, 2007). In this study, there were varying degrees of growth with decreasing temperature found in the free proline of three grasses. Meanwhile, the free proline content of *E. comosum* was highest at all temperatures. These results indicated that these three grasses can improve anti-cold ability through accumulating the proline content, and the anti-cold ability was best in *E. comosum*. The study of Li et al. (2011) also found that free proline content of different *Medicago sativa* species showed upward trends under low temperature stress (Li, 2011).

The central role of chlorophyll in photosynthesis is to harvest light energy, and converse it to chemical energy. The chlorophyll content direct affects the photosynthesis capability (Mauzerall, 1976). Chlorophyll *a* alone exists in the core complex of photosystem. Its organization and composition have highly conservation. Chlorophyll *b* is an ubiquitous accessory pigment, whose biosynthesis plays a key role in the adaptation to various light environments (Tanaka et al., 1998). In current study, as the temperature dropping, both Chlorophyll *a* and Chlorophyll *b* contents in measured plants decreased. It meant that photosynthesis in all of the three plants was expected to be negatively affected by these changes. Under the different temperatures, *C. dactylon* had the lowest contents of Chlorophyll *a* and Chlorophyll *b* suggested that the photosynthesis of *C. dactylon* was the weakest among these three grasses.

5. Conclusion

In conclusion, plants can adapt to low temperature through adjusting the content of osmotic adjustment materials. *E. comosum* had the highest content of soluble sugar, soluble protein and free proline, higher Chlorophyll contents among three plants, and lowest electrolyte osmotic rate. Additionally, the semilethal low temperature in *E. comosum* was also the lowest. From the results of the present experiment, it was concluded that cold resistance of these three revetment plants was decreased in the order of *E. comosum*, *P. paniceum*, and in *C. dactylon*.

Acknowledgements

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The Role of *Spirulina platensis* (Arthrospira platensis) in Growth and Immunity of Nile Tilapia (*Oreochromis niloticus*) and Its Resistance to Bacterial Infection

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Abstract

The current study was designed to optimize the dietary levels of *Spirulina platensis* in *Oreochromis niloticus;* this was tested via graded levels. Six isonitrogenous and isocaloric rations containing graded levels of dried spirulina 0, 5, 7.5, 10, 15 and 20 g/kg diet were fed separately to six equal groups of *O. niloticus* fingerlings for 3 months. Growth performance, non-specific immune parameters, tissue reactions and resistance of tilapias post challenge infection with *Pseudomonas fluorescens* were estimated monthly. There were significant increase in growth performance parameters and survival rates in spirulina-supplemented groups at concentration level of 10 g/kg for 2 months. Significant increases in hematocrit, nitroblue tetrazolium and lysozyme activity were observed in most of the supplemented groups. Bacterial challenge infections resulted in significantly lower mortality rate in all Spirulina groups with remarkable increase in protection of fish received 10 g/kg. In sum, it advisable to incorporate 10 g/kg diet of spirulina for 2 months for maximum growth performance, immunity and disease resistance in *O. niloticus*.

Keywords: *Spirulina platensis*, growth performance, hematocrit, nitroblue tetrazolium, lysozyme activity, challenge infection

1. Introduction

The increase in aquaculture production could be accomplished either by increasing the cultured area or intensification of production. Under such extreme conditions; several growth and immune suppressors can take place (Yousefian & Amiri, 2009), in addition, aquatic organisms are in constant contact with a plethora of bacteria, many of which are pathogenic (Kaizu et al., 2011).

Several sectors of the aquaculture industry would benefit if cultured organisms were conferred with enhanced feed efficiency, growth performance, and disease resistance without environmental conflicts (Gatlin et al., 2006). Functional feed additives strategy has recently gained considerable attention. From nutritional point of view; it does not only provide the essential nutrients required for normal physiological functioning, but also serve as a medium by which fish receive other components that may positively affect their health (Ibrahem et al., 2010) . Researches on diets optimization to enhance fish health and production are still in infancy.

There are increasing recognition for the importance of aquatic macrophytes as feed in aquaculture. Tilapias are omnivorous that can utilize cyanobacterial blue-green algae (Chow & Woo, 1990). *Spirulina platensis* (SP), a filamentous cyanobacterium, possesses diverse biological and nutritional significance. It has the potentiality to produce large numbers of antimicrobial substances; therefore, it is considered a suitable candidate for exploitation as bio-control agent against pathogenic micro-organisms (Ozdemir et al., 2004). In aquaculture, different forms of SP were tested in various fish and shellfish species (Ungsethaphand et al., 2010).

Optimization of dried form of whole *S. platensis* concentration in fish diets is still questionable, regarding its optimum concentration needed to reach its desired effects on growth performance, feed utilization, immune responses, and resistance of tilapias to infections.

SP is well known for its anti-oxidant and anti-cancerous properties. A hot water extract of SP has been orally administered to patients and proved as an anti-cancer agent. SP hinders the growth of oral cancer in Syrian hamster cheek (Grawish, 2008). Such an inhibitory effect may be attributed to the repair of carcinogen- damaged DNA, meanwhile, SP has been suggested as an efficient radical scavenger (Romay et al., 1998). Several studies reported the unique role of SP polysaccharides in enhancing the cell nucleus enzyme activity and the process of DNA repair (Kaji et al., 2002).

Therefore, the overall goals of the present study are to investigate the optimum dietary concentration and duration of dried *S. platensis* needed to exert its potential effects on growth performance, immunomodulator, chemoprotective agent, in addition to the resistance of *O. niloticus* supplemented groups to *Pseudomonas fluorescens* infections.

2. Materials and Methods

2.1 Fish

A total of 2400 *O. niloticus* fries (mean individual initial weight 4 ± 1.0 g) were obtained from Abbasa hatchery, they were divided into six equal groups, each consisting of four replicates (100 fry/replicate) in 6 separate earthen ponds, Fish in each replicate were reared in a hapa made of cotton mesh like a cage (3 × 2 × 1 m, each) that was fixed in an earthen pond (for each group, a total of 4 hapas were equally arranged in 4rows). The whole experiment was done at the experimental units of The World Fish centre, Abbasa, Sharkia, Egypt. The Fish were fed twice daily on a basal diet of 35% protein at 10% of body weight per day. The feed was placed in plastic trays fitted in the hapas (one per hapa). The water was partially renewed daily and monitored regularly; the water temperature was maintained at 25 ± 1°C.

2.2 Spirulina Platensis

Pure dried S. platensis (*Arthrospira platensis*) tablets were obtained from Lake Heath Products Co., Ltd. Liyang City, Jiangsu Province, China. It was dark pure green in colour with smooth surface. The tablets were grounded to a powder form before usage.

2.3 Rations

A standard commercial ration containing crude protein, crude lipid, vitamins and minerals met the basic dietary requirements of Nile tilapia was prepared (Table 1). The ingredients were mixed mechanically by the horizontal mixer (Hobarts model D300-T, Troy, OH, USA). The pellets were then prepared using a pellet-machine (California Pellet Mill, Roskamp Huller Co.) with 0.5 cm diameter and pellets were left for 24 h for air-drying at room temperature (28°C), broken into small pieces and sieved to obtain the appropriate size. The rations were transferred into plastic bags and stored in a refrigerator at 4°C until used. Six experimental rations were prepared. The first five groups were prepared by mixing separately a graded concentration of *S. platensis 5*, 7.5, 10, 15 and 20 g *S. platensis* /kg diet. The last group was assigned as control ration which consisted of the standard commercial ration without any treatment. The required diet was prepared biweekly and stored in a refrigerator (4°C) for daily use.

Table 1. Composition of the Oreochromis niloticus basal diet used throughout the experiment

Ingredients	Diet (%)	Protein (%)		Metabolic energy (Joules)	
		ingredients	Feed	Ingredients	feed
Fish meal	7.95	0.72	5.76	4000	32000
Soybean meal	52.8	0.48	25.39	2870	151823
Ground corn	29.1	0.10	3.17	1240	36084
Wheat flour	5.00	0.13	0.67	2700	13500
Vegetable oil	2.00	0.00	0.00	9100	18200
Cod liver oil	2.00	0.00	0.00	9100	18200
Di calcium phosphate	1.00	0.00	0.00	0.00	0000
Mineral mix.	0.07	0.00	0.00	0.00	0000
Vitamin mix.	0.05	0.00	0.00	0.00	0000
Total	100	0.00	34.99	0.00	269807

2.4 Pathogen

Pseudomonas fluorescens was previously isolated from naturally infected *O. niloticus* and identified according to the standard bacteriological tests. The pathogen was cultured in Tryptic Soya broth (TSB) (Oxoid) for 24 h at 37°C. The broth culture was centrifuged for 10 min at 3000 rpm. The supernatant was discarded and the pellets were re-suspended in phosphate buffered saline at pH 7.4 (PBS 7.4) and the optical density (OD) of the solution was adjusted to 0.5 at 456 nm, which correspond to 1×10^8 cells mL^{-1}. This bacterial suspension was serially diluted using standard dilution technique with PBS 7.4 and used for the challenge experiment.

2.5 Experimental Design

To evaluate the efficacy of SP *on* cultured *O. niloticus*; three month feeding study periods were conducted. The pre-acclimated fish were divided into 6 equal groups. Group 1 was fed on a basal diet (control) and the five groups were dietary supplemented with single graded concentration of dried SP 5, 7.5, 10, 15 and 20 g kg^{-1} diet fed, respectively. Groups were evaluated for growth performance expressed as survival, specific growth rate, and condition factor. Blood samples were collected after 1, 2, and 3 months for hematological analysis and immunological investigations. At the end of each month, the mortalities as well as the relative level of protection were estimated post challenge infections using *P. fluorescens*.

2.6 Growth Performance and Survival

Fish of all replicates were weighed individually and their body weight gain was measured. Specific growth rate (SGR) and condition factor (CF) were calculated according to Goodwin et al. (1983). The survival percentage was recorded along the period of experiment.

$$\text{SGR} = \text{Ln [final mean body weight (g)]-Ln [initial mean body weight (g)]} \div \text{time interval (days)} \times 100$$

$$\text{CF} = \text{weight (g)} \div [\text{length (cm)}]^3$$

3. Hematological and Immunological Analysis

3.1 Blood Sampling

Twenty fish were randomly collected from each group and were anesthetized via immersing in water containing tricaine methane sulfonate (MS-222) neutralized by sodium bicarbonate. Whole blood (0.5 ml) pooled samples were collected from the caudal vessels of each fish using syringes (1 ml) and 27-gauge needles rinsed with heparin (15 unit/m).

A further 0.5 ml blood sample was centrifuged at 2000 rpm for 5 min in order to separate the plasma. The latter was stored at -20°C.

3.2 Hematocrit (HCT) Values

Hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min and the percentage of the packed cell-volume was determined by the hematocrit tube reader.

3.3 Nitroblue Tetrazolium (NBT) Activity

The production of oxygen radicals from phagocytes in the blood was measured using nitroblue tetrazolium (NBT) dye as described by Anderson and Siwicki (2005). Briefly, blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1 ml N,N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The supernatant fluid was measured in a spectrophotometer at 620 nm in 1 ml cuvettes.

3.4 Adherence/NBT Assays

Nitroblue tetrazolium-glass adherent assays (NBT-glass adherent assay) were performed by placing single drops of blood (0.1 ml) on 2 glass cover slips and incubating them for 30 min at room temperature. The cover slips were then gently washed with phosphate buffered saline (PBS). Drops (0.1 ml) of 0.2% NBT were placed on microscope slides and covered by a cover slip, then incubated at room temperature for 30 min with the NBT solution. The activated neutrophils were then counted under electric light microscope (×400).

3.5 Lysozyme Activity

The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard and 0.2 mg/ml lyophilized *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.75) was used as a substrate. Plasma (50 μg) samples were added to 2 ml of bacterial suspension and the reduction in the

absorbance at 540 nm was determined after 0.5 and 4.5 min incubation at 22ºC. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}.

3.6 Challenge of Fish

For the challenge experiments; The supplemented and the control groups were subjected to three challenge infections, post 1, 2 and 3 months feeding trials; for this purpose; a total of 72 fish from each treatment (18 from each replicates) were used for challenge test, these fish were divided into two groups (each 36). Each group was subdivided into 3 subgroups (replicates, each 12) each subgroup reared in glass aquaria (50 × 60 × 70 cm). The groups were injected intraperitoneally with 0.5 ml of 4×10^8 *P. fluorescens.* The challenged fish, from each aquarium, were observed for 10 days in order to record the daily mortality.

The relative level of protection (RLP) among the challenged fish was determined

RLP % = 1 – (percent of mortality in treated groups/ percent of mortality in control group) × 100.

4. Statistical Analysis

The mean and standard error were calculated for each variable. The data were analyzed by analysis of variance (ANOVA) to identify the significantly different groups at ($P < 0.05$) by one way ANOVA with post hoc LSD multiple comparison test using SPSS software statistical program (SPSS for windows ver.15.00, USA).

5. Results

5.1 Growth Performance

Our study revealed that the growth performance expressed as body weight gain, specific growth rates, and condition factor, were significant ($p \leq 0.05$) in groups 3 & 4 in all periods versus the control group, with higher mean value level is in group 4 (Table 2).

Table 2. Growth performance and survival of experimental tilapia at end of the second month of feeding supplemented diet with Spirulina*, **

Month	Group	Spirulina Dose	Parameter							
			Body Gain (g)		Specific Growth Rate %		Condition Factor %		Survival %	
			Main	± SE	Main	± SE	Main	± SE	Main	± SE
First month	Gp. 1	Control (Zero Dose)	8.48	0.68^{C}	1.88	0.10^{C}	1.67	0.02^{C}	87.67	1.45^{A}
	Gp. 2	5g/kg	12.56	0.78^{B}	2.29	0.11^{BC}	1.82	0.02^{B}	88.67	2.40^{A}
	Gp. 3	7.5g/kg	18.79	1.28^{A}	2.39	0.13^{AB}	1.82	0.02^{AB}	91.00	2.08^{A}
	Gp. 4	10g/kg	20.33	0.99^{A}	2.63	0.09^{A}	1.88	0.02^{A}	91.67	2.03^{A}
	Gp. 5	15 g/kg	21.05	1.11^{A}	2.72	0.12^{A}	1.94	0.03^{A}	92.99	3.04^{A}
	Gp. 6	20 g/kg	21.95	0.95^{A}	2.84	0.08^{A}	1.97	0.02^{A}	94.00	3.33^{A}
Second month	Gp .1	Control (Zero Dose)	19.04	1.24^{C}	1.51	0.06^{C}	1.82	0.02^{C}	81.67	1.67^{B}
	Gp. 2	5g/kg	19.50	1.39^{B}	1.88	0.06^{B}	1.94	0.02^{B}	83.00	1.53^{AB}
	Gp. 3	7.5g/kg	31.10	2.00^{B}	2.04	0.07^{B}	1.99	0.02^{B}	85.67	1.20^{AB}
	Gp. 4	10g/kg	37.39	2.16^{A}	2.27	0.07^{A}	2.21	0.03^{A}	87.67	1.45^{A}
	Gp. 5	15 g/kg	39.61	2.11^{A}	2.40	0.06^{A}	2.24	0.04^{A}	91.00	2.04^{A}
	Gp. 6	20 g/kg	42.33	1.99^{A}	2.57	0.08^{A}	2.29	0.02^{A}	92.20	1.98^{A}
Third month	Gp. 1	Control (Zero Dose)	35.33	2.29^{C}	1.46	0.05^{C}	1.79	0.02^{B}	79.00	2.51^{B}
	Gp. 2	5g/kg	43.33	2.89^{Bc}	1.60	0.06^{Bc}	1.84	0.03^{B}	81.33^{B}	1.33^{A}
	Gp. 3	7.5g/kg	51.75	4.12^{AB}	1.74	0.06^{AB}	2.02	0.03^{A}	83.67^{B}	0.88^{A}
	Gp. 4	10g/kg	57.68	4.31^{A}	1.83	0.07^{A}	2.10	0.03^{A}	85.67	1.20^{A}
	Gp. 5	15 g/kg	59.90	5.11^{A}	1.85	0.06^{A}	2.20	0.03^{A}	88.45	1.11^{A}
	Gp. 6	20 g/kg	62.34	3.88^{A}	1.93	0.08^{A}	2.29	0.05^{A}	89.10	0.98^{A}

5.2 The Survival Rate

During the feeding experiment, the survival % expressed as mean ±SE showed significant increases in group 4 at 2 month of experiment and in all *S. platensis* supplemented groups vs. the control group after the third month of experiment (Table 2).

5.3 Hematological and Immunological Analysis

A significant ($p \leq 0.05$) increase of hematocrit (HCT) and nitroblue tetrazolium (NBT) were observed in all (with the exception of group 2 in case of NBT at 1st and 2nd month) *S. platensis* supplemented groups vs. the control group at all experimental period. On the other hand, Neutrophil Adherence values showed significant ($p \leq 0.05$) increase in all *S. platensis* supplemented groups vs. the control group (Table 3). The increase in the lysozyme activity was significant in all fish groups given basal diet supplemented with *S. platensis* at the 2nd and 3rd month (Table 2).

Table 3. Some hematological and immunological parameters of experimental tilapia at end of the first month of feeding supplemented diet with Spirulina*, **

Month	Group	Spirulina Dose	Parameter							
			HCT		NBT		Nutrophyl Adherence		Lysozyme activity	
			Main	± SE	Main	± SE	Main	± SE	Main	± SE
First month	Gp. 1	Control (Zero Dose)	27.25	1.24 ^{B}	0.23	0.02 ^{B}	9.00	1.61^{C}	8.59	1.39 ^{C}
	Gp. 2	5g/kg	30.36	1.89 ^{A}	0.29	0.02 ^{AB}	10.30	1.59^{B}	9.89	1.40 ^{BC}
	Gp. 3	7.5g/kg	31.40	1.00 ^{A}	0.33	0.03 ^{A}	10.99	1.58^{A}	11.25	1.61 ^{AB}
	Gp. 4	10g/kg	32.95	1.10 ^{A}	0.38	0.05 ^{A}	11.80	1.59^{A}	11.65	1.71 ^{A}
	Gp. 5	15 g/kg	33.00	1.3 ^{A}	0.39	0.04^{A}	12.00	1.55^{A}	11.78	1.65^{A}
	Gp. 6	20 g/kg	33.10	1.1 ^{A}	0.41	0.03^{A}	12.20	1.48^{A}	11.90	1.72^{A}
Second month	Gp .1	Control (Zero Dose)	28.42	1.58 ^{B}	0.27	0.02 ^{C}	9.56	1.37^{B}	8.97	1.39^{C}
	Gp. 2	5g/kg	31.38	1.02^{A}	0.34	0.03 ^{A}	11.30	1.52^{A}	10.26	1.45^{B}
	Gp. 3	7.5g/kg	31.90	1.23^{A}	0.40	0.03 ^{AB}	11.41	1.41^{A}	11.80	1.44^{A}
	Gp. 4	10g/kg	33.20	1.00^{A}	0.49	0.02 ^{A}	12.21	1.26^{A}	12.20	1.33^{A}
	Gp. 5	15 g/kg	33.50	1.44^{A}	0.51	0.04^{A}	12.54	1.37^{A}	12.58	1.44^{A}
	Gp. 6	20 g/kg	33.75	1.32^{A}	0.52	0.03^{A}	12.65	1.44^{A}	12.67	1.24^{A}
Third month	Gp. 1	Control (Zero Dose)	28.80	1.65^{B}	0.29	0.02^{B}	9.80	1.29^{B}	9.00	1.33^{C}
	Gp. 2	5g/kg	33.30	1.49^{A}	0.35	0.01^{AB}	10.40	1.31^{B}	10.10	1.31^{B}
	Gp. 3	7.5g/kg	33.40	1.52^{A}	0.36	0.02^{A}	11.50	1.23^{A}	10.40	1.27^{B}
	Gp. 4	10g/kg	33.80	1.47^{A}	0.38	0.03^{A}	11.70	1.26^{A}	11.90	1.43^{A}
	Gp. 5	15 g/kg	34.10	1.48^{A}	0.39	0.02^{A}	11.80	1.32^{A}	12.00	1.33^{A}
	Gp. 6	20 g/kg	34.44	1.51^{A}	0.35	0.04^{A}	12.00	1.25^{A}	12.22	1.28^{A}

5.4 The Mortality Rate

Following the challenge infection using *P. fluorescens* the mortality rate was significantly lower in all *S. platensis* supplemented groups vs. the control throughout the experimental period.

5.5 The Relative Level of Protection (RLP)

The results recorded in (Table 4) evoked significant protection in all *S. platensis* supplemented groups vs. the control.

It worth mentioned that the mean value level is in-group 4 (group received 10 g/kg) is higher in all the tested parameters with significant difference.

Table 4. Mortality and relative level of protection of experimental tilapia at end of the first, second and third months of feeding supplemented diet with Spirulina after challenged with *Pseudomonas florescence**, **

Month	Group	Spirulina Dose	*Pseudomonas florescence*		
			Mortality %		RLP %
			Main	± SE	
First month	Gp.1	Control (Zero Dose)	65.00	5.00 ^{C}	0.00
	Gp.2	5g/kg	60.00	2.89 ^{B}	8.3
	Gp.3	7.5g/kg	58.33	1.67 ^{B}	10.26
	Gp.4	10g/kg	54.67	1.67 ^{AA}	15.8
	Gp. 5	15 g/kg	52.20	1.54 ^{A}	19.69
	Gp. 6	20 g/kg	50.12	2.45 ^{A}	22.89
Second month	Gp.1	Control (Zero Dose)	66.67	1.67 ^{C}	0.00
	Gp.2	5g/kg	56.67	1.67 ^{B}	15.00
	Gp.3	7.5g/kg	53.33	1.67 ^{B}	20.01
	Gp.4	10g/kg	51.67	1.67 ^{A}	22.50
	Gp. 5	15 g/kg	49.45	1.54 ^{A}	25.83
	Gp. 6	20 g/kg	48.00	1.76 ^{A}	28.00
Third month	Gp.1	Control (Zero Dose)	63.33	3.33 ^{C}	0.00
	Gp.2	5g/kg	51.37	1.67 ^{B}	18.60
	Gp.3	7.5g/kg	48.33	1.67 ^{B}	23.70
	Gp.4	10g/kg	41.67	1.33 ^{A}	34.20
	Gp. 5	15 g/kg	40.00	1.42 ^{A}	36.84
	Gp. 6	20 g/kg	38.00	1.54 ^{A}	40.00

***** In all tables: Gp.1: First group, Gp.2: Second group, Gp.3: Third group, Gp.4: Fourth group.

******Means with the same letter in the same column are not significantly different.

6. Discussion

The primary objective in fish nutrition is to provide a nutritionally balanced mixture of ingredients to support the fish vital functions in an acceptable cost (NRC, 1993). *S. platensis* was reported to improve feed efficiency, carcass quality, and physiological response to stress in several species of fish (Takeuchi et al., 2002). None of the previous studies recommended an optimum concentration of dietary *S. platensis* b] ased on graded level study.

Spirulina appeared to be a useful tool to include in the arsenal of disease control and prevention. However, it must not replace good management techniques. The strength of spirulina appears to lie in its ability to improve growth, survival and non-specific immune function against fish pathogens as well as its chemo-protective efficiency. The algae may significantly aid the aquaculture industry.

In the current study we expanded the *S. platensis* dietary concentration to be 0.05, 7.5, 10, 15 and 20 g/kg in order to assess the optimum dietary concentration for *O. niloticus* using pure whole *S. platensis* in powder form.

Specific growth rate (SGR) and condition factor (CF) are the measuring tools reflecting the fish health status under natural and experimental conditions. In the current study pure dried *S. platensis* found to be of potential effects on growth at an optimum concentration of 7.5 and 10 g/kg. It worth mentioned that the mean value level in the group received 10 g/kg is higher in all the tested parameters with significant difference; these results cleared that the optimum dietary level of S. platensis for O. niloticus is 10 g/kg for 2 months to enhance growth performance. Duncan and Klesius (1996) reported that *Spirulina* alga was a good source of protein for animal feed, being containing high amounts of vitamins and minerals, in addition, Nakono et al. (2003) recorded that the lack of cellulose from the cellular structure of *Spirulina* render it easily digestible, thus, increase fish appetite, improve

feed intake and nutrient digestibility and in turn enhance the health of fish, increasing the ability to fight off infections through the reduction of stress levels. The results in the current study are in accordance with Watanabe et al. (1990) and Takeuchi et al. (2002) who found that feed supplemented with S. platensis powder improved the feed conversion ratio and growth rates in striped jack, Pseudocaranx dentex. Lu et al. (2002) demonstrated that raw S. platensis can be an effective uni-feed for larval tilapia at a feeding rate of 30% (on a dry basis) of body weight. Abdel-Tawwab and Ahmed (2009) recorded that the growth and feed utilization of O. niloticus were obtained at 5.0 g fresh culture of S. platensis /kg diet. On the contrary, Ungsethaphand et al. (2010) recorded that the final weight gain, specific growth rate, feed conversion ratio of hybrid red tilapia were not affected by *S. platensis* supplementation. These variations might be attributed to the difference in the *S. platensis* concentration to exert the intended effects, the form of *S. platensis*, raw or dried *S. platensis* or even its products, fish species and size in addition to the rearing conditions.

The high survival rate results in the current study are consistent with that recorded by Dernekbasi et al. (1993) who observed good survival rates in all Guppies treated with *S. platensis* using 40% *S. platensis* supplementation. However, the previous study did not mention the form of *S. platensis* used; in addition, the difference in the *spirulina* concentration could be related to the difference in the feeding habits of the fish species. In contrary to our results Abdel-Tawwab and Ahmed (2009), and Ungsethaphand et al. (2010) found no significant changes in the survival rates of fish dietary supplemented with *S. platensis* at different concentrations.

Adherence/NBT (nitroblue tetrazolium) and respiratory burst process assay spotlights on the non-specific immune response and the antibacterial mechanisms of the tested substances. The results of the current feeding trial showed a significant increase of nitroblue tetrazolium (NBT) and Neutrophil Adherence values in most of the *S. platensis* supplemented groups. The current results agree with those of Duncan and Klesius (1996) who reported enhancement of the peritoneal phagocytes from channel catfish, *I. punctatus* fed *S. platensis*, *Spirulina* algae contain carotenoids, which specifically improving fish health and increasing the ability to fight off infections through the reduction of stress levels. In addition, Watanuki et al. (1990) reported that *S. platensis* activated the functions of leucocytes, such as phagocytosis and production of superoxide, and cytokines production in common carp, *Cyprinus carpio*.

The present study found that fish fed with 10% spirulina exhibited significant haematocrit values. The increase in the immunity stimulating capacity (measure by a lysozyme activity assay) was significant in all fish groups given basal diet supplemented with *S. platensis* at the 2nd and 3rd month.

Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms. The increase in the immunity stimulating capacity could be due to the presence of C-phycocyanin in the *Spirulina* alga, which can help build the immunity capacity (Vonshak, 1997). Results were in accordance with Tayag et al. (2010) who concluded that the white shrimp *L. vannamei* that received the hot-water extract of *S. platensis* had enhanced innate immunity as lysozyme and increased resistance against *V. alginolyticus* infection.

The challenge infection revealed a significantly lower mortality percentage in the group received 10 g/kg *S. platensis* in diet for the 1, 2 and 3 months feeding trial and significantly high relative level of protection (RLP) after challenge infection using *P. fluorescens.* The disease challenge is an in vitro technique provides an opportunity to determine the performance and immunity of the fish species ebon exposure to xenobiotic (bacteria) on their natural habitats (AraKoosh et al., 2009). The results were in accordance with Watanuki et al. (2006) who recorded an increased resistance of *S. platensis* treated carp ebon artificial challenge with *A. hydrophila* than the control group. Abdel-Tawwab and Ahmad (2009) found that SP has a useful impact on fish as immuno-stimulants, they recorded that tilapia fed 5-10 g *fresh S. platensis* /kg diet increased its resistance against *A. hydrophyla.*

Finally, from the present investigation, it was concluded that optimized the dietary levels of dried Spirulina in *O. niloticus,* was useful to decrease costs of the products used and negate losses that could be encountered with improper supplementation levels. It was proved that the optimum concentration of dried *S. platensis* in the *O. niloticus* practical diet is 10 g/kg for 2 months, to positively improving health conditions*,* enhanced the non-specific immunity of *Oreochromis niloticus,* as well as its resistance to challenge by *P. fluorescens* infections. It is recommended to supplement Spirulina in the diet of Nile tilapia especially those grow in farms under immunosuppressive/stressful conditions. Additional researches are needed to study additional desired effects of the blue green algae in cultured fish.

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Desiccation Periods of *Urochloa brizantha* 'Piatã' Before Sunflower Sowing

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Abstract

In no-till farming, which integrates crops and livestock, the crop that is sown is often noted for reduced productivity. The aim of this experiment was to study the influence of a desiccation period of *Urochloa brizantha* 'Piatã' on sunflower 'Aguará 4' development and productivity. The desiccation was carried out using glyphosate herbicide at a dose of 1.92 kg·ha^{-1}. The treatments consisted of seven periods of *U. brizantha* desiccation (27, 22, 17, 10, 5, 3 and 0 days) prior to sunflower sowing (2010/11). The experimental design established randomized blocks with five replicates. The reduced period between cover desiccation and crop sowing impaired sunflower development (capitulum diameter, plant height, leaf area, and leaf and stem dry masses) over the course of the evaluations, especially for periods less than or equal to 10 days. Thus, our data suggest that sunflower producers should carry out the desiccation at least ten days prior to sowing.

Keywords: *Brachiaria brizantha*, *Helianthus annuus*, glyphosate, vegetal cover

1. Introduction

Crop-livestock integration may consist of crop sowing after pasture desiccation, which is a no-till farming method. The sunflower (*Helianthus annuus* L.) is a crop that is suited to no-till farming. Researchers have compared sunflower productivity in various cultivation systems and have shown similar levels of production with no-till and conventional-till farming methods (Silva et al., 1997; Anderson et al., 1996).

The species of the genus *Urochloa* (Syn. *Brachiaria*) are an efficient cover crop that it is able to fully protect the soil surface (Cobucci, 2001). *Urochloa brizantha* (Hochst. ex A. Rich.) Stapf cv. Piatã is a great option to use for crop-pasture integration because it displays slower initial growth than other cultivars, a suitable architecture and good mass accumulation during dry periods. Chemical-based pasture control for no-till farming is carried out using non-selective systemic herbicides. Generally, glyphosate is used due to its ability to control pasture growth and its extremely low permanency in the soil (Ashford & Reeves, 2003).

However, crop sowing on forage may cause injuries and reduced productivity. This reduction may be the result of several factors: the action of allelopathy of the cover crop or, indirectly, the desiccant herbicide (Neumann et al., 2006). The inhibitory effect that cover desiccation provides on the crop that is subsequently sown varies according to the period between pasture desiccation and crop sowing. Regarding the use of *U. brizantha* coverage, more information is needed to clarify the ideal time of desiccation such that crop productivity is not affected (Nunes et al., 2009).

A period of less than 7 days between *Urochloa* cover desiccation and soybean sowing reduces crop development (Santos et al., 2007; Nunes et al., 2009). However, (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2006) recommended that the ideal period between pasture desiccation and soybean sowing should be longer: more than 30 days. Therefore, the ideal period for desiccation prior to soybean sowing has already been studied and determined. These results led us to believe that the same effects might occur with other main crops, such as sunflowers.

The aim of this study was to verify the influence of different periods of *U. brizantha* desiccation using glyphosate on sunflower productivity when the seeds were sown using no-till methods.

2. Materials and Methods

The experiment was conducted in 2011 in a soil classified as a Clayey Red Latosol (Oxisol) that had the following chemical properties at 0-20 cm of depth: pH ($CaCl_2$) 5.3; organic matter 23 $g \cdot dm^{-3}$; P (resin) 18 $mg \cdot dm^{-3}$; 4.5, 34, 14, 28, 52.5 and 80.5 $mmolc \cdot dm^{-3}$ of K, Ca, Mg, H+Al, sum of bases and CEC, respectively; and 70% base saturation.

The area was laid out in a pasture of established *U. brizantha* cultivar Piatã. The experiment comprised seven periods (27, 22, 17, 10, 5, 3 and 0 days) between herbicide application for *U. brizantha* desiccation and sunflower sowing.

There were five replicates, and the plots were laid out in a randomized block design. The total area per plot was 45 m^2, and the net sample area consisted of two rows that were each 10-m long, forming an area of 13.5 m^2.

The cover crop was desiccated using glyphosate at a dose of 1.92 kg of acid equivalent (ae) per ha. The application was performed using a CO_2-pressurized backpack sprayer adjusted to spread a volume of 200 $L \cdot ha^{-1}$.

A complete NPK (nitrogen, phosphorus and potassium) fertilizer was spread at a rate of 300 $kg \cdot ha^{-1}$ (4-14-8 formulation) during the sunflower sowing. It was applied over two split applications. The first application during the sowing spread 300 $kg \cdot ha^{-1}$ of NPK (4-14-8 formulation). After 30 days, 90 $kg \cdot ha^{-1}$ of urea and 11 $kg \cdot ha^{-1}$ of borax were also applied. Adequate plant protection was provided to guarantee the yield potential.

To control the sunflower caterpillar (*Chlosyne lacinia saundersii*, Doubleday & Hewitson), two applications of the mixed insecticide thiamethoxam + lambda-cyhalothrin [28.2 mL + 21.2 mL of active ingredient (ai) per ha] were applied at 30 and 70 days after sowing. To prevent powdery mildew (*Erysiphe cichoracearum* DC.) infestation, a mix of the two fungicides azoxystrobin + cyproconazole (40.0 mL + 16.0 mL of ai per ha) was applied 60 days after sowing.

The sunflower hybrid Aguará 4, which is characterized by high achene productivity and oil content (45-50%), was sown on March 17th at the end of the rainy season. The row spacing for the sunflower sowing was 0.9 m, forming a population of 40,000 $plants \cdot ha^{-1}$.

After three months, an irrigation sprinkler system was installed, and water was applied as necessary to enable achene filling. The pluvial precipitation data are shown in Figure 1. The crop remained in the field for a longer period than necessary to ensure that the capitula sampled were completely dry. Dry capitula are more suited for mechanical threshing.

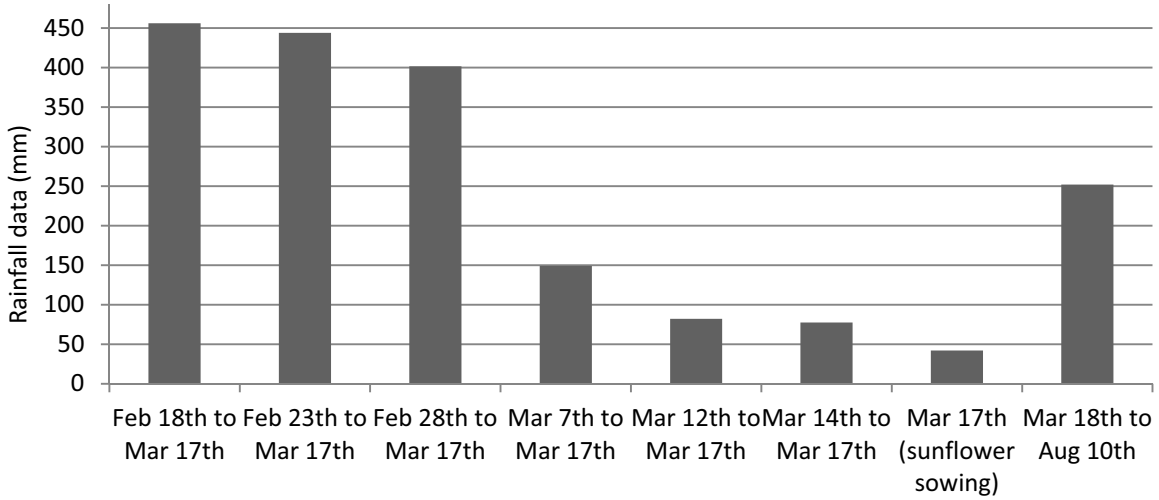

Figure 1. Pluvial precipitation (mm) during the seven periods between cover desiccation and sunflower sowing, on the day of sowing and during the period of crop growth. Source: Agricultural Station, Department of Exact Sciences of UNESP. Jaboticabal, São Paulo State, Brazil, 2011

A sample area of 0.25 m² covered by *U. brizantha* was selected for each desiccation time. A sampling frame was thrown randomly into the field four times. The material was cut at the ground level, placed into paper bags and dried with forced air oven at a temperature of 60ºC until the sample reached a constant dry matter weight. The dry matter weight of the *U. brizantha* cover present in the area was high at 14.8 $t \cdot ha^{-1}$ on average. When the

experiment was installed, the pasture was at a reproductive stage and the vegetative growth had already stabilized. There were no significant variations among the dry matter weights for the different desiccation periods (results not shown). Accordingly, all of the treatments were implemented under the same amount of cover crop matter.

At 45, 90 and 140 days after sowing (DAS), biometric evaluations were performed on the sunflower crop. At 45 DAS, 6 plants per plot were sampled, and for each plant, the heights (cm), numbers of leaves, leaf areas (cm^2) and dry masses of the stems (g) and leaves (g) were measured. At 90 DAS, the same evaluations were carried out for three plants, and the stem diameter (mm) and capitula diameter (cm) were also evaluated.

At harvest (140 DAS), 20 plants were sampled randomly and the heights, stem diameters and capitula diameters were measured once again. The achene moisture was corrected to 11% to estimate the yield ($kg \cdot ha^{-1}$), the dry mass of 100 achenes and the oil content (%). The oil content was determined by ether extraction, which was conducted at the Laboratory of Animal Nutrition at FCAV/UNESP.

The data obtained were subjected to variance analyses (F tests). To compare the means, the data were subjected to the Scott-Knott test at 5% probability. The data on achene production also underwent regression analyses (OriginPro 8 software) to model how the desiccation effect/sowing affected the achene yield.

3. Results

In the first evaluation (at 45 DAS), reduced development of the sunflower plants due to the reduced period between *U. brizantha* desiccation and crop sowing was evident (Table 1). For all of the evaluated parameters, the period of 0 days differed significantly from the others and was always the lowest value. The plants sown after the longest period (27 days after desiccation) had the highest heights, followed by the periods of 22, 17 and 10 days. In the periods between 10 and 27 days, the plants had more leaves and greater leaf areas than the plants sown after shorter desiccation periods. The leaf areas of the plants sown in the periods of 3 and 0 days were reduced to 55% and 78%, respectively, compared with the longest period. The stem dry mass was also reduced by shorter periods between desiccation and sowing. The periods of 27 and 22 days provided more stem dry mass than 17 and 10 days, followed by the periods of 5 and 3 days.

Table 1. Plant heights (cm), numbers of leaves, leaf areas (cm^2) and dry masses of leaves (g) and stems (g) of sunflower 'Aguará 4' at 45 days after sowing. The treatments were different time periods between *U. brizantha* desiccation and no-till sunflower sowing

DAS	Height (cm)	Number of leaves	Leaf area (cm^2)	Leaf dry mass (g)	Stem dry mass (g)
27	55.37 a[1]	16.08 a	1469.00 a	6.03 a	4.95 a
22	50.83 b	16.03 a	1220.02 b	5.14 a	4.59 a
17	48.66 b	16.13 a	1216.94 b	5.11 a	3.99 b
10	46.43 b	15.40 a	1177.11 b	5.05 a	3.98 b
5	42.70 c	13.83 b	831.07 c	4.05 a	2.45 c
3	41.26 c	13.20 b	667.70 c	2.71 b	2.18 c
0	34.99 d	10.78 c	321.28 d	1.28 c	1.16 d
CV (%)	8.17	6.68	18.37	22.71	19.43
F (treatments)	16.25*	21.56**	24.00**	15.14**	23.64**
F (blocks)	0.94^{ns}	2.89*	4.23**	2.52^{ns}	1.63^{ns}

*, **Significant at $\alpha = 0.05$ and 0.01, respectively. ^{ns}not significant. [1]Within a column, means followed by the same letter are not significantly different according to Scott Knott's F-protected test at $P = 0.05$.

The inhibitory effect provided by reduced periods between *U. brizantha* desiccation and sunflower sowing was in a similar manner at 90 DAS (Table 2). That trend is clearly illustrated by the capitulum dry mass. In the characteristics related to the leaves (leaf area and dry mass), stems (stem diameter and dry mass) and capitulum diameter, there were higher values when the sunflowers were sown in the periods of 27 and 22 days. There was no difference between the treatments with respect to the number of leaves. Only the period of 0 days produced plants that were smaller than for the other periods.

Table 2. Plant heights (cm), numbers of leaves, stem diameters (mm), capitulum diameters (cm), leaf areas (cm²), leaf dry masses (g) and stem dry masses (g) of sunflower 'Aguará 4' 90 days after sowing. The treatments were different time periods between *U. brizantha* desiccation and no-till sunflower sowing

DAS	Height (cm)	Number of leaves	Stem diameter (mm)	Capitulum diameter (cm)	Leaf area (cm²)	Leaf dry mass (g)	Stem dry mass (g)	Capitulum dry mass (g)
27	131.53 a[1]	24.57 a	18.95 a	14.11 a	3272.94 a	22.03 a	53.50 a	29.62 a
22	129.60 a	24.20 a	17.99 a	14.60 a	3340.20 a	21.40 a	55.25 a	26.06 b
17	127.53 a	23.44 a	16.77 b	12.17 b	2517.72 b	15.74 b	42.31 b	18.06 c
10	127.60 a	23.00 a	16.17 b	10.36 c	1793.32 c	12.88 c	39.61 b	14.87 d
5	121.53 a	23.08 a	14.99 c	10.06 c	1841.86 c	10.98 d	33.90 c	12.79 e
3	118.50 a	22.87 a	14.68 c	8.85 c	1550.47 c	9.39 e	29.16 d	12.23 e
0	96.00 b	21.17 a	12.48 d	6.71 d	1213.50 c	6.12 f	18.25 e	6.84 f
CV (%)	6.36	5.89	6.85	13.39	22.49	8.12	5.86	8.62
F (treats.)	12.49**	3.22*	19.67**	18.61**	14.19**	137.76**	167.07**	146.70**
F (blocks)	1.26[ns]	2.09[ns]	5.90**	2.17[ns]	11.01**	1.83[ns]	0.88[ns]	1.48[ns]

*, **Significant at α = 0.05 and 0.01, respectively. [ns]not significant. [1]Within a column, means followed by the same letter are not significantly different according to Scott Knott's F-protected test at P = 0.05.

At harvest, the parameters analyzed followed the same trends found at earlier developmental stages. As the period between *U. brizantha* desiccation and sunflower sowing decreased, the reductions in the crop productivity characteristics increased (Table 3). The tallest plants were sown during the two longer periods followed by the 17- and 10-day periods. The period of 0 days produced the smallest plants and differed significantly from the other periods. There was no difference between the treatments with respect to the weight of 100 achenes and the oil content. The capitulum diameter only showed a difference compared with the period of 0 days, which produced the lowest value. Although there were no marked differences between the treatments with respect to this characteristic, the low achene production could be explained by the higher number of undeveloped inflorescences.

Table 3. Heights (cm), stem diameters (mm), capitulum diameters (cm), weights of 100 achenes (g), achene production (kg·ha^{-1}) and oil contents (%) of sunflower 'Aguará 4' at harvest. The treatments were different time periods between *U. brizantha* desiccation and no-till sunflower sowing

DAS	Height (cm)	Stem diameter (mm)	Capitulum diameter (cm)	Weight of 100 achenes (g)	Achene production (kg·ha^{-1})	Oil content (%)
27	134.86 a[1]	17.37 a	17.77 a	5.72 a	1757.31 a	48.53 a
22	132.60 a	17.70 a	16.42 a	5.51 a	1641.94 a	47.95 a
17	128.52 b	16.54 a	16.05 a	5.42 a	1556.21 a	49.41 a
10	127.78 b	15.73 b	14.80 a	5.17 a	1308.35 b	48.67 a
5	120.80 c	15.04 b	15.38 a	5.11 a	1295.41 b	48.03 a
3	119.02 c	15.01 b	15.23 a	5.13 a	1112.11 b	48.51 a
0	106.00 d	13.22 c	12.49 b	4.75 a	782.41 c	48.47 a
CV (%)	3.68	5.21	9.55	9.21	18.85	2.65
F (treats.)	23.40**	17.82**	6.09**	2.14[ns]	8.71**	0.42[ns]
F (blocks)	1.20[ns]	4.01[ns]	0.51[ns]	0.20[ns]	1.36[ns]	2.78[ns]

**Significant at α = 0.01. [ns]not significant. [1]Within a column, means followed by the same letter are not significantly different according to Scott Knott's F-protected test at P = 0.05.

The variables achene production and stem diameter responded to the periods of pasture desiccation in a similar manner: these variables increased with longer desiccation periods (27, 22 and 17 days) followed by the shorter desiccation periods (10, 5 and 3 days), which differed significantly from 0 days of desiccation. Desiccation on the

same day as sunflower sowing reduced achene production by 55% compared with the longer periods between desiccation and sowing.

When analyzed quantitatively, the achene production results showed exponential behavior (Figure 2). Shortening the period between *U. brizantha* desiccation and sunflower sowing caused an exponential reduction in achene production. For an acceptable loss of 5% in sunflower productivity, *U. brizantha* desiccation must be carried out at least 23 days before no-till crop sowing.

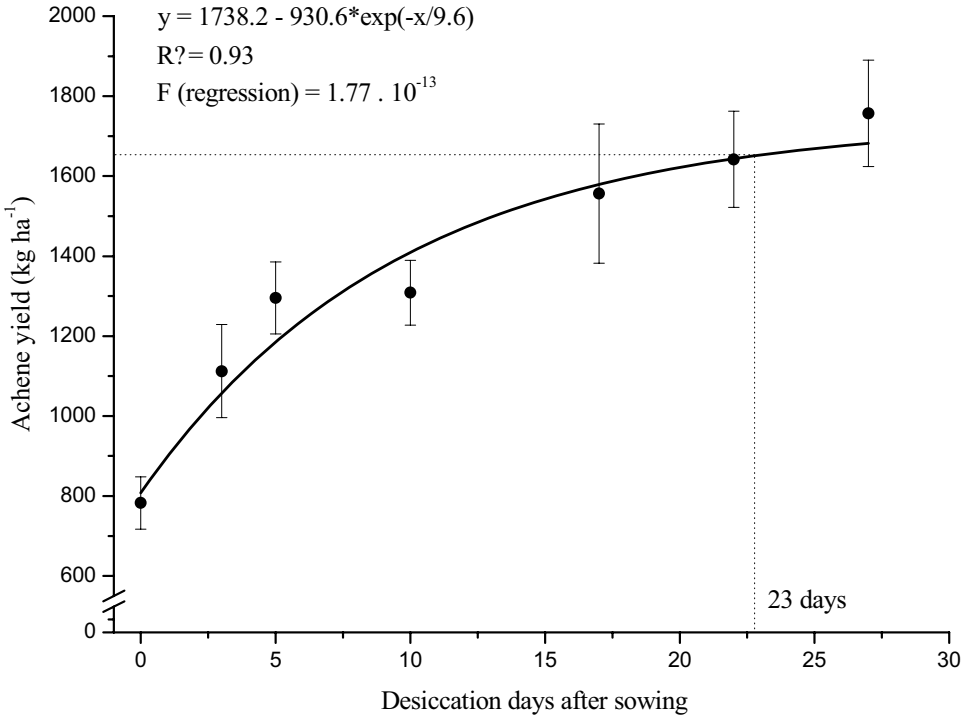

Figure 2. Achene production (kg·ha^{-1}) of sunflower 'Aguará 4' sown at increasing periods after *U. brizantha* desiccation

4. Discussion

A period equal to or longer than 17 days showed higher crop productivity. This period was similar to the period proposed by Silva (2001) for no-till corn but differed from the period of Nunes et al. (2009) and Nepomuceno et al. (2012) for soybean sowing. However, previous studies documented productivity reductions when the sowing was carried out on the same day as the desiccation. In this study, the productivity reduction provided by this treatment was similar to the 50% reduction observed by Nunes et al. (2009).

Fancelli and Dourado Neto (2004) affirmed that a high production of organic compounds and the permanence of the vegetation cover allowed the expression of allelopathic effects. Martins et al. (2006) showed the allelopathic potential of *U. brizantha*. The compound responsible for this effect has been isolated and is derived from a steroidal saponin called protodiocsin (Nepomuceno, 2011). Considering that the pasture had a high density and that the plants of this genus persisted for a long time (Cobucci, 2001), the release of *U. brizantha* allelochemicals may have caused negative effects on the sunflowers. The release of allelopathic compounds can occur during the decomposition of the plant tissues (Macías et al., 2007). Assuming that the decomposition of the dense cover was slow, this gradual release could explain the damage observed even 10 days after desiccation.

The high glyphosate dose used for *U. brizantha* desiccation could also be the cause of the reduction in sunflower development and productivity when sown at shorter periods after desiccation. Furthermore, the slow decomposition of the dense cover after treatment could prolong the phytotoxic effect of the glyphosate that is released during this process (Neumann et al., 2006; Tesfamariam et al., 2009). Due to the low Kow of glyphosate (Battaglin et al., 2005) and the heavy rains in the days after desiccation, the glyphosate was potentially released

from the vegetal residue and then became available for absorption by the sunflower seedlings.

Although nutritional features were not analyzed in this experiment, nutritional deficiencies may also have occurred in this experiment. Tesfamariam et al. (2009) found manganese nutritional deficiencies in sunflowers even when the sowing occurred longer than 21 days after the pasture desiccation. This potential of the herbicide to cause a nutritional deficiency even when the sowing is carried out many days after desiccation may perhaps explain the productivity reduction found after the 10-day interval in our study.

In addition to the nutritional questions, there is also the problem of the low availability of nitrogen to crops sown on grass. This fact is mainly due microbial immobilization of the nutrient. Although no-till farming methods provide an increase in the total nitrogen content of the soil, lower absorption of nitrogen by crops in this system of farming has frequently been observed.

5. Conclusion

U. brizantha desiccation (with a dose of 1.92 kg of acid equivalent per ha for a dry mass of cover of 14.8 $t \cdot ha^{-1}$) in a period equal to or less than 10 days before no-till sowing of sunflower 'Aguará 4' caused a reduction in crop development and productivity. The period of desiccation influences the sunflower productivity, either by *U. brizantha* allelopathy or by a glyphosate phytotoxic effect. One or both of these factors were responsible for a 50% reduction in sunflower production when sowing occurred at the same time as *U. brizantha* desiccation. Thus, it is clear that sunflower producers should perform desiccation at least ten days before sowing.

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Position and Density of Pistillate Inflorescences of Some Hazelnut Cultivars Grown in Iran

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Abstract

Little work has been done to examine the position and density of pistillate inflorescences (PIs) in hazelnut. In this study, characteristics of PIs of three Iranian cultivars ("Pashmine", "Tabestane", and "Shastak") and three imported cultivars ("Barcelona", "Merveille", and "Prostorika") were examined over one year and compared. Overall, results showed strong similarities between cultivars in density of flowering shoots per branch with the density of PIs notably higher in the upper section of the branches. Significant variation was observed between cultivars in the percent of terminally and laterally located PIs and average number of pistillate flowers per inflorescence. However, more PIs per inflorescence did not necessarily result in more nuts per cluster or higher yield. These preliminary results indicate that the Iranian and imported cultivars share many similar PI characteristics. However, they also suggest that other genetic and environmental factors contribute to nuts produced per cluster and total crop yield. The results provide a foundation from which further studies can be completed on the relationships between PIs and yield in hazelnut.

Keywords: *Corylus avellana* L., filbert, floral density, inflorescence, pistillate flowers

1. Introduction

Flowers, the reproductive parts of most plants, are essential to the fruiting process. Therefore, in tree crops including hazelnut (*Corylus avellana* L.), they are of great economic importance. Efficient commercial production largely depends on the quantity and quality of flower buds produced (Hanke, Flachowsky, Peil, & Hättasch, 2007). According to Werner, Mowrey and Chaparro (1988), the initial number of flowers differentiated in the previous season is the first component of yield in fruit trees and has a significant impact on productivity. This component of yield has been found to be genotype dependent with large variations in bud density observed in peach (*Prunus persica* L.) (Okie & Werner, 1996).

In almond, flower density, fruit set, fruit density, and productivity, are highly affected by the cultivar, year, and environment (Kodad & Socias i Company, 2006; Socias i Company, Alonso, & Aparisi, 2004). Similarly, Alburquerque, Burgos and Egea (2004) suggested that in apricot (*Prunus armeniaca* L.), flower bud density, bud drop, and yield efficiency are also influenced by cultivar. They demonstrated that early flowering cultivars typically had the highest flower bud density. Thus, estimation of flower density and subsequent fruit set is important in assessment of a cultivar's performance (Socias i Company, Felipe, Aparisi, García, & Dicenta, 1998). Seifi, Guerin, Kaiser and Sedgley (2011) reported that in olive (*Olea europaea* L.) cultivars, the section of shoot (upper, middle, and basal) affected the inflorescence length and the number of flowers per inflorescence, but not the percent of perfect flowers present. They also found that shoot orientation (north, south, east, and west) did not have any influence on these characteristics in all studied cultivars. This type of information can be helpful for breeders when selecting cultivars as parents in breeding programs to produce progeny with higher cropping efficiency.

Hazelnut is an obligate out-crossing, monoecious, wind-pollinated shrub with a sporophytic self-incompatibility system (Mehlenbacher, 1997). During flowering season, which occurs in the late winter through early spring depending on the cultivar, it is possible to simultaneously observe staminate (catkins) and pistillate inflorescences (PIs) on the leaf scar axils of one-year-old shoots. This species has a unique reproduction process where pollen lands on the stigma, germinates, and grows down the style, but then remains dormant for approximately five months before fertilizing the egg cell (Thompson, Lagerstedt, & Mehlenbacher, 1996; Germain, 1994). Pistillate inflorescences are located singly on shoots, on catkin peduncles, or adjacent to vegetative buds (Figures 1 to 4). (Germaine, 1994). The relative amounts of PIs in these positions varies between the cultivars (Thompson et al., 1996). It has been shown that the highest-yielding cultivars, such as Ennis, Casina, Montebello, and Willamette, generally have most of their PIs born on catkin peduncles (Thompson et al., 1996). The number of PIs produced is affected by three major factors, which includes available light, origin of PIs on the stem, and the overall vigor of growth of the one-year-old shoots (Germain, 1994). Since the number of flowers is strongly related to yield in hazelnut, an important objective in breeding programs is selecting cultivars with heavy annual flowering capacity (Thompson et al., 1996).

Notwithstanding its importance, there has been little emphasis on describing the position and density of hazelnut PIs in the literature. The objective of this preliminary study was to investigate and document these traits in local hazelnut cultivars in comparison to imported cultivars grown in Iran. This data would then be compared to nuts per cluster and total yield to draw conclusions on the relationship between PI characteristics, fruit set, and crop potential in Iran.

Figure 1. Pistillate inflorescence (PI) on catkin peduncle

Figure 2. A single, lateral PI on the leaf scar axil of one- year-old shoot

Figure 3. A single, terminal PI

Figure 4. Two lateral PIs in one node adjacent to a vegetative bud

2. Materials and Methods

This study was carried out during the winter of 2011, at the Astara Hazelnut Research Station in Astara, Guilan province, Iran (lat. 38˚ 25′ N, long. 48˚ 52′ E). The average annual precipitation is 1350 mm, and the annual minimum and maximum temperatures are -5˚C and 36.5˚C, respectively. Six hazelnut cultivars were selected, including three local cultivars - "Pashmine", "Tabestane", and "Shastak"-and three imported cultivars - "Barcelona" (USA), "Merveille" (syn. Merveille de Bollwiller) (France), and "Prostorika" (Russia). Each cultivar was represented by three trees. All trees were planted between 2000 and 2003. The trees were grown as multi-trunk shrubs on a spacing of 5 × 5 m and were irrigated using a drip irrigation system. Trees were grown in full sun, with ample light exposure available to most branches including the inner parts of the canopy.

Four branches in different orientations (north, east, south, and west) were selected for each of the three trees per cultivar. Each branch was divided into equal thirds: basal, middle, and upper. The following characteristics were recorded for each branch section: number of flowering shoots (one-year-old shoots carrying PIs), number of flowering nodes (nodes carrying PIs), total number of PIs, percent of terminal (located at the end of the shoot) and lateral (located attached to the shoot) PIs, position of PIs on shoots [beside vegetative buds, on catkin peduncles, or single (no other buds nearby)], and number of pistillate flowers per inflorescence (from 40 inflorescences collected randomly from each tree). Information on the yield of these cultivars was assembled at the end of the season to investigate the relationship between PI characteristics and yield. Recorded data was analyzed using GENSTAT statistical software (Ver. 7, Lawes Agricultural Trust, Rothamsted Experimental Station) and the means were compared using LSD (0.05).

Table 1. Density and position of pistillate inflorescences (PIs) on branches of six hazelnut cultivars

	Pistillate flowering shoots[y] (no./1.0 m of branches)	Flowering nodes[z] (no./1.0 m of branches)	PIs (no./1.0 m of branches)
Cultivar	$P \leq 0.001$	$P = 0.125$	$P = 0.214$
"Barcelona"	9.03 ± 1.53 [b]	16.39 ± 3.19	20.51 ± 4.23
"Merveille"	13.37 ± 1.67 [a]	21.28 ± 3.61	24.79 ± 4.22
"Prostokarika"	8.26 ± 1.24 [b]	15.21 ± 2.87	19.29 ± 3.92
"Pashmine"	10.38 ± 1.88 [b]	21.45 ± 4.60	22.09 ± 4.8
"Shastak"	10.38 ± 2.41 [b]	29.12 ± 8.40	34.72 ± 9.7
"Tabestane"	13.73 ± 1.63 [a]	22.71 ± 3.14	26.28 ± 3.91
Origin of cultivar	$P = 0.809$	$P = 0.366$	$P = 0.571$
Local	10.22 ± 0.88	17.63 ± 1.87	21.53 ± 2.37
Imported	11.50 ± 1.16	24.43 ± 3.33	27.70 ± 3.82
Branch orientation	$P = 0.957$	$P = 0.857$	$P = 0.826$
North	10.94 ± 1.35	20.61 ± 3.13	23.73 ± 3.8
South	10.62 ± 1.45	19.87 ± 3.9	22.55 ± 4.3
West	10.66 ± 1.58	21.02 ± 4.6	25.03 ± 5.2
East	11.21 ± 1.46	22.63 ± 3.7	27.12 ± 4.6
Branch section	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$
Upper	20.94 ± 1.1[a]	46.09 ± 4.0 [a]	53.94 ± 4.6 [a]
Middle	7.90 ± 0.83 [b]	11.54 ± 1.39 [b]	13.67 ± 1.92 [b]
Basal	3.73 ± 0.79 [c]	5.44 ± 1.32 [c]	6.21 ± 1.62 [c]

Mean ± SEM. Means within a column followed by the same letter are not significantly different (LSD, 0.05).

Data represents average amounts from three trees of each cultivar.

In order to achieve a more normal distribution, data were transformed to square roots prior to analysis.

[y] Pistillate flowering shoots: one-year-old shoots carrying pistillate inflorescences that arise from main branches which constitute growth two years old and older.

[z] Flowering nodes: individual nodes where pistillate inflorescences are located.

3. Results and Discussion

The results showed surprising similarities between the cultivars in terms of PIs position and density. While "Tabestane" and "Merveille" had a significantly higher number of pistillate flowering shoots than the group (13.73 and 13.37 per 1.0 m of branch, respectively; $P \leq 0.001$), all of the other cultivars were found to be similar (Table 1). Additionally, there were no significant differences between the cultivars in the density of pistillate flowering nodes or the total number of PIs per branch. Furthermore, no significant differences between the four geographic orientations in the density of flowering shoots, flowering nodes, and total number of PIs per branch were found in each cultivar ($P > 0.05$ in all of them) (Table 1). Therefore, the amounts of flower induction and formation, and consequent fruit set, are probably similar in all orientations under the conditions present in this study.

For all cultivars, the three sections of the branches differed in their densities of flowering shoots, flowering nodes, and total number of PIs per branch ($P \leq 0.001$). On average, there were 21.0 flowering shoots in every 1.0 m of the upper third, and only 7.9 in the middle and 3.7 in the basal sections. Similarly, the density of flowering nodes and total number of PIs were also much higher in the upper third (46.09 and 53.94 per m, respectively) compared to the middle (11.54 and 13.67) and basal (5.44 and 6.21) sections. This finding was expected as the increased amount of light received in the upper canopy has been reported to have a positive impact on flower formation (Germain, 1994). For this reason, it is suggested to avoid removing the upper sections of branches as much as possible during pruning to retain the highest areas of productivity.

Table 2. Percentage of pistillate inflorescences (PIs) found in different positions on flowering shoots of six hazelnut cultivars

	Terminal PIs (%)[y]	Lateral PIs (%)[y]	PIs beside vegetative bud (%)[z]	PIs on catkin peduncles (%)[z]
Cultivar	$P \leq 0.001$	$P \leq 0.001$	$P = 0.015$	$P = 0.015$
"Barcelona"	40.61 ± 6.40 [abc]	59.39 ± 6.40 [abc]	69.61 ± 6.53 [b]	30.39 ± 6.53 [a]
"Merveille"	61.41 ± 5.03 [a]	38.59 ± 5.03 [c]	74.87 ± 4.78 [b]	25.13 ± 4.78 [a]
"Prostokarika"	37.77 ± 5.95 [bc]	62.23 ± 5.95 [ab]	70.65 ± 6.85 [b]	29.35 ± 6.85 [a]
"Pashmine"	24.35 ± 7.29 [c]	75.65 ± 7.29 [a]	91.92 ± 4.74 [a]	8.08 ± 4.74 [b]
"Shastak"	32.82 ± 6.50 [bc]	67.18 ± 6.50 [ab]	59.95 ± 7.67 [b]	40.05 ± 7.67 [a]
"Tabestane"	51.38 ± 5.68 [ab]	48.62 ± 5.68 [bc]	77.01 ± 4.45 [b]	22.99 ± 4.45 [a]
Origin of cultivar	$P = 0.112$	$P = 0.112$	$P = 0.329$	$P = 0.329$
Local	38.58 ± 3.93	61.42 ± 3.93	77.07 ± 3.38	22.93 ± 3.38
Imported	47.57 ± 3.49	52.43 ± 3.49	71.95 ± 3.43	28.05 ± 3.43
Branch orientation	$P = 0.425$	$P = 0.425$	$P = 0.607$	$P = 0.607$
North	48.68 ± 5.16	51.32 ± 5.16	73.37 ± 4.90	26.63 ± 4.90
South	38.18 ± 5.25	61.82 ± 5.25	79.90 ± 3.99	20.10 ± 3.99
West	41.67 ± 5.49	58.33 ± 5.49	71.69 ± 5.21	28.31 ± 5.21
East	44.09 ± 5.26	55.91 ± 5.26	72.75 ± 5.17	27.25 ± 5.17
Branch section	$P = 0.004$	$P = 0.004$	$P = 0.104$	$P = 0.104$
Upper	32.17 ± 2.89 [b]	67.83 ± 2.89 [a]	73.07 ± 2.92	26.93 ± 2.92
Middle	49.11 ± 4.78 [ab]	50.89 ± 4.78 [ab]	72.23 ± 4.93	27.77 ± 4.93
Basal	60.51 ± 7.02 [a]	39.49 ± 7.02 [b]	82.07 ± 5.45	17.93 ± 5.45

Mean ± SEM. Means within a column followed by the same letter are not significantly different (LSD, 0.05).

Data represents average amounts from three trees of each cultivar.

In order to achieve a more normal distribution, data were transformed to square roots prior to analysis.

[y] Terminal PIs + Lateral PIs = 100%.

[z] PIs beside vegetative bud + PIs on catkin peduncles = 100%.

The position of PIs located upon the flowering shoots are summarized in Table 2. There were highly significant differences between the cultivars in the percent of terminal and lateral PIs present ($P \leq 0.001$). "Merville" had the highest number of inflorescences in terminal positions (61.41%). In contrast, "Prostorika", "Pashmine", and "Shastak" all had a greater percent of lateral inflorescences compared to terminal inflorescences. In all cultivars, the number of PIs located beside vegetative buds was higher than the PIs located on catkin peduncles. "Pashmine"

and “Tabestane” had a significantly higher number of PIs located beside vegetative buds compared to other cultivars (P = 0.015). The lowest percentage of PIs located on catkin peduncles (8.08%) was observed in “Pashmine”. Overall, there was no significant difference between Iranian and imported cultivars in the percent of PIs in different positions of the flowering shoots. Further, in all cultivars examined, most PIs were found in a lateral position and were located adjacent to vegetative buds.

Our results also showed that the orientation of the branch did not have a significant influence on the percent of terminal and lateral PIs present or the percent of PIs located beside vegetative buds and on catkin peduncles. Similarly, branch section was not found to have significant influence on the percent of PIs located beside vegetative buds and PIs on catkin peduncles; however, branch section was shown to have a significant influence on the percent of terminal and lateral PIs (P = 0.004). Generally, there were a higher percentage of terminal PIs at the basal section of the branches (60.51%) compared to the other two sections. In contrast, there were more lateral PIs on upper section of the branches (67.83%).

On flowering shoots of hazelnut, each node may contain one or more PIs. A summary of the percentage of nodes carrying one to five PIs for each cultivar is found in Table 3. In all cultivars, the majority of pistillate flowering nodes contained only one inflorescence (78.94% - 96.17%). It was followed by two and three inflorescences per node. Results showed that there were no significant differences between the cultivars in the percentage of nodes carrying one and three to four PIs, with significant influence found only on the percentage of nodes with two PIs (P = 0.049). “Barcelona” had the highest number of nodes with two PIs (20.46%), while “Pashmine” and “Shastak” had the lowest number (2.98% and 3.68%, respectively). Furthermore, nodes with four inflorescences were rarely found in “Prostorika” (2.88%) and “Shastak” (0.20%), and “Shastak” was the only cultivar found containing nodes with five inflorescences (0.44%). Further, no significant differences were observed among the four branch orientations and three branch sections in the percent of nodes with 1 to 5 PIs.

Table 3. Percentage of nodes with 1-5 pistillate inflorescences (PIs) on six hazelnut cultivars

	Nodes with 1 PI (%)	Nodes with 2 PIs (%)	Nodes with 3 PIs (%)	Nodes with 4 PIs (%)	Nodes with 5 PIs (%)
Cultivar	P = 0.076	P = 0.049	P = 0.243	P = 0.417	P = 0.217
“Barcelona”	78.94 ± 5.90	20.46 ± 6.40 [a]	2.78 ± 1.59	0	0
“Merville”	88.65 ± 3.40	9.54 ± 3.39 [ab]	1.81 ± 1.40	0	0
“Prostokarika”	85.10 ± 5.30	8.56 ± 3.40 [ab]	3.46 ± 2.41	2.88 ± 2.88	0
“Pashmine”	96.17 ± 2.32	2.98 ± 2.30 [b]	0.76 ± 0.76	0	0
“Shastak”	91.38 ± 2.87	3.68 ± 2.25 [b]	4.30 ± 2.15	0.20 ± 0.20	0.44 ± 0.44
“Tabestane”	90.35 ± 2.44	8.54 ± 2.25 [ab]	1.12 ± 0.69	0	0
Origin of cultivar	P = 0.187	P = 0.266	P = 0.868	P = 0.994	P = 0.885
Local	84.65 ± 2.77	12.40 ± 2.56	2.64 ± 1.05	0.95 ± 0.95	0
Imported	92.52 ± 1.50	5.64 ± 1.31	1.83 ± 0.68	0.05 ± 0.05	0.11 ± 0.11
Branch Orientation	P = 0.339	P = 0.141	P = 0.084	P = 0.584	P = 0.388
North	85.89 ± 3.47	13.15 ± 3.87	2.12 ± 1.35	0.10 ± 0.10	0
South	88.54 ± 2.74	7.75 ± 2.49	3.99 ± 1.58	0	0
West	87.54 ± 3.37	11.41 ± 3.17	1.05 ± 1.05	0	0
East	91.98 ± 3.01	3.96 ± 1.64	1.87 ± 1.02	1.97 ± 1.97	0.22
Branch section	P = 0.096	P = 0.199	P = 0.233	P = 0.322	P = 0.570
Upper	86.15 ± 2.56	10.12 ± 2.07	2.68 ± 0.96	1.10 ± 1.10	0.12
Middle	90.10 ± 2.32	8.15 ± 2.48	2.69 ± 1.27	0	0
Basal	91.29 ± 4.09	8.44 ± 4.06	0.27 ± 0.27	0	0

Mean ± SEM. Means within a column followed by the same letter are not significantly different (LSD, 0.05).

Data represents average amounts from three trees of each cultivar.

In order to achieve a more normal distribution, data were transformed to square roots prior to analysis.

Significant differences were observed between the cultivars in terms of the number of pistillate flowers per inflorescence ($P < 0.01$). "Merveille" (10.12) and "Barcelona" (7.62) had the highest and lowest average number of pistillate flowers per inflorescence, respectively (Table 4). Thompson et al. (1996) described the hazelnut PI as typically consisting of 4 to 14 small flowers, which are formed at the apex of a compound bud (Thompson et al., 1996). Interestingly, in this study, some inflorescences of "Merveille" had only two flowers and all of the cultivars except for "Barcelona" also had PIs containing 15-20 flowers. Table 4 also presents the average number of nuts per cluster and the recorded yield (kg inshell nuts/tree) of all cultivars [data derived from Hosseinpour, 2011]. While "Merveille" had the highest number of pistillate flowers per inflorescence, it was found to produce on average few nuts (1-2) per cluster (Hosseinpour, 2011). Thus, more pistillate flowers per inflorescence will not necessarily lead to more nuts set in the cluster. Further, based on our data, no direct relationship between the pistillate flowers per inflorescence and the total yield collected could be resolved for each cultivar.

Table 4. Average number of pistillate flowers per inflorescence, nuts per cluster, and yield for six hazelnut cultivars

Cultivar	Average number of pistillate flowers per inflorescence [y]	Average number of nuts per cluster [y,z]	Yield (kg inshell nuts/ tree) [z]
"Barcelona"	7.62 ± 0.18 c	2.42 ± 0.12 c	3.4
"Merville"	10.12 ± 0.27 a	1.30 ± 0.06 d	2.5
"Prostorika"	9.62 ± 0.32 ab	3.15 ± 0.2 ab	3.7
"Pashmine"	8.48 ± 0.19 bc	1.40 ± 0.09 d	3.0
"Shastak"	9.07 ± 0.21 ab	2.75 ± 0.17 bc	1.5
"Tabestane"	8.78 ± 0.22 abc	2.37 ± 0.15 c	2.3

[y] Mean ± SEM. Means within a column followed by the same letter are not significantly different (LSD, 0.05). 40 inflorescences per cultivar were analyzed.

[z] Data on nuts per cluster and yield are from Hosseinpour 2011.

4. Conclusion

With the exception of "Tabestane" and "Merveille", results showed a strong similarity between cultivars in the density of flowering shoots per branch, with all cultivars similar in terms of the number of flowering nodes and PIs within the branches. Further, across the cultivars, geographic orientation did not have a significant influence on the density of flowering shoots, flowering nodes, and PIs. However, significant variability was observed between cultivars in the percent of terminally and laterally located PIs and the average number of pistillate flowers found per inflorescence. Interestingly, an increased number of pistillate flowers per inflorescence did not necessarily result in more nut set per cluster. In general, the density of PIs was significantly higher in the upper section of the branches when compared to the middle and basal portions. Most PIs found in this section were lateral, while the majority of the PIs located in the basal section were terminal. Across all cultivars, most of the PIs found in nodes were singles, with only a few of the nodes carrying two or more inflorescences. Overall, these preliminary results suggest that the Iranian and imported cultivars do not differ much in the characteristics of their PIs. This finding indicates that additional factors such as successful pollination must play a significant role in nuts produced per cluster, as well as total yield, on which notable differences are present between the cultivars. Further investigations are needed in additional years to draw strong conclusions on the relationship between hazelnut pistillate inflorescences and their impact on total yield. It is hoped that the results presented here provide a foundation from which additional, longer-term studies can be completed on the relationships between PI characteristics and yield in hazelnut.

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Old Cultivars and Populations From Traditional Pepper-Growing Regions of Serbia as Breeding Potential

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Abstract

In order to collect and preserve the pepper gene fond (*Capsicum annuum* L.) material was collected in locations in South Serbia and Central Kosovo with long tradition of growing old pepper varieties. The area around the town of Aleksinac (villages Moravac, Prčilovac, Nozrina and Lužane) has a long tradition of growing old varieties and populations of pepper. The Žabarka and Čokotanka varieties are typical representatives hereof, and have been included in this study. From the area near the town of Leskovac (village Donja Lakošnica) we included the variety Lakošnička. This variety is very much grown here and is intended for grinding. In Central Kosovo, material was gathered in Laplje selo and Preoce, and the varieties collected were Rošajka and Bobinka crvena. These varieties have very specific colour, shape, size, aroma, intensity of heat and mode of usage. The different varieties will be stored in the Serbia Gene Bank and become valuable material in the selection and breeding of pepper in programs for obtaining varieties suitable for drying. The characteristics of the plants and fruits were studied and according to these traits grouping was done. Correlation of genotypes and traits was done by PCA (Principal Component Analysis). PCA grouped genotypes according to their traits (morphological and chemical) in two groups each. Genotypes grouped in this way represent the basic material for program of breeding pepper varieties suitable for drying. The selected varieties on the basis of superiority of characteristics can be proved a valuable material for any pepper breeding of Serbia which can be recommended for commercial cultivation.

Keywords: *Capsicum annuum*, old-time varieties, gene-fond, quality

1. Introduction

The fruit of *Capsicum annuum* belongs to the longum group, popularly known as red pepper. The dry fruits are used in cooking to give a mild flavour and red colour (Maiti et al., 2007). It is also high in vitamins A and C, which are important antioxidants and part of today's healthy lifestyles. Carotenoids, the largest group of plant pigments, function as antioxidants and as vitamin A precursors (Guzman et al., 2010). Vitamin C is hypothesized to prevent cancer by inhibiting the formation of nitrous compounds in the stomach, and also by stimulating the immune system (Doglin & Yasunori, 2003). Pepper producers mostly grow newly selected varieties and hybrids of pepper, produced in Serbia or abroad. However, there are many local populations and varieties grown in yards and green gardens. The seeds of these are maintained and reproduced by local producers. They are very heterogeneous and many specific traits make them very highly valued by the consumers. Numerous specifics and magnificent adjustment to local ecological conditions make local populations very interesting for pepper breeding (Ilić et al., 1997). Many pepper varieties differ in shape, size, aroma, intensity of heat etc. Pepper fruits are usually consumed fresh or dried (as spice) but the range of application is numerous: food, pharmaceutical and cosmetic industry (Sousa et al., 2006), to name just a few. The different applications of pepper products has to have the base in corresponding variety, which suits the function of the product with its traits (Costa et al., 1989; Viana et al., 2006). The genus *Capsicum* consists of many varieties that are grown for their fruits, making it one of the most popular vegetables in the world. It originates from South America, including tropical and subtropical regions (Pickersgill, 1997) and represents the result of selective breeding after its domestication. It is cultivated worldwide, and has become a key element in many regional cuisines, and the selection of the autochthone genotypes has been guided by traditional fruit usage (MacNeish, 1964). Within the *Capsicum* genus, many breeding programs include specific

crossing of several different varieties of *Capsicum annuum*. Hot and sweet pepper genotypes (*Capsicum frutescens* L. and *Capsicum annuum* L.) are very rich in genetic variability creating in this way a new, economically important variety. Traits that define a variety represent a set of a large number of traits, mostly polygene, that correlate and create the identity of a genotype (He & Wang, 1989; Basavaraja & Hulamani, 2001). Enrichment of the genefond by finding autochthone and domesticated genotypes represents a continuous task, together with its evaluation and characterization (Ortiz et al., 2010).

One of the primary reasons to sustain conservation of plant genetic resources in gene banks is to prevent the loss of genetic diversity (Maggioni, 2004). The main pepper collections can be found in the Russian Federation (2313), Germany (1504), France (1400) and Hungary (1400). A duplicate of the Capsicum Genetic Cooperative collection, including morphological and physiological markers, is conserved at the University of Torino, Italy. European collections hold pepper accessions originating from all over the world, as well as characterized genetic stocks and resources evaluated for disease resistance genes (Maggioni, 2004).

Systematic collection of old and local populations can be a basis for:

a) Breeding of new varieties with desirable morphological characteristics (such as intensive colour).

b) Selection towards better yield and resistance to diseases (Bezuneh, 1974).

The existing commercial assortment in Serbia (there are 40 officially registered varieties and hybrids of pepper) does not fully meet the demands of industry and contemporary vegetable production. It is therefore necessary to collect genotypes suitable for drying (for pepper powder) and for stuffing (with meat, rice, beans etc.). Breeding of new genotypes and creation of assortment for these purposes, as well as collection of old varieties are very significant tasks.

2. Material and Method

Within the project "*South East European* Development: Network on *Plant Genetic Resources* (SEEDNet)", and within the working group for vegetables from the Republic of Serbia, field research was conducted in order to note, register, collect, classify and evaluate old varieties and populations. The collection list and passport data were in accordance to descriptors for *Capsicum* genus. Five old varieties intended for traditional use (drying) were analysed:

Table 1. The analyse of five old varieties intended for traditional use

Local name of variety	Botanical origin	Locality
Rošajka	*C. annum ssp. macrocarpum var. longum*	Central Kosovo (Laplje selo, Gračanica)
Lakošnička	*C. annum ssp. macrocarpum var. longum*	South Serbia, Leskovac (Lakošnica)
Žabarka	*C. annum ssp. macrocarpum var. dolma*	Near Aleksinac (Moravac, Žitkovac)
Čokotanka	*C. annum ssp. macrocarpum var. longum*	South Serbia, from Aleksinac to Niš
Bobinka	*C. annum ssp. microcarpum var. cerosiforme*	South-east Serbia, Central Kosovo

2.1 Plant Material

The experiments were performed in an experimental garden located in the village of Moravac near Aleksinac (longitude: 21°42' E, latitude: 43°30' N, altitude 159 m) in the central area of south Serbia. Pepper plants were produced from seedlings (sowing during mid-March and planting late May) in the open field, during 2009-2011.

All necessary crop management measures were applied: preparation of the land, fertilizing with mineral and organic fertilizers, weed, disease and pest control, inter-row tillage, irrigation and foliar nutrition. During the vegetation, morphological and biological traits of every variety – population (with the existing descriptors), yield, as well as biochemical traits (dry matter content, ash content, total sugar content of ascorbic acid and β-carotene content) were followed.

2.2 Quality Parameters

Total dry matter was determined by drying procedure at the temperature of 105°C up to the level of constant mass. Approximately 0.5 g of freeze-dried sample was weighed into porcelain crucibles that had previously been heated for 3h at 550°C, and the sample was converted to white ash at this same temperature for over 12-18h.

Total and reducing sugars content was determined by the Luff–Shoorl's method, vitamin C content by Tillman's method, and carotenoid content was measured using the method based on pigment extraction by petrol- ether and pigment isolation by column chromatography, as well as by determining the colour intensity using spectrophotometric measurement.

2.3 Data Analysis

Correlation of genotypes and traits was determined by multi-variation technique, PCA (Principal Component Analysis) and using Statistical software: XLSTAT Version 2012.4.02 Copyright Addinsoft 1995-2012. The analysis was performed on the basis of the average values of the investigated parameters.

3. Results and Discussion

3.1 Chemical Composition of Old Pepper Varieties and PCA Analysis

Our results show that the Bobinka crvena cultivar stands out for its total content of: ash (1.03%), vitamin C (140.8 $mg \cdot 100g^{-1}$) and β-carotene (471.2 μg/g DM). Lakošnička has the highest dry matter content (13.65%), high content of total sugars (5.94%) as well as high level of β-carotene (416.2 $\mu g \cdot g^{-1}$ DM), which is especially significant for drying and grinding (Table 1).

Table 1. Chemical composition of some autochthons cultivars and old populations of pepper from Serbia

Cultivar	Dry matter %	Total sugar %	Ash %	Ascorbic Acid ($mg \cdot 100g^{-1}$)	β-karoten ($\mu g \cdot g^{-1}$ D.M.)
Rošajka	11.62	3.59	0.90	95.27	149.22
Lakošnička	13.65	5.94	0.84	98.59	416.70
Čokotanka	7.96	4.28	0.54	105.63	106.18
Žabarka	11.97	5.54	0.66	115.05	241.61
Crvena bobinka	13.03	4.03	1.03	140.84	471.23

The first two components explain 83.23% of the variability of chemical traits of the fruit. The third main component explains 15.06%, while the fourth explains only 1.72% of total variance of chemical traits.

Table 2. Traits (eigen) values and the percentage of total variance

PC	Eigen values	Percentage of variance	Cumulative (%)
1	2.81	56.20	56.20
2	1.35	27.03	83.23
3	0.75	15.06	98.29
4	0.09	1.72	100.00

The content of β-carotene and dry matter are two of the most important variables building the first principal component. Total sugar content is the most significant variable of the second principal component (Table 3).

Table 3. The percentage share of each feature in the first four principal components

Trait	PC1	PC2	PC3	PC4
Dry matter	28.83	5.89	10.68	35.11
Total sugar	1.23	67.99	6.13	0.43
Ash	25.21	10.26	20.26	0.63
Ascorbic Acid	12.36	14.26	59.70	12.28
β-Carotene	32.38	1.60	3.24	51.55

The results in Table 3 show that the content of the ascorbic acid is the variable which is to 59.70% comprised in the third principal component. The fourth component further separates the genotypes according to dry matter and β-carotene content. The total content of sugar, ash and ascorbic acid were not correlated to other chemical traits in this study.

Based on the results in Table 2, the graphical representation of the variability of chemical properties of five pepper genotypes was based on the ratio of the first and second principal component (Figure 1).

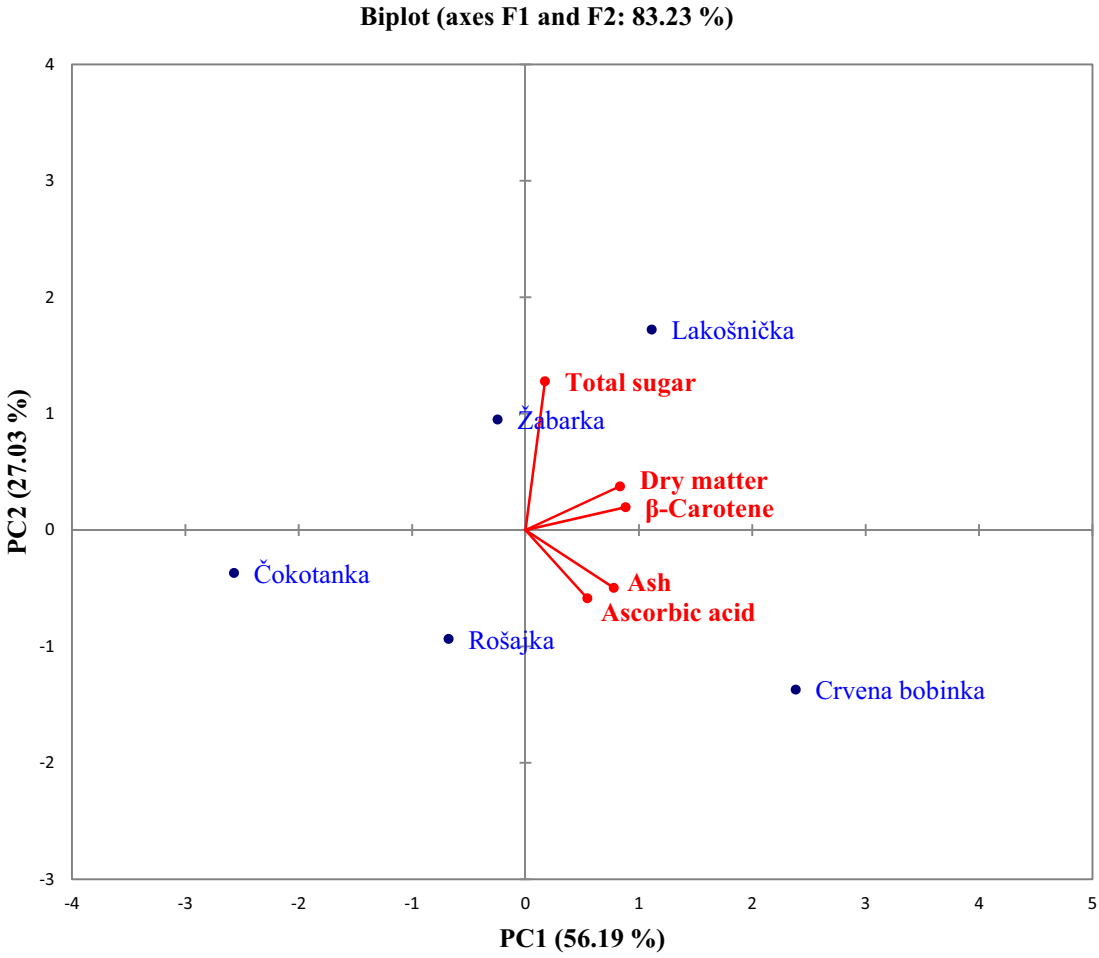

Figure 1. The relationship of the first and second principal components (PC1 and PC2) in the analysis of 5 pepper genotypes

The first principal component separates the genotypes according to dry matter content and β-carotene. The second principal component separates the genotypes according to total sugar content. Genotypes in the biplot positive side, compared to the other principal component, are characterized by high sugar content. Genotypes with high dry matter content and β-carotene are on the positive side of the biplot. Dry matter and β-carotene contents are in positive correlation. Genotypes with high dry matter content are high in β-carotene.

The genotype Lakošnička is in the positive quadrant of the biplot for PC1 and PC2, which means that it has a high content of dry matter, total sugars and β-carotene. The Žabarka genotype also has a high content of total sugars but the dry matter and β-carotene contents are lower. Bobinka crvena has the highest level of dry matter and β-carotene, but low level of total sugars.

The genotypes Čokotanka and Bobinka crvena give the largest contribution to the first principal component. These two genotypes are in negative correlation regarding two traits with the highest participation in building of the first principal component, dry matter and β-carotene contents. The largest contribution to the second principal component comes from the genotype Lakošnička with its high level of total sugar. The Rošajka genotype participated with 57.54% in building of the third principal component, while Žabarka was the most significant genotype in building of the fourth component (Table 4).

Table 4. The percentage share of genotypes in the principal components

Genotype	PC1	PC2	PC3	PC4
Rošajka	3.27	12.92	57.64	6.17
Lakošnička	8.82	43.93	7.17	20.08
Čokotanka	47.05	2.01	12.50	18.43
Žabarka	0.44	13.36	12.03	54.17
Crvena bobinka	40.42	27.78	10.66	1.14

Varietal characteristics of interest to the general production were low capsaicin, low moisture, thin pericarp (suitable for short period of drying), uniform and concentrated maturation (facilitates the mechanical harvest), high yield and resistance to diseases, resistance to drastic temperature changes, salinity of soil and water for irrigation (Hornero-Méndez et al., 2000).

Table 5. Traits of the vegetative parts of plants

Variety	Branching type	Growth	Plant height (cm)	Plant width (cm)
Rošajka ljuta	cupped	medium	45-55	40-45
Lakošnička	cupped	medium	40-50	35-40
Venčara žabara	cupped	medium	45-55	45-55
Čokotanka	cupped		40-45	45-50
Bobinka crvena	transient		40-55	35-40

3.2 Traits of Plants and Fruits (PCA Grouping)

Čokotanka is a pepper variety with large (100-150 g) erected fruits, with 10-12 fruits per plant. The pericarp is 4-6 mm thick, and fruits are intended for fresh consumption and proceeding (Table 6). Rošajka has sweet and hot populations. Their common trait is *nutans* type of fruit. Usually, after harvesting, they are dried and used as spices.

Table 6. Fruit characteristics and yield per plant

Variety	Fruit shape	Position the fruit on the plant	Number of fruits per plant	Pericarp thickness (mm)	Fruit weight (g)	Yield of the fruits per plant (g)
Rošajka	Hornshaped	(nutans)	12-18	2-3	20-40	450
Lakošnička	elongated	(nutans)	10-15	2	50-70	500-800
Žabarka	irregular	(nutans)	7 -12	2	60-80	550
Čokotanka	Conical, long	(erectum)	12-15	4-6	100-150	1625
Bobinka	Cherry-like	(seminutans)	20-40	2-3	2-9	180

When analysing the characteristics of plants and fruits, the first two principal components comprise 89.20% of the variability properties. The third principal component explains 9.38%, while the fourth explains 1.41% of total variance of plant and fruit traits (Table 7).

Table 7. Eigenvalues and the percentage of total variance

PC	Eigenvalues	Percentage of variance	Cumulative (%)
1	3.79	63.17	63.17
2	1.56	26.04	89.20
3	0.56	9.38	98.59
4	0.09	1.41	100.00

Characteristics: fruit yield per plant, fruit weight and pericarp thickness were the most important variables included in the first principal component. Number of fruits per plant, plant height and width were the most important parts of the second principal component. The third principal component further branches genotypes according to fruit width, in two groups. The fourth principal component was in the best way explained by pericarp thickness (Table 8).

Table 8. The percentage share of each feature in the first four principal components

Trait / osobina	PC1	PC2	PC3	PC4
Number of fruits per plant	8.02	32.33	31.36	17.17
Pericarp thickness	18.01	10.67	22.45	28.70
Fruit weight	25.41	0.98	1.99	12.46
Fruit yield per plant	25.78	1.16	0.03	5.56
Plant height	12.73	26.95	14.39	18.36
Plant width	10.06	27.92	29.77	17.76

As with the chemical properties, a figure of the properties of plants and fruits was based on a comparison of the first and second principal components (Figure 2).

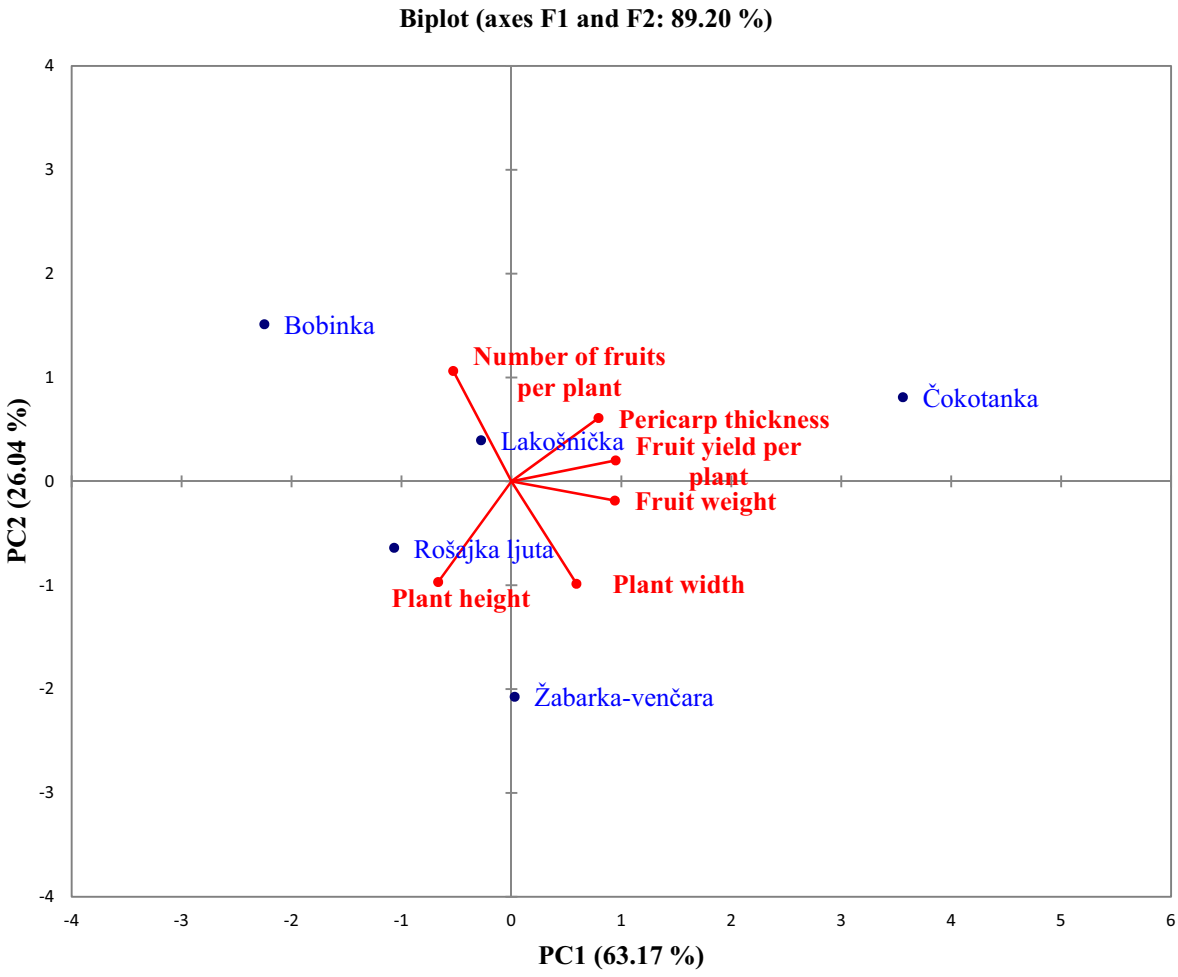

Figure 2. Ratio of the first and the second principal component (PC1 and PC2) in analysing plants and fruits of 5 pepper genotypes

The first principal component separates genotypes according to characteristics of fruit yield per plant and fruit weight. The second principal component separates genotypes according to plant height. The plant height varies between 42.5 and 50 cm. Genotypes on the positive side of the PC2 biplot were on average 50 cm high. Genotypes with higher fruit weight and higher yield per plant were located on the positive side of the biplot compared to the first principal component (Čokotanka).

The Čokotanka genotype is in the positive quadrant of the biplot for PC1 and PC2, which means that it has a high yield per plant, a higher fruit weight and an average plant height of 50 cm. The genotype Žabarka also has an average plant height of 50 cm, but its fruit yield per plant is 3 times lower than for the Čokotanka.

The Čokotanka genotype gives the largest contribution to the first principal component. This genotype has the highest values for fruit weight and fruit yield per plant of the genotypes studied. The Žabarka genotype, which has the largest and widest plant, gives the largest contribution to the second principal component. The Lakošnička genotype participated with 77.03% in the third principal component, while Rošajka was the most significant genotype for the fourth principal component (Table 9).

Table 9. The percentage share of the principal components of genotypes

Genotype	PC1	PC2	PC3	PC4
Rošajka	6.007	5.253	1.900	66.840
Lakošnička	0.403	1.992	77.025	0.580
Žabarka	0.004	55.116	2.229	22.651
Čokotanka	66.936	8.388	4.463	0.214
Crvena bobinka	26,650	29,250	14,384	9,716

Phenotype divergence of five varieties from the *Capsicum s*p. germplasm collection grown in Serbia (in regions with long tradition of pepper growing and nutrition) represents a rich source and potential for obtaining valuable recombination in pepper breeding programs (Bozokalfa et al., 2009), pepper being the economically most significant vegetable species in Serbia. This genetic potential, especially in the field of resistance to diseases and abiotic factors, as well as big range of shapes – from small and round, to large bell type shape (Zewdie et al., 1997), represents a very significant potential in selection programs regarding the morphological and chemical traits and stability in pepper production. The variation rank for most major morphological characteristics, including thickness of the pericarp and content of vitamin C is in accordance with Martinez et al. (2005). These authors also studied the capsaicin content and determined the variability, which depends on the divergence of genotype, environment influence, growing conditions, production practices and stages of maturation, which affect fruit quality traits.

The evaluation of the phenotype divergence was justified by applying PCA analysis, since the grouping can be performed according to desirable traits. For both groups of traits, morphological and biochemical, genotypes were classified in two groups of genotypes according to principal components. Bozokalfa et al. (2009) have shown that PCA analysis is suitable for grouping genotypes in *Capsicum annuum* L according to their different traits. They classified the entire collection of Turkish pepper consisting of 48 genotypes, into 7 groups. Zou et al. (2007) characterized 26 pepper genotypes using nine flower traits, classifying the Chinese genotypes in 6 groups. Portis et al. (2006) classified the 19 ecotypes originating from the Italy gene fond, which were divided into 9 clusters by using multivariate quantitative techniques. According to Lahbib (2012), the Tunisia local varieties (11) divided by 7 traits for yield and its components, were analysed with PCA and the 3 main components of variability that make up 87% of the total variance were established. Combining different multi-variation analyses to determine the divergence of pepper genotypes originating from Brazil, it was found that the thickness of the pericarp was a trait that accounted for the largest part of variance (Do Rego et al., 2003). In our study, the variance of the pericarp thickness was only 29.698% of the total variance. These results can be explained by a thin pericarp and by small differences among the researched genotypes. The studied old varieties belong to a group of pepper intended for drying (to be used as spice (powder), or for stuffing) since they have a thin pericarp.

The percentage of variation of biochemical properties of β-carotene for the fourth component (PC4) was 51.553% in this study. These properties in pepper fruits have previously been shown to be a major source of variation (Wall et al., 2001). Carotene accumulation in pepper causes a different colour in the biological maturity of the fruit. It is

considered that the lack of the gene for capsanthin, Capsorubin synthase is a precondition for the formation of yellow pepper fruits (Hurtado-Hernandez & Smith, 1985; Ha et al., 2007). The influence on colour of immature fruits and leaves is a combination of β-carotene and anthocyanins (Lightbourn et al., 2008). In Serbia, there are a small number of varieties that are labelled as genotypes "suitable for drying" and are therefore preserved varieties for drying and processing. They are a favourite choice for this purpose among local growers. The character and method of processing in traditional cooking defined in the fruit characteristics of different genetic constitution in some regions of Serbia.

4. Conclusion

A large number of tested pepper varieties differ in morphological and sensor characteristics (including colour), which determines the possibility and the mode of their usage. Not all pepper varieties can be recommended for commercial production, but must first be verified in production, through a series of agronomic and industrial demands. The greatest value of the species tested, is their high content of carotenoids, since the commercial value depends on the capacity of coloration that is directly related to the relative wealth of colours.

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Viability Selection of Bovine Oocyte Using Annexin-V Assay

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Abstract

Morphological characteristic is the primary reference for selecting viable cumulus-oocyte-complex (COCs). Annexin-V assay has been suggested for isolating these viable cells based on apoptosis status. This study compared fertilization rate between Annexin-V selected COCs with morphologically healthy selected COCs. Minimal binding time between Annexin-V and the COCs was determined to ensure slight disruption. Before observation with florescent microscope, eighty-two cattle's COCs were incubated with the Annexin-V assay for 5 min, 15 min and 24 hours. Further, 157 COCs were separated into 2 groups; positively (n=76) and negatively (n=81) tagged with annexin-V-FITC and PI fluorescent signals. Once separated, control comprised of non-stained COC (n=68) and the two afore mentioned groups were matured, fertilized and cultured (IVM-IVF-IVC). After 72 hpi, cleavage rate was determined. Results shows the binding between annexin-V assay and the nonviable COC had occurred as early as 5 min with positively and negatively tagged COCs were 44 (53.7%) and 38 (46.3%) respectively. The fertilization rate of control group (66%), negatively tagged (79%) and positively (50%) tagged COCs was significantly different (χ^2 (2) = 14.60, $p < 0.05$) between the groups. In conclusion, oocyte selection using annexin-V-FITC had significant differences in cleavage rate compared with oocytes assumptive as morphologically healthy. Based on COCs Annexin-V assay, COC that are assumed as morphologically healthy is a mixture of viable and nonviable oocytes.

Keywords: annexin V-FITC, propidium iodide, apoptotic cumulus-oocyte complex, cleavage rate, *In vitro* fertilization (IVF)

1. Introduction

Viable oocyte selection prior to maturation process is an important step in achieving high successful *in vitro* fertilization rate (Camargo et al., 2006). To date, this selection of viable oocytes is solely based on morphological assessment (Plourde et al., 2012). The selection procedure which is consisted of assessment on follicle size, morphology of cumulus cells and homogeneity of the cytoplasm has been reported to cause biased (Blondin, Bousquet, Twagiramungu, Barnes, & Sirard, 2002) and false interpretation. COCs that morphologically identified as early signs of atresia in contrast had shown good nuclear maturation and development potential than those considered to be morphologically viable (Bilodeau-Goeseels, 2001; De Wit, Wurth, & Kruip, 2000). In addition, this assessment also takes longer time to assess especially when it involves high number of sample to be assessed in one particular day. Any further delay in placing the COC into the nutrient media for the maturation process will result in failure of fertilization.

A scientific approach is required such as annexin V assay which postulated to be an objective evidence in assessing oocyte quality that could offer less biasness and also shorten assessment time. The ability of annexin V fluoroscein isothiocyanate (FITC) to bind to expose phosphotidyl serine (PS) as an early indicator of apoptosis process is well studied (van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998). Even though there were several studies conducted in assessing the apoptotic oocyte using annexin v FITC (Anguita, Vandaele, Mateusen, Maes, & Van Soom, 2007; Li et al., 2009) but none of these studies had tested on its efficacy as an assessment method to

isolate good quality oocyte by fertilizing them. Therefore, the aim of the present study was to demonstrate the ability of annexin V assay as an alternative assessment of oocyte quality.

2. Materials & Methods

2.1 Materials

Tissue culture media-199 with Earl's Salts (TCM-199), bovine serum albumin (BSA) EFAF, phosphate buffer saline (PBS), penicillin-streptomycin solution, Sodium pyruvate, Gentamycin sulphate, B-estradiol, fetal bovine serum (FBS), mineral oil, epidermal growth factor (EGF), follicle stimulating hormone (FSH), luteinizing hormone (LH), Annexin-V FITC, sodium chloride, potassium chloride, Magnesium chloride hexahydrate, sodium bicarbonate, calcium chloride dehydrate, Hypotaurine, D-Penicillamine, epinephrine, lactic acid (sodium salt), sodium metabisulphite, potassium dihydrogen fosfat, L-Glutamine, BME amino acid, MEM amino acid, Myo-Inositol and phenol red.

2.2 Oocyte Collection

All procedures have followed Institutional guideline. A total of 46 cattle's ovaries were collected at a local abattoir and placed in warm phosphate buffer saline (PBS) supplemented with 1% of antibiotic (10,000 IU penicillin and 10 mg streptomycin). The ovaries were transported back to the laboratory within 3 hours after the animal was slaughtered. Oocytes recovery was done by slicing the ovaries on a petri dish containing 10mg/ml of bovine serum albumin (BSA) dissolved in Dulbecco's phosphate buffered saline and 0.01% of penicillin and streptomycin mixtures. Later, morphologically healthy oocytes were selected using a dissecting microscope. Selection of the oocytes was based on these characteristics: 1) COCs with compact multilayer cumulus cells that are tightly adherent to the zona pellucida; 2) presence of homogenous appearing ooplasm with general appearance of transparent COCs. At completion. Once completed, a total of 239 healthy oocytes were obtained.

2.3 Sperm Collection

Bovine semen sample were collected through artificial vagina technique and cryopreserved. Briefly, the extended samples were chilled at 4°C for 3 hrs prior to loading into 0.25 ml French straw. Straw were then exposed to liquid nitrogen vapour for 9 min before plunging into liquid nitrogen for storage until required.

2.4 Optimization of Staining Incubation Time

A total of 83 COCs classified as morphologically healthy were washed twice in washing solution and twice in maturation (IVM) media. Lastly, these COC were then transferred into droplets containing IVM media, 1X binding buffer, 5 µL Annexin V-FITC and 10 µL PI. IVM droplets were prepared by mixing TCM 199 and 'heat stressed' serum at a ratio 9:1 and 1% of antibiotic. A total of 82 COC were then incubated at 38°C for five minutes in 5% CO_2 humidified air. They were then observed under a fluorescent microscope at 5 min, 15 min and 24 hours intervals. Florescent results were visualized under a florescent microscope. Optimum incubation time was determined when florescent signal for Annexin V-FITC and PI was detectable using the naked eye of the microscope.

2.5 In vitro Maturation (IVM)

A total of 157 morphologically healthy COC was incubated for five minutes (optimized incubation time) with Annexin V FITC and PI. The COC were then grouped based on their fluorescent signals. Oocytes without any definitive fluorescent signal were sorted into the negative group-in which it is assumed as viable COC. While those with very prominent green and red fluorescent signals were grouped into the positive group. This group was classified as non-viable COC. Control group were COC with healthy morphology characteristic but non-stain. Oocytes in each group were later matured by transferring them into new IVM droplets prior to its incubation at 38°C in 5% humidified CO_2 for 24 hrs. In all, control, positive and negative group was represented by 68, 76 and 81 oocytes respectively.

2.6 In vitro Fertilization (IVF)

Frozen-thawed bull semen was mixed and centrifuged twice with sperm washing solution modified from Bracket and Olliphant (BO) media. The media was supplemented with heparin and antibiotic. Sperm suspension was then diluted in BO media supplemented with 2 mg BSA/ml and 30 µL calcium ionophore. Matured oocytes from both groups (positive and negative) were washed and partially denuded in oocyte washing solution prior to culture with sperm suspension at a final concentration of 1 x 10^6 spz/ml. The mixed oocytes and sperm were then finally incubated at 38°C in 5% CO_2 for 18 hrs.

2.7 In vitro Culture (IVC)

At 18 hrs post-insemination (hpi), presumptive zygotes were mechanically denuded from the remaining cumulus cells and excessive sperm cells. Groups of 10 embryos were then placed in each 100 µL droplets of CR1aa media supplemented with amino acid and FCS. The embryo cultures were then incubated at 38°C in 5% CO_2 for 3 days.

3. Results

Identification of negative and positive fluorescent signals is as shown in Figure 1. Figure 1(a) is an image of morphologically healthy oocytes under bright filed microscope. When the fluorescent light was on, Annexin V FITC had produce green florescent signal while PI a red florescent signal. General positive florescent signals are COC which express green and red signals (Figure 1(b)-A). Negative COC are cells that do not radiate any fluorescent signal (Figure 1(b)-B).

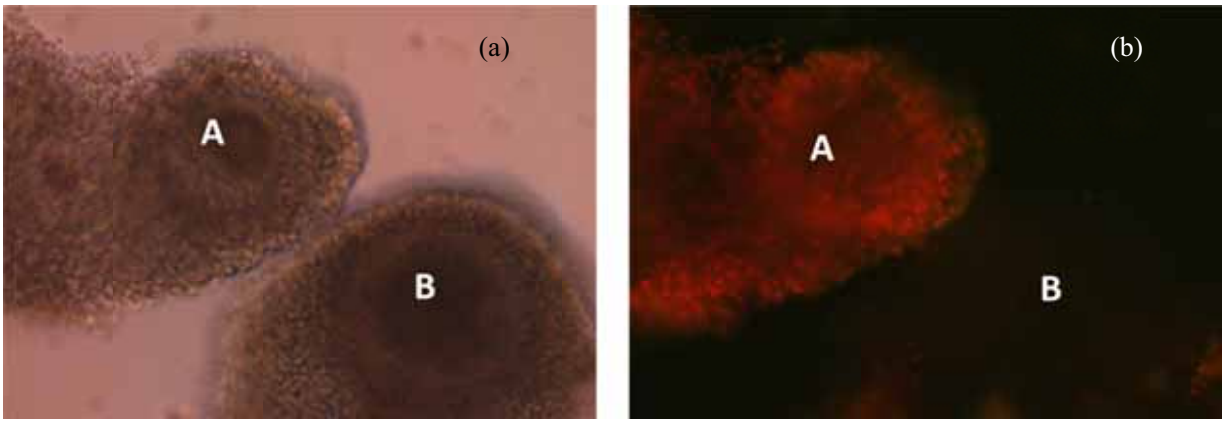

Figure 1. Cumulus oocytes complexes stained with Annexin V-FITC and PI. (a) is magification at X40 under bright field phase while (b) is fluorescent phase

A & B: Relative position of each cell in relation to the images.

Table 1. Number and percentage of COC with positive and negative fluorescent signal according to their binding time

Binding time (min)	No. Of COC with fluorescent signal (%)	
	Positive	Negative
5	44 (54)	38 (46)
15	48 (56)	36 (44)
1440	-*	-*

-* indicate no Annexin V- FITC and PI fluorescent signal.

The number of oocytes that had shown positive and negative fluorescent signals according to incubation time is presented in Table 1. Even though there was an increased number of COC that were positively tagged with Annexin V and PI over the time, but statistically the increase was insignificant. At 24 hrs no fluorescent signal was seen and was reported as such.

Table 2. Rate of cleavage of inseminated oocytes that negative and positive tagged with Annexin V and those morphologically healthy

Group	No. of oocyte	Cleavage rate (%)
Control	68	45 (66)
Positive	76	38 (50)*
Negative	81	64 (79)*

* indicates significant differences with (χ^2 (2) = 14.60, $p < 0.05$).

Cleavage rate is a termed used to measure the percentage of inseminated oocytes which have cleaved to 2, 4 and up to 16 cells before progress to morula stage. Number of fertilized oocytes in control and experimental (positive and negative) groups that show cleavage on day-3 after fertilization is shown in Table 2. Statistical analysis had indicated that there was a significant difference (χ^2 (2) = 14.60, $p < 0.05$) of cleavage rate between the three groups. A greater percentage of cleavage was observed among oocytes under negative (79%) group followed by control (66%) and positive (50%) groups.

4. Discussions

One of the major cell physiological activities is to maintain an asymmetric distribution of its components between the inner and outer leaflets of the plasma membrane (Copenhagen, 2009). During apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer leaflets. This exposure, function as a marker for macrophages to phagocyte the non-viable cells (Greenberg et al., 2006). In a fertility lab, identification of this exposed PS is facilitated by Annexin V labelled with fluoroisothiocyanate (FITC) which has been known to specifically bind to PS in the presence of calcium ion (Lang et al., 2003). When using Annexin V-FITC, PI is also used together as it is used to detect non-viable cells.

In early stages of apoptosis, membrane integrity of the oocyte remains unchanged. During this stage PS are already expressed on the oocyte cell wall. This is detected using Annexin V-FITC which is represented as a fluorescent green signal under the fluorescence microscope. As the oocyte reaches late stage apoptosis or necrosis, loss of membrane integrity would have occurred. This would enable PI to enter the cells. Presence of PI within the cells is represented as a red fluorescent signal under a fluorescence microscope (Figure 1(b)-A).

To date, various incubation duration and temperature are used to allow Annexin V to bind onto the non-viable oocytes. As an example, study by Li et al. (2009) had incubated denuded goat's oocytes with Annexin V staining for 30 min at 4°C while Anguita et al. (2009; 2007) had incubated bovine's oocytes for 15 min at 37-38°C. Both afore mention methods had extremely long incubation periods as further development of oocyte were not their main priority. In this study, optimization of Annexin V staining binding time was done to minimize the exposure of COCs towards ambient temperature and also to avoid any delay in placing the cells to receive necessary nutrient for their development process. COC exposed to low temperature during recovery process have shown significant decrease in quantity and also quality of embryo produced (Matsushita, Tani, Kato, & Tsunoda, 2004). Therefore the minimal binding time between the Annexin V and the translocated phosphatidylserine (PS) on the surface of non-viable cells is one of the crucial elements prior to its application as an assessor.

In this present study, development of oocytes was our main priority and so binding time were shorten as short as possible. Result indicated that suitable florescent signal from Annexin V-FITC can be obtained after a minimum incubation time of five minutes. In this period, Annexin V assay was able to bind with 44 non-viable oocytes. As these oocytes were initially classified as morphologically healthy, presence of the Annexin V-FITC had indicated that some morphologically healthy oocytes are biologically unhealthy. When binding time was increased to 15 min, this assay had identified 48 non-viable cells. Increasing binding time to 24 hrs had resulted in an absence of fluorescent signal. This may due to photobleaching, although further investigation is required to elucidate this mystery (Lavagnino, Zanacchi, & Diaspro, 2011).

The efficacy of Annexin V assay in isolating the good quality (viable) oocytes from the non-viable oocytes are measured in the number of inseminated oocytes that had successfully cleave into 2 to 4 cells on day-3 of post-insemination. Results found that oocytes from the negative signal (viable oocytes) had a greater cleavage rate (79%) followed by control group (morphologically healthy oocytes-66%) and lastly positive group (non-viable oocyte - 50%).

We would like to note that this study had its limitation. Staining used was an indirect assessment to the oocytes quality because the stain was only able to stain the surround cell (cumulus cells). The stain was unable to penetrate the thick cumulus layer and stain the oocyte. Identification cleaves cell stage to determine whether the zygotes were in a 2-cells stage of 4-cells stage was also not presented.

5. Conclusion

As a conclusion, oocytes that are morphologically healthy are actually composed of a mix of viable and non-viable oocytes. Therefore, this assay should be considered to be an alternative assessment in oocyte quality to improve in vitro fertilization simultaneously in enhancing the in vitro embryo production. But prior to this application, any toxic effect of the stains mentioned above towards the genetic development should also be investigated seriously.

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Effect of Dietary Supplementation With Fibrolytic Enzymes on the Productive Performance of Early Lactating Dairy Cows

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Abstract

This study was conducted to investigate the impacts of exogenous fibrolytic enzymes (Fibrozyme, *Alltech inc* company, USA) supplementation for 12 weeks on milk production and composition as well as blood metabolites in early lactating dairy cows. Total of 120 multiparous Holstein dairy cows at early lactation (57±4.2 days in milk) were randomly assigned into two groups according to lactation period "stage of lactation" and lactation season. The first group (control, n=60) were fed total mixed ration (TMR) without a supplement of exogenous fibrolytic enzymes. The second group (treatment, n=60) were fed TMR supplemented with a commercial exogenous fibrolytic enzymes at the rate of 15 g/cow/d for 12 weeks. Each group was placed in a shaded pen equipped with free stalls. An exogenous fibrolytic enzymes was added to the TMR at the time of feeding once per day at 10 am. Cows were fed as a group open feed, with free access to water.

The results of this experiment on dairy cows showed that there were not significant changes in dry matter intake of lactating dairy cows with or without a supplement of exogenous fibrolytic enzymes at early lactation. Exogenous fibrolytic enzymes supplementd to lactating dairy cows improved ($P<0.003$) milk yield (41.0 vs. 39.5 kg/cow/d) compared to untreated dairy cows. Also, the fat corrected milk was increased ($P<0.025$) as a response to exogenous fibrolytic enzymes supplementation to lactating dairy cows compared to un-supplemented dairy cows. In addition, the supplementation of exogenous fibrolytic enzymes enhanced ($P<0.006$) the energy corrected milk (40.6 vs. 39.4 kg) and feed efficiency in early lactating dairy cows compared to the control group. The results revealed that supplementation of exogenous fibrolytic enzymes had no significant effect on milk fat, protein lactose and solid not fat (SNF) percentage compared to the control group of dairy cows. While, the quantities of milk protein (1.36 vs. 1.30kg), lactose (2.0 vs. 1.92kg) and SNF (3.47 vs. 3.31kg) in supplemented-dairy cows were improved significantly compared to the control group except quantity of milk fat ($P<0.096$). Serum glucose, albumin, urea and triglycerides were not affected ($P>0.05$) but total protein, globulin and cholesterol were declined ($P<0.05$) due to fibrozyme inclusion compared to control group of dairy cows. The supplementation of exogenous fibrolytic enzymes to early lactating dairy cows achieved higher net profit by 0.93 US$ per cow than control group. It is concluded that exogenous fibrolytic enzymes supplementation to early lactating dairy cows improved significantly milk production, SNF and energy corrected milk.

Keywords: fibrolytic enzymes, productive traits, blood metabolites, dairy cow

1. Introduction

The ruminant production systems are dependent worldwide on forage as the main nutritional components (Wilkins, 2000). The digestion of forage occurs through the microbial fermentation as a result of the presence of microorganisms at the reticulo-rumen and its adaptation to digest lignocellulosic components. The microbial mode of digestion allows ruminants to better unlock the unavailable energy in the plant cell wall components than other herbivores (Krause et al., 2003). This gives ruminant animals the ability to convert low nutritive and resistant lignocellulosic biomass to milk, meat, wool and hides (Weimer et al., 2009). However, most forage plants are high in cell walls and low in nitrogen (N) and energy content (Romney & Gill, 2000). Despite the importance of fibrous components in forages for salivation, rumen buffering and efficient production of ruminal end products (Mertens, 1997) only 10 to 35% of energy intake is available as net energy (Varga & Kolver, 1997). This is because the ruminal digestion of plant cell walls is not complete (Krause et al., 2003).

The use of exogenous fibrolytic enzymes (EFE) to enhance quality and digestibility of fibrous forage is on the

verge of delivering practical benefits to ruminant production systems. In this regard, 2 cellulases and xylanases are respectively amongst the two major enzyme groups that are specified to break ß1-4 linkages joining sugar molecules of cellulose and xylans found in plant cell wall components (Beauchemin et al., 2003). Several studies with EFE have made mention of the increase of microbial activities in the rumen, which resulted in an enhancement of animal performance traits. Despite the increase in feed digestibility and subsequent production traits, the relationship between the improvement in forage utilization and enzymatic activities is yet to be explained in ruminant systems (Eun et al., 2007). In addition, results with EFE addition in ruminant systems are variable and somewhat inconsistent (Beauchemin et al., 2003; Colombatto et al., 2003), making their biological response difficult to predict.

Some studies have shown substantial improvement of feed digestibility and animal performance traits (Cruywagen & Goosen, 2004; Bala et al., 2009; Arriola et al., 2011), while others reported either negative effects or none at all (Baloyi, 2008). If the potential intake and/or the density of available nutrients of forages can be increased with EFE as feed additives, then poor quality forages can be economically and successfully converted into meat and milk for human consumption. Moreover, an increase in the input costs in the dairy industry has demonstrated the need for methods to increase production efficiency. One way of increasing efficiency would be to increase the bioavailability of nutrients in a feedstuff, which might be accomplished through the exogenous fibrolytic enzymes supplement. Therefore, the objective of the undertaken experiment was to evaluate the effect of exogenous fibrolytic enzymes "Fibrozyme" supplementation on dry matter intake, feed efficiency, blood metabolites, milk production and milk composition in early lactating dairy cows.

2. Materials and Methods

2.1 Animals' Management and Experimental Design

This experiment was conducted at Alexandria commercial company of agriculture at Alexandria governorate during March to May, 2011 for 12 weeks. Total of 120 multiparous Holstein dairy cows at early lactation (57± 4.2 days in milk) were randomly assigned into two groups. The first group (control, n=60) were fed total mixed ration (TMR) without a supplement of exogenous fibrolytic enzymes. The second group (treatment, n=60) were fed TMR supplemented with a commercial fibrolytic enzymes (Fibrozyme is enzymes blend, which is prepared from fermentation extract and fermentation soluble of *Aspergillus Niger and Trichoderma longibarachiatum,* and having xylanase activity by minimum 100 XU/g, *Alltech inc* company, USA) at the rate of 15 g/cow/d according to the guide of the manufacture for 12 weeks. Fibrozyme was added and mixed to the TMR at the time of feeding once per day. Each group was placed in a shaded pen equipped with free stalls. Cows in the two groups fed a total mixed ration (TMR, Table 1), which composed of alfalfa hay, corn silage, green clover, soybean meal, yellow corn, limestone, vitamins, minerals mixture, protected fat (magnapac), sodium bicarbonate and mono calcium phosphate. The proximate analysis and calculated nutritive value of the TMR is given in Table 2.

Table 1. Ingredients of the experimental total mixed ration (TMR) in the lactation trial

Items	% of DM
Alfalfa hay	10.7
Green clover	7.3
Corn silage	26.0
Ground yellow corn	25.8
Soy bean meal	24.6
Limestone	1.2
NaCl	1.0
Minerals mixture and vitamins	0.3
Magnabac (protected fat)	2.7
Sodium bicarbonate	0.1
Mono calcium phosphate	0.30

Roughage concentrate ratio was 44:56%.

Table 2. Proximate analysis (%) and calculated nutritive value of the experimental total mixed ration (TMR) in lactation trial

Nutrients	As fed basis (%)	As dry matter basis (%)
Oragnic matter	51.17	94.0
Crude Protein	10.39	19.1
Neutral detergent fiber	18.51	34.0
Acid detergent fiber	8.71	16.0
Hemicellulose	9.79	18.0
Cellulose	7.35	13.5
Lignin	2.45	4.5
Ash	3.27	6.0
Non Fiber carbohydrates	19.9	36.6
Ether Extract	2.4	4.4
Nutritive value		
TDN, %	38.71	71.1
ME (M cal/kg DM)	1.71	3.13
NE_L (M cal/kg DM)	0.88	1.62

TDN: Total digestible nutrients; ME: Metabolizable energy; NE_L: Net energy for lactation.

Cows were fed as a group open feed, with free access to water. Amount of TMR delivered was measured with electronic scales on mixer–feeder wagon. The TMR was mixed and fed using Delaval mixer wagon. The diet was formulated using Gavish computer operated cattle feeding system 2008 to cover or exceed NRC recommendations (NRC, 2001) as in Table 2. Cows were milked three times daily at 4 am, 12 pm. and 8 pm in a Dobell 20-parallel milking parlor equipped with automatic cow identification, milk recording system, and automated detacher milker units.

2.2 Sampling Analysis

During the entire experiment, representive samples of TMR were collected weekly and stored at -20C^{o} until chemical analysis for dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fiber (CF), neutral detergent fibers (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to Goering and Van Soest., (1970); Van Soest et al., (1991); AOAC, (2006). Cellulose and hemicellulose were calculated by differences among NDF, ADF and ADL. Total digestible nutrients (TDN), metabolizable and net energy for lactation were calculated according to NRC (2001).

Milk samples were collected biweekly and analyzed immediately for fat, protein, lactose and SNF content using infrared method by Milk Analyzer (Milko tester Instruments Inc, Bulgaria). Average fat and CP yields were calculated by multiplying milk yield by fat and CP content of milk on an individual cow basis. Milk energy (MJ/kg) was calculated on an individual cow basis using the milk fat, CP and lactose content of the milk (Tyrrell & Reid, 1965).

Blood samples were collected from Jugular vein (10 cows per group were randomly selected) prior to morning feeding monthly. Serum were obtained by centrifugation the blood tubes for 20 min, 3000xg and stored at -20^{o}C until blood metabolites analysis. Concentrations of serum total protein, albumen, urea, glucose, triglycerides and cholesterol were determined using commercial kits manufactured by Stanbio Diagnostic Company, Germany. The concentrations of serum total protein, albumin, urea, glucose, triglycerides and cholesterol were measured calorimetrically according to Henry (1974); Doumas et al. (1971); Patton and Crouch (1977); and Tietz et al. (1995), respectively. The concentration of globulins in each serum sample was obtained by subtracting the value of albumin from the total blood serum protein concentration. The ratio of A: G was calculated.

2.3 Economic Efficiency

Economic efficiency expressed as the daily feed and supplement cost and price of milk. The price of one ton of TMR was 400 US$, while the price of fibrozyme was 12.73 US$/kg and milk price (fat=3.5% and SNF=8.5%) was 0.524US$ according to Egyptian prices of year 2011.

2.4 Statistical Analysis

Statistical analysis was completed using a completely randomized design with a 2 factorial arrangement to analyze the data. The MIXED procedure of SAS (Version 9.2 SAS. 2002) and a model containing treatment,

week (repeated measure), and all interactions of these terms and as the random effect was used to analyze the data from measurements that were repeated weekly. Contrast statements were used to determine the effects of enzymes application (control vs. enzymes) and the interaction (enzymes treatment vs. time).

3. Results

The mean values of the proximate analysis of TMR are shown in Table 2. The results showed that the organic matter (OM), crude protein content (CP) and ether extract (EE) were 94.0, 19.1 and 4.4%, respectively. The fiber fractions content of TMR were 34, 16, 18, 13.5, 4.5 and 36.6% for NDF, ADF, hemicellulose, cellulose, lignin and non-fiber carbohydrates, respectively. The calculated total digestible nutrients (TDN), metabolizable energy (ME) and net energy for lactation (NE_L) in TMR were 71.1%, 3.13 Mcal/kg DM and 1.62 Mcal/kg DM, respectively.

The effects of fibrolytic enzymes supplementation as a nutritional manipulation to lactating dairy cows at early lactation on dry matter intake (DMI), milk yield, fat corrected milk (FCM), energy corrected milk (ECM) and feed efficiency are shown in Table 3. The response to fibrolytic enzymes supplement to dairy cows on milk yield profile within the treatment period (12 weeks) is presented in Figure 1. The results indicated that there were not significant changes in DMI of lactating dairy cows with or without a supplement of exogenous fibrolytic enzymes at early lactation. Exogenous fibrolytic enzymes supplement to the diet of lactating dairy cows improved ($P<0.003$) milk yield (41.0 vs. 39.5 kg/cow/d) and feed efficiency ($P<0.001$) compared to untreated dairy cows. Also, the fat corrected milk was increased ($P<0.025$) as a response to exogenous fibrolytic enzymes supplement to lactating dairy cows compared to un-supplemented dairy cows.

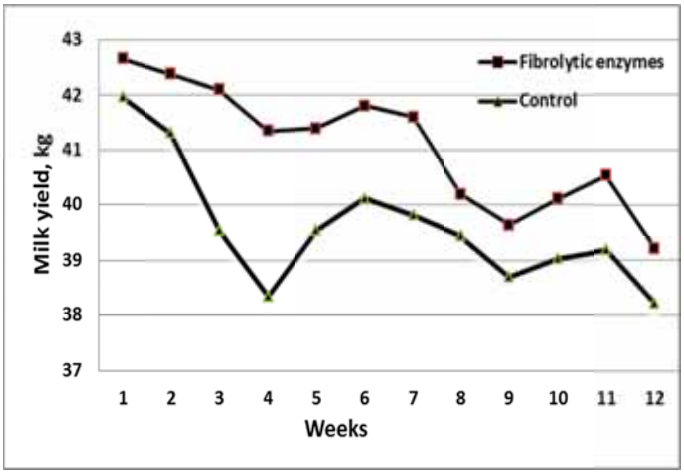

Figure 1. Milk production profile of early lactating dairy cows supplemented with exogenous fibrolytic enzymes

Table 3. Dry matter intake, milk yield, fat corrected milk, energy corrected milk and feed efficiency of lactating dairy cows at early lactation fed total mixed ration with or without exogenous fibrolytic enzymes (EFE) (± SE)

	Exogenous fibrolytic enzymes		*P values*		
Items	(-)	(+)	W	EFE	W*EFE
DMI, kg/d	24.78±0.17	24.73±0.17	0.845	0.845	0.954
Milk yield	39.5±0.31 [b]	41.0±0.31 [a]	0.018	0.003	0.947
FCM, kg/d	35.67±0.285 [b]	36.58±0.285 [a]	0.018	0.025	0.950
ECM, kg	39.4±0.32 [b]	40.6±0.32 [a]	0.018	0.006	0.945
Milk: Feed ratio	1.58±0.01 [b]	1.64±0.01 [a]	0.001	0.001	0.930

Different letters (a, b) in the same row indicate significant differences.

SE: standard error; SNF: solid not fat; W: weeks.

Fat Corrected milk (FCM) = milk yield*0.4+ fat yield*15

Energy corrected milk (ECM) = 0.327*milk yield (kg)+12.95 *fat (kg)+7.20*protein (kg) (Tyrrell & Reid, 1965).

In addition, the supplementation of exogenous fibrolytic enzymes enhanced (P<0.006) the ECM (39.4 vs. 40.6 kg) in dairy cows compared to un-treated group. The significance contrasts in Table 3 showed that there were no interaction between treatment and time, while time had significant effects on milk yield, FCM, ECM and feed efficiency in treated group compared to the control group.

Effects of exogenous fibrolytic enzymes supplement to the diet of lactating dairy cow at early lactation on milk composition are given in Table 4. The results denoted that supplementation of exogenous fibrolytic enzymes had no significant effect on milk fat, protein lactose and solid not fat (SNF) percentage compared to the control group of dairy cows. While, the quantities of milk protein (1.36 vs. 1.30 kg), lactose (2.0 vs. 1.92 kg) and SNF (3.47 vs. 3.31 kg) in supplemented-dairy cows were improved significantly compared to the control group of dairy cows except quantity of milk fat (P<0.096).

Table 4. Milk composition of lactating dairy cows at early lactation fed total mixed ration with or without exogenous fibrolytic enzymes (EFE) (± SE)

	Exogenous fibrolytic enzymes		*P* values		
Items	(-)	(+)	W	EFE	W*EFE
Fat, %	3.43±0.16	3.38±0.16	0.520	0.384	0.858
Fat, kg	1.34±0.01	1.36±0.01	0.017	0.096	0.954
Protein, %	3.32±0.02	3.37±0.02	0.001	0.277	0.809
Protein, kg	1.30±0.01 [b]	1.36±0.01 [a]	0.018	0.001	0.939
Lactose, %	4.92±0.20	4.95±0.190	0.897	0.897	0.978
Lactose, kg	1.92±0.015 [b]	2.00±0.015 [a]	0.018	0.004	0.984
SNF,%	8.49±0.09	8.59±0.09	0.132	0.864	0.648
SNF, kg	3.31±0.027 [b]	3.47±0.027 [a]	0.018	0.001	0.945

Different letters (a, b) in the same row indicate significant differences.

SE: standard error; SNF: solid not fat; W: weeks.

The significance contrasts in Table 4 showed that there were no interaction between treatment and time, while time had significant effects on yield of milk fat, protein, lactose and SNF in treated group compared to the control group. Effects of exogenous fibrolytic enzymes inclusion to lactating dairy cows at early lactation on blood metabolites are presented in Table 5.

Table 5. Least square means (± SE) of blood metabolites of lactating dairy cows at early lactation fed total mixed ration with or without exogenous fibrolytic enzymes (EFE)

	Exogenous fibrolytic enzymes		*P values*		
Items	(-)	(+)	M	EFE	M*EFE
Glucose (mg/ dL)	52.0±2.68	51.3±2.71	0.532	0.802	0.197
Total protein (g/dL)	12.8±0.49 [a]	10.4±0.47 [b]	0.016	0.001	0.263
Albumin (g/dL)	4.14±0.25	4.04±0.25	0.089	0.758	0.364
Globulin (g/dL)	8.9±0.59 [a]	6.3±0.59 [b]	0.212	0.002	0.143
A/G ratio	0.50±0.29	1.2±0.28	0.543	0.085	0.416
Urea (mg/dL)	34.4±1.01	35.0±1.01	0.007	0.678	0.707
Triglycerides (mg/dL)	28.8±2.98	23.4±2.98	0.888	0.195	0.710
Cholesterol (mg/dL)	242.0±15.2[a]	193.7±15.2[b]	0.956	0.002	0.224

Different letters (a, b) in the same row indicate significant differences.

SE: standard error; M: months.

Table 6. Economic evaluation of the exogenous fibrolytic enzymes supplementation at early lactation of dairy cows

Items	Control	Treatment
DMI, kg/d	24.78	24.73
Feed Cost, US$	9.912	9.892
Supplement cost, US$	0	0.191
Milk yield, kg/d	39.5	41.0
Milk Price, US$/kg	0.524	0.527*
Return, US$/cow/d	20.70	21.61
Profit, US$/cow	10.79	11.72
Net profit, US$/cow	0	0.93
Economic efficiency	2.09	2.19

Price of TMR = 0.4 US$/kg.

Supplement cost (Exogenous fibrolytic enzymes, Fibrozyme) = 12.73 US$ /kg.

* The difference in milk price between two groups was due to higher 0.1% in SNF.

The results revealed that exogenous fibrolytic enzymes supplementation caused significant decline in serum total protein (12.8 vs. 10.4 g/dL), globulin (8.9 vs. 6.3 g/dL), A/G ratio content (0.81 vs. 0.54) and cholesterol (242.0 vs. 193.7 mg/dL) compared to control group of dairy cows. While, there were no significant changes regarding glucose, albumin, urea and triglycerides when exogenous fibrolytic enzymes was supplemented to the diet of lactating dairy cows at early lactation compared to control group. The results of statistical analysis showed no interaction was detected between treatments and months in the blood metabolites, while only values of total protein and blood urea N were highly affected by months.

Economic evaluation of the exogenous fibrozyme enzymes supplementation at early lactation of dairy cows is given in Table 5. The results showed the inclusion of 15 g of exogenous fibrolytic enzymes will cost 0.191 US$ /cow/day, which will increase both milk production and SNF by 1.5 kg and 0.1% per cow/day. The daily profit of individual cow was 10.79 and 11.72 US$in control and treated group of lactating dairy cow at early lactation, respectively excluding the labor, veterinary medicines and other management's costs. Moreover, the net profit for exogenous fibrolytic enzymes inclusion in diet of early lactating dairy cows was higher 0.93 US$ than untreated cows and economic efficiency increased from 2.09 to 2.19 by the treatment.

4. Discussion

Feeding high-producing cows continues to challenge dairy farmers and nutritionists. Also, dairy profit margins vary as milk prices and feed costs shift yearly. Feed additives are a group of feed ingredients that can cause a desired animal response in a non-nutrient role, such as pH shift, metabolic modifier, or performance (Hutjens, 1991). The use of exogenous fiber-degrading enzyme additives for ruminants was first examined in the 1960s, as reviewed by Beauchemin and Rode (1996). Enzyme products for ruminant diets are of fungal (mostly *Trichoderma longibrachiatum, Aspergillus niger* and *A. oryzae*) like our tested product (Fibrozyme); bacterial (*Bacillus* spp., Pendleton, 2000) or rumen bacterial (Gado et al., 2009) origin. According to Sheppy (2001), there are four main reasons for using enzymes in animal feed: 1) to break down anti-nutritional factors; 2) to increase the availability of starches, proteins and minerals enclosed within fiber-rich cell walls; 3) to break down specific chemical bounds in raw materials which are not usually broken down by the animals' own enzymes, thus releasing more nutrients, and. 4) to supplement the enzymes produced young animals.

Beauchemin et al. (1999) used lactating and cannulated Holstein cows to investigate the effects of grain source and fibrolytic enzymes supplementation on ruminal fermentation, nutrients digestion in the rumen and in intestine, and milk production. Two grains (barley and hull-less barley) were combined with and without enzymes. They proposed three ways for the arrival and action of the enzymes into the intestine: enzymes applied to dry feed may enhance the binding of the enzyme to the substrate, which may increase the resistance of the enzymes to proteolysis and prolong their residence time within the rumen; enzymes applied to silage or TMR immediately prior to feeding, may be released into the ruminal fluid and may pass through the rumen quickly before they can be effective, which would provide larger intestinal effects and exogenous enzymes may alter

digestion and nutrient absorption in the small intestine. Digestion of plant cell walls, which is carried out by ruminal microorganisms, provides a large amount of energy for ruminants. Fibrolytic enzymes isolated from fungi fermentation cultures have been utilized to improve DMI and forage digestibility; particulate passage rate; and digestibility of DM, NDF, and ADF in beef steers (McAllister et al., 1999).

In consistence with our finding on DMI, Ahn et al. (2003); Bernard et al. (2010) and Arriola et al. (2011) reported that adding fibrolytic enzymes supplementation to dairy cow diet did not enhance DMI and no difference was found between cows fed supplemented diet or un-supplemented diet with fibrolytic enzymes. On the other hand, several researchers recorded an increase in DMI of dairy cows when fibrolytic enzymes was applied to forage before mixing with other ingredients (Lewis et al., 1999) or applied to TMR or concentrate portion of the diet (Bowman et al., 2002; Ware & Zinn, 2005). However, the effects of fibrolytic enzymes on DMI appear to be vary among enzymes products and the method of applying of enzymes (Bowman et al., 2002)

A number of studies have examined the effects of fibrolytic exogenous enzymes on digestibility and milk production in dairy cows. In some studies, dietary addition of fibrolytic enzymes either to forages or concentrate portion increased milk production from 5- 16% (Lewis et al., 1999; Gado et al., 2009; Holtshausen et al., 2011) as noticed at the current study but no milk response was reported in others (Elwakeel et al., 2007; Bernard et al., 2010). Furthermore, these enhancement in milk yield at the current study are in line with those found by Guerra et al. (2007) who used Fibrozyme in diet containing alfalfa hay and they reported that Fibrozyme supplementation increased milk yield, which may be due to improved utilization of nutrients in digestive tract and in rumen and increased gain of net energy. Differences in enzyme activity, form and application rates, and diets across these studies complicate elucidation of the reasons for these discrepancies and highlight the need for caution when comparing studies involving different enzyme preparations.

The improvement in feed efficiency observed in the current lactation study might be attributable to greater NDF digestibility in the rumen and the similar trend was concluded by Holtshausen et al. (2011). Improvements in feed conversion efficiency were due to lower DMI rather than a change in milk yield. Improved feed efficiency indicates better utilization of nutrients when TMR was treated with enzymes, with the magnitude of improvement being a linear function of enzymes dosage.

There were no responses in the percentages of milk fat, protein, lactose and SNF for supplementing exogenous fibrolytic enzymes to early lactating dairy cows under the conditions of the current study, but the yield of milk protein, lactose and SNF was enhanced significantly by fibrozymes inclusion in the early lactating dairy cows. In agreement with our finding, several studies has been reported that fibrolytic enzymes supplementation to Holstein dairy cows did not affect ($P>0.05$) on milk composition (Lewis et al., 1999; Knowlton et al., 2002; Reddish & Kung, 2007; Elwakeel et al., 2007; Bernard et al., 2010; Arriola et al., 2011). On the other hand, Yang et al. (1999) and Mansour (2009) found that milk fat increased when adding fibrolytic enzymes. The increase in fat percentage may be due to the increase in available energy and fatty acids for fat synthesis. Gado et al. (2009) concluded that milk protein yield for Brown Swiss cows was ($P<0.05$) increased (0.57 %) for cows fed ZADO® supplemented diet compared with 0.45 kg/h/day for cows fed control diet. The variability in responses among studies may be attributed to variety of enzyme products and experimental conditions.

Contents of albumin, glucose, triglycerides and urea were not differing significantly due to fibrozymes supplementation to early lactating dairy cows (Table 5). Broderick et al. (1997) treated alfalfa silage with four levels of fibrolytic enzymes mixture (xylanase and cellulase) and fed dairy cows, they found that the adding of fibrolytic enzymes did not influence blood glucose and urea in all levels. Also Viktor (2006) concluded that there was no significant difference in blood plasma glucose concentrations for Holstein dairy cows fed diet supplemented with fibrolytic enzyme (xylanase). On the other hand, total protein and globulin and cholesterol were declined significantly by fibrozymes supplementation, which is similar to that found by Abd El-Kareim (2004). A/G ratio indicated that the treated animals were in good health since A/G ration was more than 1.00. Exogenous fibrolytic enzymes inclusion in TMR fed to the early lactating dairy cows improved economic efficiency and achieved daily net profit 0.93 US$ per cow over control cows. These results agreed with those obtained by Tozer et al. (2003) who found that although costs per kilogram of milk produced were lowest for pasture-concentrate cows, cows on TMR had the highest net income per cow per day because of higher yields of milk and milk components, but cows on the pasture-concentrate had lower daily net income due to lower yields of milk and milk components.

5. Conclusion

The results of the current study concluded that feeding a fibrozyme applied to the total mixed ration of dairy cows in early lactation has the potential to increase milk production, SNF and economic efficiency.

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Genetic Analysis of Yield and Yield Contributing Quantitative Traits in Bread Wheat Under Sodium Chloride Salinity

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Abstract

The genetic basis of salt tolerance was investigated in six bread wheat cultivars (Local white, Pavon, Pasban 90, Frontana, Tobari 66 and Chakwal 97) differing in salinity tolerance, and their F_1 crosses made in a half diallel mating design. The F_1s and parents were germinated in pots, and were subjected to 200 mM NaCl salt stress after one month. Most of the crosses had high heterosis for yield suggesting that breeding for high yield under salt stress is possible. Narrow sense (h^2_N) heritability estimates ranged from 0 to 51%, whereas broad sense (h^2_B) heritability estimates ranged from 25 to 84 % for the studied traits. Additive genetic effects were significant for days to heading, days to maturity, plant height and fertile tillers plant^{-1}, suggesting that early selection could be useful to bring desirable changes in these characters under NaCl stress. Dominance effects were significant for yield and yield contributing traits, indicating that selection for yield under NaCl stress would be effective in later generations.

Keywords: diallel analysis, quantitative traits, bread wheat, salinity tolerance

Abbreviations: GCA, general combining ability SCA, specific combining ability AD, Additive-Dominance AUP, Adjusted Unbiased Prediction MPH, mid parent heterosis BPH, better parent heterosis

1. Introduction

Salinity is the oldest and severe abiotic stress which limits crop production in arid and semi arid areas of the world. Over 800 million hectares of land are salt affected throughout the world (Munns, 2005). In Pakistan, about six million hectares are salt affected (Chatrath et al., 2007). One of the possible ways to bring saline marginal soils under crop cultivation is to develop salt tolerant crop cultivars instead of pricey engineering approaches (Qureshi et al., 1990). Furthermore heterosis breeding approaches offers the way to surmount the yield barriers (Kumar et al., 2011).

Salt tolerant crop varieties can be developed by conventional methods if genetic variation for salinity tolerance exists in the available germplasm. Genetic variation with high heritability is desirable for direct phenotypic selection. Only a few cycles of selection could result in significant improvement in salinity tolerance. Diallel cross designs are frequently used in plant breeding research to obtain information on genetic effects for a fixed set of parental lines or estimates of general combining ability (GCA) and specific combining ability (SCA), variance components and heritability for a population from randomly chosen parental lines. Diallel analysis is a reliable mechanism to understand the type of gene action controlling complex genetic traits of economic importance (Inamullah et al., 2006). Knowledge of broad sense heritability, narrow sense heritability, GCA and SCA is useful in the choice of parental genotypes. Combining ability studies assist in the identification of parents with greater GCA values and parental combinations with greater SCA values.

High heritability values of traits which confer salinity tolerance in spring wheat indicated that a major progress in salinity tolerance may be possible through selection by the imposition of high selection pressure (Ashraf, 1994). Selection of plants exhibiting better combination of desirable traits is easy if variation is controlled by additive gene effects. Stuber (1994) reported that assessment of additive and non-additive gene action could be helpful in determining the possibility of commercial utilization of heterosis and isolation of pure lines among the progenies of the good hybrids. S. Singh and M. Singh (2000) reported that salinity sensitive parent response was partially dominant, whereas the tolerant parent showed partial dominance for yield potential. Salinity tolerance and yield potential appeared to be controlled by different gene complexes.

The present study was initiated to estimate the morphological traits related to salt tolerance and to determine the type of gene action controlling salt tolerance in wheat under 200 mM NaCl stress.

2. Materials and Methods

2.1 Plant Material and Growth Conditions

Seeds of the six bread wheat varieties (Local white, Pavon, Pasban 90, Frontana, Tobari 66 and Chakwal 97) differing in salinity tolerance were taken from the gene bank of Plant Genetic Resources Program, NARC Islamabad. These cultivars were crossed in a one-way diallel mating design to obtain a total of 15 [(6(6–1)/2] cross combinations. F1 seeds along with their parents were sown in pots in a randomized complete block design (RCBD) having three replications. After one month of germination, 200 mM NaCl salt stress was given to all F_1 and parents. Two plants were maintained in each pot. One pot represented one replication. Data were recorded for plant height, No. of tillers plant-1, days to heading, days to maturity, spike length, number of spikelets spike^{-1}, number of grains spike^{-1}, 100 gain weight and grain yield plant^{-1}.

2.2 Data Analysis

The Mixed Procedure in SAS (SAS Institute, 2003) was used to analyze the data. Likelihood ratios were computed as described by Iqbal et al. (2007). Diallel analysis was carried out using the average of parental values and F_1 crosses values by utilizing an Additive-Dominance (AD) model following Zhu (2003). Components of genetic variance were calculated following Rao (1971). Genetic effects were estimated using the Adjusted Unbiased Prediction (AUP) method of Zhu and Weir (1996). Estimates of narrow-sense heritability were obtained by the formula $h^2_N = V_A/V_P$, and those of broad-sense heritability by $h^2_B = (V_A+V_D)/V_P$. The significance of components of variance was tested by means of one-tailed t-test, and those of genetic effects by two-tailed t-tests. Genetic analyses were done using the software "QGA Station 1.0" developed by Chen and Zhu (2003). Heterosis was determined for each cross as the percentage deviation of F_1 means from mid parent means (MP) and better parent (BP) following the formulae given by Dreisigacker et al. (2005).

$$MPH\ (\%) = (F_1 - MP)/MP \times 100$$

$$BPH\ (\%) = (F_1 - BP)/BP \times 100$$

The F_1 hybrid performance, MPH and BPH was tested for significance by an ordinary *t*-test (Dreisigacker et al., 2005). Genetic and phenotypic correlations among the traits, and their standard errors, were estimated using multivariate REML implemented in the MIXED procedure of SAS (SAS Institute, 2003).

Table 1. Analysis of variance of nine traits of a half diallel cross among six spring wheat varieties differing in salt tolerance

SOV	Days to heading	Days to Maturity	Plant Height	Fertile tillers plant^{-1}	Spike length	Spikelets spike^{-1}	Grains spike^{-1}	100 grain weight	Yield plant^{-1}
Block	ns	ns	7	5	ns	ns	5	ns	ns
Genotype	65**	74**	83**	39**	57**	26*	58**	75**	74**
Parents (P)[a]	55**	68**	72**	81**	75**	73**	12 [ns]	23**	45**
Crosses (C)[a]	45**	32**	28**	19 [ns]	25*	27 [ns]	88**	77**	55**
P vs. C	ns	ns	ns	ns	**	ns	ns	**	**
Residual	35	26	16	56	43	74	37	25	26

**,* Significant at $P < 0.01$, and $P < 0.05$, respectively, on the basis of likelihood ratio test.

[ns] not significant ($P \geq 0.05$), [a] Proportions of the sum of parents and crosses.

Table 2. Best linear unbiased predictors (BLUPs) of genotypes for nine traits in a half diallel cross among six spring wheat varieties differing in salt tolerance

Genotypes	Days to heading	Days to maturity	Plant height	Fertile tillers plant^{-1}	Spike length	Spikelets spike^{-1}	Grain spike^{-1}	100 grain weight	Yield plant^{-1}
Frontana	113.9	149.4	70.4	3.7	7.8	14.7	30.6	2.7	2.6
Local white	116.5	154.8	84.5	5.5	9.2	16.3	28.1	2.2	4.9
Pavon	108.3	145.6	55.1	3.5	9.4	16.1	35.5	2.7	4.2
Tobari 66	106.6	138.4	57.9	3.5	7.4	15.5	35.8	2.1	2.6
Pasban 90	108.0	134.2	51.7	4.6	9.3	16.2	32.6	2.3	4.7
Chakwal 97	106.3	136.9	57.6	3.0	7.8	14.6	30.3	2.0	1.9
Frontana × Local white	116.2	159.0	66.7	4.6	9.1	15.8	35.7	4.1	6.0
Frontana × Pavon	113.4	145.6	60.1	2.6	7.9	15.3	28.6	3.8	4.1
Frontana × Tobari 66	110.0	147.3	73.3	4.4	8.3	15.1	32.7	3.9	5.6
Frontana × Pasban 90	107.4	140.5	53.6	4.2	8.6	15.7	29.3	3.9	4.8
Frontana × Chakwal 97	111.7	147.0	60.7	3.5	9.2	15.9	33.8	3.8	4.9
Local white × Pavon	118.2	153.3	70.1	3.5	8.9	15.3	22.5	4.2	5.6
Local white × Tobari 66	106.6	146.7	77.9	4.2	10.1	17.5	46.7	4.0	8.9
Local white × Pasban 90	104.6	139.6	55.4	4.2	9.1	15.5	34.9	3.9	6.1
Local white × Chakwal 97	109.7	146.1	66.1	3.5	8.2	15.0	27.5	3.8	6.2
Pavon × Tobari 66	107.2	141.1	57.3	3.3	9.2	15.9	33.7	3.6	4.6
Pavon × Pasban 90	106.3	138.1	51.4	3.0	9.8	16.1	35.0	3.8	5.3
Pavon × Chakwal 97	109.4	142.9	58.2	3.3	9.4	16.7	22.0	3.5	5.1
Tobari 66× Pasban 90	110.3	142.0	55.4	4.4	8.8	16.5	25.1	1.9	1.9
Tobari 66 × Chakwal 97	106.6	139.6	60.4	3.7	9.6	17.1	43.4	3.2	7.0
Pasban 90 × Chakwal 97	110.3	146.7	54.8	3.5	9.9	16.4	33.1	3.9	5.8
SE*	2.3	3.0	3.3	0.7	0.5	1.0	3.9	0.4	0.8

* Standard error of the difference between BLUPs.

3. Results

Genotypes (F_1 and parents) differed significantly ($P < 0.05$) for all traits studied. Variance due to genotypes was more than 50% for all the studied traits except fertile tillers plant^{-1} and spikelets spike^{-1} (Table 1). The effects of parents were significant ($P < 0.05$) for all traits but grain spike^{-1}. Genotypic variance for crosses were also significant ($P < 0.05$) for all traits but fertile tillers plant^{-1} and spikelets spike^{-1} (Table 1).

Local white was the latest of the six cultivars followed by Frontana, Pavon, Pasban 90, Chakwal 97 and Tobari 66 in descending order of days to heading and maturity. The F_1 crosses involving Frontana and Local white also matured later than other crosses (Table 2). Local white was the tallest parent followed by Frontana. Pasban 90 and the crosses involving it had the shortest plants. The F_1 crosses involving Local white and Frontana were also taller than the crosses not involving these parents. Local white and Pasban 90, and the crosses involving these produced the maximum fertile tillers plant^{-1}. The longest spikes were produced by Pavon, Local white and Pasban 90. The F_1 crosses involving Pavon, Local white and Pasban 90 also produced the longest spikes (Table 2). Local white and its crosses had the maximum spikelets spike^{-1}. Tobari 66 and two of its F_1 crosses had maximum grains spike^{-1}. Pavon and Frontana, and the crosses involving these had the highest 100 grain weight. The F_1 crosses of these parents also produced the highest grain spike^{-1}. Local white and its crosses had the highest yield plant^{-1} (Table 2). Local white, Pavon and Pasban 90, and the crosses involving one of these performed better than the other three parents under 200 mM NaCl stress.

Additive genetic effects were significant ($P < 0.01$) for days to heading and maturity, plant height and fertile tillers plant^{-1} (Table 3). Additive genetic effects were > 50% of the total phenotypic variation in days to maturity and plant height. Dominance effects were significant ($P < 0.05$) for all traits studied. Dominance effects were > 75 % of the total variability in 100 grain weight and yield plant^{-1} (Table 3).

Narrow sense (h^2_N) heritability estimates were significant ($P < 0.01$) for days to heading and maturity, plant height and fertile tillers plant^{-1} (Table 3). Narrow sense (h^2_N) heritability estimates ranged from 0 to 51 %. Broad sense (h^2_B) heritability estimates were significant ($P < 0.01$) for all the traits studied and ranged from 25 to 84 %. Broad sense heritability estimates were relatively low for spikelets spike^{-1} (27%) and fertile tillers plant^{-1} (41%), but higher for the rest of the traits studied (Table 3).

Table 3. Percent proportions of variance components to total phenotypic variance for nine agronomic traits in F_1 of a half diallel cross among six spring wheat varieties differing in salt tolerance

	Days to heading	Days to Maturity	Plant Height	Fertile tillers plant^{-1}	Spike length	Spikelets spike^{-1}	Grains spike^{-1}	100 grain weight	Yield plant^{-1}
V_A/V_p	27**	51**	64**	33**	ns	ns	ns	ns	ns
V_D/V_P	37**	23**	20**	8*	53**	25**	64**	77**	76**
V_E/V_P	36**	26**	16**	59**	43**	75**	36**	23**	24**
h^2_N	27**	51**	64**	33**	ns	ns	ns	ns	ns
h^2_B	64**	74**	84**	41**	57**	25**	64**	77**	76**

**, * Significantly different at $P < 0.01$ and $P < 0.05$, respectively, from zero; ^{ns} Non-significant ($P \geq 0.05$)

h^2_N and h^2_B are heritabilities in narrow and broad-sense, respectively, V = Variance, P = Phenotypic, A = Additive, D = Dominance, E = Residual.

Analysis of the relative importance of general combining ability (GCA) and specific combining ability (SCA) effects provides an indication of the type of gene action involved in the expression of traits. Frontana and Local white had significant ($P < 0.01$) positive GCA (2.12 and 2.33, respectively) for days to heading. High positive SCAs for days to heading were recorded for the crosses 'Local white × Pavon' (7.10) and 'Tobari 66 × Pasban 90' (4.17). The highest positive GCA effects for days to maturity were observed for Local white (5.56) and Frontana (3.46). Significant ($P < 0.05$) SCAs were found for days to maturity for the crosses 'Pasban 90 × Chakwal 97' (8.31) and 'Frontana × Local white' (7.51). Significant ($P < 0.01$) positive GCA effects were observed for Local white (9.20) and Frontana (2.84). Significant ($P < 0.05$) positive SCAs for plant height were observed for the crosses 'Frontana × Tobari 66' (8.62) and 'Local white × Tobari 66' (7.92).

Local white (0.74), Pavon (-0.66) and Chakwal 97 (0.50) exhibited significant ($P < 0.01$) GCA for fertile tillers plant^{-1}. Significant ($P < 0.05$) positive SCAs were found for spike length for the crosses 'Frontana × Chakwal 97' (0.96), 'Local white × Tobari 66' (1.53), 'Pavon × Pasban 90' (0.55), 'Tobari 66 × Chakwal 97' (1.12) and 'Pasban 90 × Chakwal 97' (0.98). Significant ($P < 0.05$) positive SCAs for spikelets spike^{-1} were observed for the crosses 'Local white × Tobari 66' (2.10), 'Pavon × Chakwal 97' (1.35) and 'Pasban 90 × Chakwal 97' (0.73). Significant ($P < 0.01$) positive SCAs were found for grains spike^{-1} for the crosses 'Local white × Tobari 66' (15.07) and 'Tobari 66 × Chakwal 97' (11.26). Significant ($P < 0.01$) positive SCAs were observed for 100 grain weight for the crosses 'Frontana × Tobari 66' (0.83), 'Local white × Pavon' (0.75), 'Local white × Tobari 66' (0.99), 'Local white × Pasban' (0.78), and 'Pasban 90 × Chakwal 97' (0.96). Significant ($P < 0.05$) positive SCAs for yield plant^{-1} were detected for crosses 'Local white × Tobari 66' (3.92), 'Pavon × Pasban 90' (0.80), 'Tobari 66 × Chakwal 97' (2.88) and 'Pasban 90 × Chakwal 97' (1.42).

Table 4. Mid parent heterosis values for some quantitative wheat traits in 15 hybrids

Cross	DH	DM	PH	FTP	SL	SS	GS	TGW	YPP
Frontana × Local white	0.7	5.0	-14.7	-14.7	8.9	3.3	26.9	74.0	67.7
Frontana × Pavon	2.2	-2.9	-4.8	-4.8	-10.8	-1.1	-16.1	46.1	22.7
Frontana × Tobari 66	-0.5	2.6	14.7	14.7	11.0	0.4	-1.8	74.5	144.5
Frontana × Pasban 90	-4.0	-1.1	-13.1	-13.1	0.0	3.0	-8.8	68.5	34.4
Frontana × Chakwal 97	1.4	3.0	-5.2	-5.2	23.2	18.5	13.7	75.5	148.6
Local white × Pavon	5.9	2.2	0.2	0.2	-5.9	-11.1	-35.6	82.2	26.0
Local white × Tobari 66	-5.4	0.0	9.7	9.7	27.2	18.8	56.1	103.1	158.2
Local white × Pasban 90	-8.3	-3.8	-19.7	-19.7	-1.5	-9.0	18.4	93.0	30.8
Local white × Chakwal 97	-2.1	0.2	-7.2	-7.2	-4.0	-6.6	-7.0	96.8	94.5
Pavon × Tobari 66	-0.3	-0.9	0.9	0.9	11.4	1.5	-7.8	55.2	39.9
Pavon × Pasban 90	-2.2	-1.6	-4.4	-4.4	5.6	-0.3	3.2	60.9	21.6
Pavon × Chakwal 97	2.2	1.2	3.6	3.6	11.8	17.2	-39.8	56.3	77.2
Tobari 66 × Pasban 90	3.1	4.6	0.9	0.9	5.7	8.2	-31.9	-14.8	-56.7
Tobari 66 × Chakwal 97	0.0	1.5	4.9	4.9	33.6	27.2	37.8	64.0	270.6
Pasban 90 × Chakwal 97	3.1	9.3	0.6	0.6	19.4	13.0	6.2	96.8	80.0

DH=Days to heading; DM=Days to maturity; PH=Plant height; FTP=Fertile tillers per plant; SL=Spike length; SS=Spikelets per spike; GS=Grains per spike; TGW=Thousand grain weight; YPP=Yield per plant.

Mid-parent heterosis (MPH) estimates for days to heading, days to maturity, plant height and fertile tillers plant^{-1} were less than 15% (Table 4). This indicated that improvement in these traits through hybridization is difficult. The cross 'Tobari 66 × Chakwal 97' had highest (33.6%) MPH for spike length. This cross also had the maximum (27.1%) MPH for spikelets spike^{-1}, 37.8% for grains spike^{-1}, 64% for 100 grain weight and 270.6% yield plant^{-1}. Highest (56%) MPH for grains spike^{-1} was recorded for cross 'Local white × Tobari 66'. This cross also had high MPH for 100 grain weight (103%) and yield plant^{-1} (158%). Fourteen crosses had >50% MPH for 100 grain weight. This indicated that there is a great potential in the cultivars to improve 100 grain weight which is an important yield contributing trait. Eight crosses showed > 50% MPH for yield plant^{-1}. Four crosses including 'Frontana × Tobari 66' (141%), 'Frontana × Chakwal 97' (107%), 'Local white × Tobari 66' (89%) and Tobari 66 × Chakwal 97 (213) showed > 50% BPH for yield plant^{-1}. The highest heterosis (271%) for yield was recorded for cross 'Tobari 66 × Chakwal 97'. High heterosis estimates for yield suggested that breeding for high yield under salt stress is possible if parents with better combining ability are crossed.

Generally genotypic correlation coefficients were higher than phenotypic correlation coefficients (Table 5). All the studied traits were positively correlated with yield plant^{-1} except days to heading. Similar findings were also reported by previous workers (Ali, 2001; Yagdi, 2009).

Table 5. Phenotypic (p) and genotypic (g) correlation coefficients among some quantitative traits in wheat

	Days to Maturity	Plant Height	Fertile tillers plant^{-1}	Spike length	Spikelets spike^{-1}	Grains spike^{-1}	100 grain weight	Yield plant^{-1}
Days to heading	p 0.80**	p 0.47**	p 0.25*	p- 0.12	p -0.16	p -0.31**	p 0.07	p -0.04
	g 0.89**	g 0.63**	g 0.24	g -0.14	g -0.29	g -9.57**	g 0.11	g -0.04
Days to Maturity		p 0.65**	p 0.23**	p 0.05	p 0.00	p -0.06	p 0.28**	p 0.23**
		g 0.76**	g 0.43**	g 0.07	g 0.16	g -0.20	g 0.32**	g 0.26**
Plant Height			p 0.31**	p -0.01	p 0.03	p 0.08	p 0.08	p 0.28**
			g 0.62**	g -0.07	g -0.03	g 0.02	g 0.07	g 0.34**
Fertile tillers				p 0.25	p 0.21	p 0.12	p -0.14	p 0.20*
plant^{-1}				g 0.19	g 0.32	g 0.03	g -0.26	g 0.17
Spike length					p 0.73**	p 0.39*	p 0.21**	p 0.52**
					g 0.93*	g 0.34	g 0.40**	g 0.74**
Spikelets spike^{-1}						p 0.42**	p -0.03	p 0.37**
						g 0.43	g 0.10	g 0.64*
Grains spike^{-1}							p 0.07	p 0.40**
							g 0.14	g 0.49**
100 grain weight								p 0.61**
								g 0.65**

**, * Significantly different at $P < 0.01$ and $P < 0.05$, respectively.

4. Discussion

The present study revealed that additive genetic effects are important in the inheritance of days to maturity and heading, plant height and fertile tiller plant^{-1} under 200 mM NaCl stress. This suggested that early selection could be useful to bring desirable changes in these characters under NaCl stress. The contribution of additive genetic effects in the inheritance of days to maturity was also reported by Iqbal et al. (2007). Estimates of broad sense heritability for most of the yield components were within the requisite range for the improvement of salinity tolerance in wheat. The high heritability estimates for a number of variables indicated that significant advances in salinity tolerance in wheat may be possible through selection. High SCA estimates indicated dominance gene effects and high GCA suggested major role of additive gene effects. Non significant GCA and SCA values indicate epistatic gene effects (Fehr, 1993). However, in wheat, additive × additive interaction component is fixable in later generations. Number of fertile tillers directly contributes to crop yield. 'Local white' proved to be the best general combiner to increase fertile tillers plant^{-1}. Negative GCA effects of two parents 'Tobari 66' and 'Pasban 90' indicated that these parents could be used to incorporate early maturity in wheat.

Early heading is desirable because it provides sufficient time for grain formation and filling. Seven crosses showed negative heterosis for days to heading. Genotypes with early maturing habits are generally required to avoid stress conditions. Therefore, negative heterosis for days to heading and maturity is useful. Five F_1 crosses had negative MPH and could be used to incorporate earliness. Workers (Dreisigacker, 2005; Sadeque, 1991; Inamullah, 2006) also reported negative heterosis for days to heading and maturity and reported the importance of heterotic studies for incorporating earliness in wheat. Tall plants expected to lodge quite often. They require more energy to translocate solutes to the grain and have lower grain weight. Short stature wheat is therefore preferred and negative heterosis is desirable. Seven crosses showed negative heterosis for plant height. Dreisigacker (2005) and Sadeque (1991) had reported negative as well as positive heterosis for plant height.

Spike length is directly associated with number of grains. Positive MPH has been reported for spike length (Thakur, 1991), while negative MPH was reported for spike length in wheat genotypes (Sadeque, 1991). Most of the crosses had positive MPH for grains spike^{-1}, 100 grain weight and yield plant^{-1}. This showed the effectiveness of heterosis for increased grain yield under salinity stress. Similar findings were reported by Afia (2000). Above 50% MPH of eight crosses and BPH for four crosses suggested that hybrids could yield better under salt stress conditions. Our results showed high heterosis for yield plant^{-1} which was particularly observed under salinity stress. Earlier,

Walton (1971) reported 92% MPH for grain yield. Our results showed that heterosis could improve grain yield and provide sufficient chance to select the desired combinations under salinity stress.

Positive correlation of days to heading and maturity with plant height and fertile tillers plant^{-1} suggested that delayed crop maturity leads to better crop growth. Negative correlation between days to heading and yield plant^{-1} suggested that early maturity helps plant to avoid stress and increases crop yield under salinity stress. All the yield attributing traits displayed positive correlation at both genotypic and phenotypic level with yield plant^{-1}, indicating that all these traits contributed towards yield under NaCl stress.

Three parents, 'Local white', 'Pavon' and 'Pasban 90', and their F_1 crosses performed better on the basis of yield and yield components under NaCl stress. These parents can, therefore, serve as donor parents for developing salt tolerant wheat varieties. Significance of additive effects for days to heading, days to maturity, plant height and fertile tillers plant^{-1} suggested that selection in early breeding generations could be effective to bring positive changes in these traits under salt stress. High dominance effects for yield and yield contributing traits indicated that selection for yield and yield components under salt stress could be effective in later generations. Above 50% MPH of eight crosses and BPH for four crosses suggested that hybrids could yield better under salt stress conditions. Narrow sense (h^2_N) heritability estimates ranged from 0-51%, whereas broad sense (h^2_B) heritability estimates ranged from 25-84% for the studied traits. On the basis of yield and yield components, crosses 'Local white × Tobari 66', 'Tobari 66 × Chakwal 97', 'Frontana × Local white', 'Local white × Pasban 90', 'Pavon × Pasban 90' might be better choice to get high yield under NaCl stress conditions.

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Property Rights and Access: the Case of Community Based Fisheries Management in Bangladesh

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Abstract

The revenue oriented approach has not been able to involve poor fishers in the inland fisheries management in Bangladesh. A community-based fisheries management (CBFM-2) approach was implemented over a period of 6 years (2001-2006) to improve access to fishing rights of the poor and to improve productivity as well as sustainability of fisheries resources. This study investigates the changes in fisher's access to livelihoods in the various types of water bodies such as closed beels (deeper depressions in the floodplain), open beels (lake), rivers and floodplains to enhance their livelihoods. Data for the study was obtained from two questionnaire-based field surveys conducted by the Bangladesh CBFM project office: a baseline study carried out in 2002 and an impact study in mid-2006. A total of 2,826 households were randomly selected from several regions in Bangladesh, comprising 1,994 households at 34 (51%) CBFM project water bodies and 832 households at 10 (59%) control water bodies. This study found that the CBFM fishers have obtained greater access to fisheries and improved livelihoods than non-CBFM fishers. The fishers have now changed their attitudes, have greater awareness of fisheries rules and are able to resolve conflicts much easier in the CBFM water bodies. Long term access rights over fisheries resources should be considered as the priority for a sustainable inland fishery and livelihoods of fishers in Bangladesh.

Keywords: community based management, inland fisheries, livelihood assets, social capital, access rights, poor fishers

1. Introduction

The importance of securing fisher's access to fisheries is being increasingly recognized in many parts of the world. In Bangladesh about 80% of rural households rely on fisheries as the important source of food and income (Thompson & Hossain, 1998) and fish alone supply 60-80% of the total animal protein needs of the country (Muir, 2003). The economic value of fish and other aquatic resources (mainly plant products) from wetlands has been found to be more than double the return from a single rice crop (Colavito, 2002). Poor households catch small wild fish for their own consumption, which are inexpensive and are not included in official statistics. In the monsoon (rainy) season in Bangladesh about 4 million hectares of land are inundated, and more than half the country is under water in an exceptional flood year (Ali, 1997). The floodplains are rich in nutrients and provide good habitat for fish. However, excess fishing efforts in these fisheries are now a major threat to the livelihood of poor fishers. Fish production and catch per fisher has declined (DOF, 2006; ICF, 2006), the low per capita consumption has caused great concern to the poor fishing households (Sultana & Thompson, 2007).

Fishing rights on public water bodies is determined by conferring lease. However, the lease usually goes to the elites who can acquire exclusive rights to determine fishing access to the water body by using their social power (Toufique, 1997). The leaseholders had less incentive to conserve the natural habitat. Maximizing profit from fisheries was the prime aim for the lease holders.

Property rights in inland fisheries are complex and poorly defined in Bangladesh. The main reason for this complexity is due to the heterogeneity in the characteristics of water bodies, types of fishers, types of gear used, varieties of species caught and state regulation (Toufique, 1999; Smith et al., 2005). The majority of fisheries are

treated as common pool resources during the monsoon, while in the dry season when fish are trapped in floodplain ditches they however becomes the property of the private ditch owner. Thus property rights arrangements can change their features by fishing seasons in these floodplains. The characteristics of the property rights described by Scott (1988) as exclusivity, durability, security and transferability do not fit into the inland fisheries of Bangladesh.

The government, NGOs and donors have initiated to implement a community based approach in inland fisheries in Bangladesh since 1990s. Community Based Fisheries Management Phase 2 (CBFM-2) projects (2001 – 2006) has developed an alternative fisheries management system where the responsibilities of fisheries are shared by the government, non-government organizations (NGOs) and the fishers[Note 1]. The ultimate goal of the CBFM is to organize poor fishers in securing use rights, protect their resources from outsiders (lease holders).

The main thrust of the CBFM-2 project was to test a range of community managed and co-managed approaches considering the diversity of resource systems and local circumstances. The Bangladesh government through the Ministry of Land transferred 116 water bodies including 77 *jalmahals* (public water bodies) to the Department of Fisheries (DoF) for CBFM-2 project. The CBFM project has adopted a flexible management approach in the water bodies suitable to local circumstances. The protection of freshwater biodiversity is increasingly recognized as a major conservation priority (Abell et al., 2002); conservation measures helps to reduce poverty and resource degradation (Brown et al., 2005).

The access issue has been raised and debated in all successive regimes in Bangladesh. The complexity of securing property rights over fisheries has been an issue for more than a decade (Metzner, 2008). The aim of the study is to assess the impact of community based approach on establishing user rights to the inland fisheries in Bangladesh. This study addresses how the fishers are able to enforce their fisheries rules to reduce fishing effort and conserve natural stock, and how they cope with livelihood maintenance and sustain their livelihoods when fish catches are on continuous decline.

This paper is organized into six sections: Section 2 reports the property rights, livelihoods approach and CBFM project; Section 3 describes the methodology and data sources of the study; Section 4 describes livelihoods of fisher households; Section 5 presents access to various assets: fishing, land, micro credit, and social assets for fishing household in the CBFM and control areas. This paper concludes with a summary of findings and recommendations.

2. Property Rights, Livelihood Approach and CBFM Project

In Bangladesh there are different types and characteristics of inland water bodies: closed *beels*, open *beels*, rivers and floodplain *beels*[Note 2]. The government (Ministry of Land) transferred 77 *jalmahals* to the DoF for the CBFM-2 project. The DoF was primarily responsible in pursuing local administration to handover of the water bodies officially to the organized fisher groups. The organized fishers took several years to establish access rights to some of the CBFM water bodies after formal handover of water bodies from MoL to the DoF (Thompson, 2004).

In the CBFM approach, the DOF and NGOs had worked jointly in identifying fishers, according to their poverty criteria[Note 3] and to ensure that these poor fishers are not excluded from the fisher groups. The NGOs had formed fisher groups in all CBFM sites and provided them with training and credit. The CBFM fishers were offered leases to government owned water bodies (*jalmahals*) from the MoL for 10 years. Management committees (Beel or River Management Committee) representing fisher groups were formed in all CBFM water bodies. They introduced fisheries rules which are tailored to local conditions in reducing fishing effort. The fishers in the closed *beels* (CB)[Note 4] established use rights and paid lease fees annually to the government revenue department. However, there are now conflicts over fisheries access between the new and the previous leaseholders in some of the CB sites. Conflicts were severe in some water body areas where local elites were involved in the management (Islam & Dickson, 2006).

Open *beels* (OB) are relatively bigger where a large number of people from different socio-economic classes participate to fish for their livelihoods. Fishers in OB paid lease fees annually to the government and established use rights. These *beels* remain submerged during the monsoon seasons when subsistence fishing is allowed free of lease costs. Conflicts occurred between the lease holders and the new fisher groups in the OB during the monsoon seasons.

Fishing in sections of rivers are year-long operations in Bangladesh. Since 1995, most of the previously leased river-sections have been made open-access. However, previous leaseholders have invested their capital by erecting *katha* (brush piles) in the deeper and more productive areas of the river. Poor fishers are hired to harvest fish from

katha and are paid a minimum price from their catch, while the majority of the benefits go to the investors. Thus poor fishers are gradually losing their fishing rights over the river sections.

In Bangladesh, around 6.3 million hectares of agricultural land is regularly inundated for about 4 to 5 months (Master Plan Organization, 1987; Ali, 1997). The lands in the floodplain *beels* (FPB) are privately owned and fishing access to these fisheries is free during the monsoon seasons. Fishing in FPB is thus seasonal, primarily operated for subsistence needs of the surrounding fishers based on their customary use rights. However, the landowners are concerned about damages to their crops on their lands during these fishing seasons. The fisher groups are encouraged to conserve brood fish stocks in the floodplains, by excavating dry season refuges for fish.

Fishers in Bangladesh are generally divided into three categories, namely full time fishers, part-time fishers and subsistence fishers (Thompson et al., 2003). The full time fishers are the poorest group who mainly fish for income; part time fishers fish for both income and own consumption, while subsistence fishers only fish for their own consumption. In Bangladesh, fishers generally have limited assets and their livelihoods are crucially dependent on fisheries. The livelihood approach (Carney, 1998; Scoones, 1998) is increasingly being used by many development agencies and NGOs in order to achieve a better understanding of natural resource management systems (Ashley & Carney, 1999). The analyses of the livelihood approach are based on the belief that people require a range of assets to achieve positive livelihood outcomes in the rural livelihood framework (Ellis, 2000). These assets include natural, human, physical, financial and social/political assets. A single asset category on its own is not sufficient to yield diverse livelihood outcomes (Haan & Lipton, 1998). The household combines these various asset endowments to sustain their livelihoods. Livelihood diversification can play key role in reducing fisher poverty who have limited livelihood options (Ellis, 1998).

The inabilities of the fishers to actively participate in fisheries management are due to the lack of various livelihood assets. This paper reports the CBFM organized fishers' access to livelihood assets that contribute to their household income and non-income benefits using a livelihood asset framework.

3. Methodology

Table 1. Category of households by income and fishing profiles

Category	Household type	Characteristics
I	Poor fisher	Fishes for income or for both income and food, usually possesses no agricultural land.
II	Poor – Non-fisher	Does not fish for income, has no agricultural land, usually does labouring work.
III	Moderately poor fisher	Fishes for income, has some agricultural land but less than 100 decimals (0.4 ha), or if occupation includes service or professional job and has thatched house
IV	Moderately poor – Non-fisher	Does not fish for income, has some agricultural land but less than 100 decimals (0.4 ha), or if occupation includes service or professional job and has thatched house
V	Better off	May or may not fish for income, has land more than 100 decimals (0.4 ha), has someone with a service and a tin or a pucca (concrete) house

Note: fisher include category I & III, non fisher include category II & IV, and category V is better off.

Data used in this study were obtained from CBFM (phase 2) project. The project office had conducted two questionnaire-based field surveys: the first was in 2002 shortly after the start of the CBFM-2 project and the second was carried out in mid-2006, just before the conclusion of the project. Prior to the first survey, partner NGOs had carried out household census in all project and control areas. Random sample of more than 6,000 households was selected from the census list for the first survey. For the second survey, 1994 households from 34 (51%) CBFM project water bodies and 832 households from 10 (59%) control water bodies have been randomly selected. These selected households in the second survey were also being interviewed in the first survey. The questionnaire used in both surveys covered a wide range of socio-economic and livelihood parameters, aquatic resource use, fishing involvement, access, compliance, existing NGO support and indicators of various livelihood assets. The questionnaires were administered to five categories of households based on their income and fishing

profiles (as shown in Table 1) in both project water bodies and control sites. The surveys were conducted through face to face interview by a team of ten experienced local enumerators. Training was given to acquaint them with the nature and purpose of the questionnaire prior to the fieldwork. The respondents were informed about the schedule of the survey through DOF and NGO field officers to ensure their presence during the survey. This paper reports the descriptive statistics on livelihood and income as well as access to various livelihood assets of fisher households in Bangladesh.

4. Livelihoods of Fisher Households

4.1 Household Incomes from Fishing

Fishing is the primary source of income for the majority of the fishers. Table 2 shows that the overall fishers' income from fishing increased by 21 percent (from Tk 15,035 to Tk 18,189) over 2002-2006 (Table 2). Income from fishing has significantly increased in FPB and rivers (104 percent and 60 percent respectively), and slightly increased in OB (9 percent). However, fishing income has decreased in CB (23 percent). These results show that the implementation of CBFM partly contributed to raising income of fishing households in Bangladesh. Fishing income from CB has reduced because fishers have to pay up-front for leasing fees and costs of stocking fish. The organized fishers have failed to harvest their stock due to conflicts and litigations[Note7] with previous leaseholders (Islam & Dickson, 2006).

In the control sites, the non fishers have increased their income from fishing in OB, CB and FPB, while fishing income has significantly reduced in rivers from 2002 to 2006 (Table 2). This indicates that fishers' access to fishing in OB, CB and FPB is less restricted. Although the river fisheries are open access in Bangladesh, increased in fishing activities has reduced household fishing income due to overexploitation of fisheries in rivers.

Table 2. Households fishing incomes by water body type and occupation in CBFM and control sites, 2002-2006

Water bodies		Project			Control		
		2002	2006	% change	2002	2006	% change
Open Beel	Fisher	15,917	17,256	+9	14,585	18,859	+30
	Non fisher	913	629	-32	609	2,125	+249
	Better off	1,867	1,386	-26	2,441	4,012	+65
Closed Beel	Fisher	12,967	9,973	-23	9,956	7,378	-26
	Non fisher	731	826	+13	553	1,257	+128
	Better off	2,377	2,431	+3	1,150	809	-30
Flood Plain	Fisher	15,599	31,761	+104	13,817	12,314	-7
	Non fisher	5,023	1,590	-69	2,458	2,801	+14
	Better off	7,682	5,855	-24	5,910	6,230	+6
River	Fisher	14,573	23,271	+60	22,379	20,797	+7
	Non fisher	1,097	1,980	+81	3,687	666	-820
	Better off	3,542	3,943	+12	668	1,050	+58
All	Fisher	15,035	18,189	+21	15,076	17,286	15
	Non fisher	1,316	1,015	-23	1,509	1,773	18
	Better off	2,811	2,443	-13	2,392	3,304	38

4.2 Income Diversification

Fisher households earned their incomes from a wide range of sources. Reduction in fishing effort can be supplemented by other income generating activities. In the CBFM project, partner NGOs provided training and credit support to the organized fishers. The fishers were able to invest in alternative income generating activities to improve their livelihoods. Table 3 shows that among the income sources, the share of income from fishing for the CBFM fisher households reduced from 49 percent to 46 percent over 2002-2006. However, fisher's income from farming increased significantly from 12 percent to 16 percent over 2002-2006 (Table 3). Fisher who previously worked as labourer in agriculture are now involved in own farming activities.

Table 3. Households' income from different sources in CBFM and Control sites, 2002-2006

Source of Income	Project (percentage)		Change	Control (percentage)		Change
	2002	2006	(+/-)	2002	2006	(+/-)
Fishing Income	49.2	45.8	- 3.4	48.2	41.6	- 6.6
Farm Income	11.9	15.6	+ 3.7	11.1	15.4	+ 4.3
Wage Labour	16.3	12.5	- 3.8	20.7	16.4	- 4.3
Business	11.4	12.3	+ 0.9	10.5	10.3	- 0.2
Other Self employment	5.7	5.9	+ 0.2	4.5	3.9	- 0.6
Remittance	1.9	4.1	+ 2.2	1.9	9.0	+ 7.1
Other	3.7	3.8	+ 0.1	3.1	3.4	+ 0.3
All	100	100		100	100	

Fishers in the CBFM sites were able to increase their income from non fishing sources. As shown in Table 3, incomes from farming and remittances have increased for fishers in the CBFM project sites. Households in CBFM areas were less reliant on wage laboring as their income from this source showed a significant decline (-3.8) from 2002 to 2006. Similar pattern has observed in the control sites. Many poor people migrate to other regions in Bangladesh for seasonal employment, such as paddy harvesting, road and building construction, rickshaw pulling and other wage laboring activities (Hossain & Nargis, 2010). The poor household members are increasingly residing temporarily away from their villages to find better work (CARE/LMU, 2005).

Table 4. Number of fishers in CBFM project and control sites

Water bodies	Project			Control		
	2002	2006	% change	2002	2006	% change
Closed Beel fishers						
Fish in single (main) water body	199	196	-1.5	67	62	-7.5
Fish in multiple water bodies	109	77	-29.4	13	17	33.3
All	308	273	-11.4	80	79	1.3
Open Beel fishers						
Fish in single (main) water body	383	437	14.0	157	174	10.8
Fish in multiple water bodies	171	147	-14.0	18	67	272.2
All	554	584	5.4	175	241	37.7
Floodplains fisher						
Fish in single (main) water body	36	56	55.0	43	60	39.5
Fish in multiple water bodies	68	36	-47.1	39	24	-38.5
All	104	92	-11.5	82	84	2.4
Rivers fisher						
Fish in single (main) water body	117	103	-12.0	45	58	29.0
Fish in multiple water bodies	37	50	35.1	19	12	-36.8
All	154	153	-0.6	64	70	9.4

5. Household Access to Various Assets

5.1 Access to Fishing

The involvement of fishers in fishing activities is highly determined by resource availability, property rights arrangements and their livelihood options. Accessibility to fishing in various fisheries determines households' income and consumption. Fishers usually catch fish in the water bodies close to their homes. A good number of fishers fish in multiple water bodies to increase their income from fishing. As shown in Table 4, the number of CBFM participating fishers in all types of water bodies except for OB has decreased from 2002 to 2006. The number of fishers fishing in their main water body increased in OB and FPB which indicates that fishing access was relatively less restricted in these water bodies. The number of fishers has decreased in CB which indicates that access was more restrictive for these sites. This is consistent with the fact that closed beels are stocked by a limited number of fishers and they usually share the costs and benefits from fisheries. Rivers are managed by local elites who employ limited number of people to fish in the water bodies which indicate that access to the CBFM river sites was more restrictive due to the influence of previous leaseholders. Table 4 also shows that the number of fishers who fish in multiple water bodies has significantly reduced in all the CBFM water bodies, except in the rivers. This indicates that besides fishing in their respective main water body fishers tend to move their fishing to rivers (Table 4).

In the control areas the number of fishers has increased in all the water bodies except CB (Table 4). These water bodies are stocked by lease-holders where access to fishing is restricted. However, there is less restriction to fish in other water bodies (OB, FPB, and river) in the control sites. Table 4 shows that the number of fishers who fish in multiple water bodies have reduced in FPB and rivers while there was a significant increase in the number of fishers in OB followed by CB. This indicates that the lease-holders in these water bodies aimed to maximize their fishing income by employing poor people to fish. The FPB and rivers are open access, these fisheries have been severely overexploited, and thus catch has reduced. Fishers are less likely to go for fishing in these water bodies.

5.2 Access to Land

Majority of the households live in the rural areas and rely mainly on agriculture. Fishers are largely poor in Bangladesh and 56 percent of rural households are classified as functionally landless (that is they own less than 0.2 ha of land). Table 5 shows that the size of the landholdings for households from both project and control sites has increased from 2002 to 2006 through renting or share cropping.

Table 5. Household land holdings in CBFM and control areas

Household category	Project			Control		
	2002	2006	% increase	2002	2006	% increase
Fisher						
Own Land (decimal)	34	39	16	31	36	16
Lease/share (decimal)	31	43	40	33	54	62
Total (decimal)	65	83	27	65	90	39
Non fisher						
Own Land (decimal)	53	56	5	47	56	19
Lease/share (decimal)	36	35	0	35	37	6
Total (decimal)	89	92	3	83	94	14

However, the size of land holdings for the fisher households is smaller compared to the non fisher households. Fishers in the CBFM sites increased their land areas from 65 decimals in 2002 to 83 decimals in 2006, while in the control sites, fishers increased their land from 65 decimals in 2002 to 90 decimals in 2006 (Table 5). The increasing land holdings for fisher households show that farming has become increasingly more important for them.

5.3 Access to Micro-Credit

Poor fishers lack access to credits in the rural areas of Bangladesh. The main source of credit for majority of the poor fishers comes from *mohajons* (money lenders). The NGOs play a crucial role in providing micro-credit for the poor people in Bangladesh. The CBFM project had facilitated credit support to the organised fishers. As shown in Table 6 that the CBFM project households received considerably greater amount of credit from NGOs

(both CBFM partner NGOs and other NGOs) and from relatives compared to the households in the non-CBFM control sites (Table 6). The CBFM fishers had received higher amount of credit (Taka 6,759/household) from other NGOs as compared to the amount of credit received from partner NGOs (Taka 3,624/household)[Note 5]. The findings indicate that the organised fishers were able to source out multiple credit agencies in the project areas. Community based approach could facilitate the poorest fishers in building links with various NGOs for sourcing out credit. The respondents report that they have invested a large portion of their micro-credit to obtain land (rent/share cropping) for crop cultivation, which was considered as the main supplementary income earning activity for the fishers.

Table 6. Sources and amount of credits (Taka) received by the households in CBFM and control sites, 2005

Source of micro-credit	Project			Control			t-value
	Mean	N	SD	Mean	N	SD	
NGO (CBFM)	3,624	24	1,611	4,750	4	1500	-1.30
Other NGOs	6,759	41	4,142	4,803	38	4327	2.05**
Banks	15,271	14	17,376	13,525	12	14,411	0.28
Relatives/neighbour	4,711	28	5,671	2,488	25	2,785	1.78*
Mohajans (money lender)	5,669	31	5,768	5,088	25	6,232	0.36
Samity/society	3,468	28	2,924	3,718	17	2,773	-0.28
Total	5,242	166	4,441	4,797	121	5,814	0.74

5.4 Access to Social Capital

Social capital emphasizes the different ways of mobilizing social relationship in a society to manage various resources and to engage with other actors. Such relationship can play important roles in securing access and defend private as well as group natural resource property rights, and thus seclude those resources from other users (Islam et al., 2011). The CBFM fishers have had more interactions with NGO staffs, government officials, local politicians and elites during meetings and public gatherings to discuss various social issues and exchange information on various sources of credit support and income generating potentials in their localities. These interactions may have improved the confidence of poor fishers to participate in the CBFM project activities. In order to measure changes in social assets, an index was constructed using the Principal Component Analysis[Note 6]. Six variables load highly on a single common factor (Table 7).

The scores of six separate variables were aggregated to form the social capital index. In social capital index the most important factor found is household's active participation in fisheries management (0.876). The organized fishers introduced fisheries management rules in the CBFM water bodies. They participated in fisheries enhancement activities in the water bodies and these include establishing fish sanctuaries, introducing closed seasons and gear restrictions. The second most important social capital factor is that local community has complied with fisheries rules introduced by the management committee (0.861). The fisheries rules were endorsed by the DoF and other local government agencies to be effectively implemented at water-body levels. Fishers introduced stocking of fingerlings in CB where poaching was a common threat for stocked beels. However, guarding was performed by fishers and successfully controlled poaching in most of the CB sites.

Table 7. Social capital factor

Performance Indicator	Factor Loading
Active fisheries management	0.876
Compliance with fishery rules	0.861
Ability to enforce fisheries rules	0.795
Speed of resolving conflicts	0.725
Information exchange	0.699
Knowledge in fisheries management	0.648
Total	4.604

Extraction Method: Principal Component Analysis.

The ability to enforce fisheries rules (0.795) is an important social capital factor. The CBFM activities were monitored by local staff in each water body. Rule breakers were punished by confiscating their destructive gears, fishing was banned for certain period of time and rule breakers were excluded from fisher groups. The management committees with the help of local administration were able to resolve fisheries related conflicts. The respondents reported that conflicts can now be resolved quicker as compared to five years ago. This speed of resolving conflicts has a factor loading of 0.725 (Table 7). The information exchange among the fishers and the level of knowledge factors (0.699 and 0.648 respectively) were important in social capital factors. Attainment of formal education is miserably low among the fishing households in Bangladesh. The partner NGOs had conducted trainings on leadership development and accounting management. They produced newsletters as well as audiovisual materials and organized TV talk shows in the project areas. These media activities were found to be very effective for disseminating fisheries knowledge at village levels.

It was observed that the number of court cases increased in areas with poor level of trust among the villagers. Court cases were only lodged when conflicts were severe and beyond the control of the management committee and community leaders (Islam & Barr, 2006). It was found in this study that conflicts were resolved through *salish* (village court) in the CBFM project areas (75 percent in 2002 to 78 percent in 2006), while in the control areas, the incidence of court cases have increased (13 percent in 2002 to 17 percent in 2006). This indicates that fishers are now more united and cooperative (Islam, 2006).

6. Summary of Findings and Recommendations

This paper investigated how the organized fishers under the CBFM project secure access to various assets in order to enhance their livelihoods. Comparisons between CBFM and non-CBFM fisher households fishing in closed *beels*, open *beels*, floodplains and river sections were made by using household survey data collected by CBFM project office in 2002 and 2006.

Fishing income increased by 21 percent (from Tk 15,035 to Tk 18,189) from 2002-2006. The increased in fishing income reflects better utilization of the fisheries resources at the CBFM sites. However, fishing income decreased in the CBFM CB as fishers had to incur higher operating costs by paying lease fees and stocking costs. This may have negative impact on the long-term viability of the community management of CB fisheries in Bangladesh. Although, fishing income has reduced, fishers in the CBFM project areas have also diversified their income sources. Fishers income from agricultural farming increased from 12 percent to 16 percent over 2002-2006. Therefore, CBFM fishers were able to increase income from non fishing income generating activities.

The number of participating fishers has been reduced in most of the CBFM project water bodies while their number has increased in the control sites. The CBFM fishers established user rights to the fisheries and were able to implement local management rules for achieving sustainable use of fisheries. Fishers in the CBFM sites obtained more areas of land through renting or sharecropping and used for agricultural farming. Similar increase in land areas were also found for the fishers in the non-CBFM sites. The increased areas of land holdings of fisher households indicate that agriculture has become important for many fisher households in the CBFM project.

The CBFM fishers received larger amount of credits from NGOs (not necessarily from project partner NGOs) compared to non-CBFM fishers. Fishers also obtained their credit from other NGOs and other interest free sources such as friends and relatives. The fishers are now more aware of new credit opportunities available in their locality.

The CBFM fishers have changed their attitudes, are more cooperative, have greater awareness of fisheries rules and are able to resolve conflicts much easier in the CBFM project sites as compared to the control sites. The CBFM fishers have greater access to fisheries and were able to defend themselves against outside threat. Therefore, long term securities of resource use and government support are required for sustaining the CBFM in the inland fisheries of Bangladesh.

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Notes

Note 1. The partner NGOs are mixed in size and characteristics. They include large national NGOs such as BRAC, PROSHIKA and, international NGO such as Caritas. The NGO effort has largely focused on institution-building: organizing fishers into groups, getting access to resources, providing credit, enabling them to design and carry out their own measures for resource management.

Note 2. These names appear to refer just to the physical characteristics of water bodies, each has implication for tenure and access.

Note 3. See Thompson, P. M. (2004). It was agreed by each partner NGO, the WorldFish Center and the DoF in the CBFM project that the beneficiary selection criteria was to ensure that a major share of the benefits from project activities reach the poorest members of the community. The general selection criteria were: (a) persons who catch fish by themselves for their livelihoods; (b) persons who have less than 50 decimals of land including the homestead in floodplain sites, and (c) persons who have an annual income of less than Tk. 30,000, primarily from manual work (Ahmed & Dickson, 2007).

Note 4. Beels are the deepest part of the floodplains, often with a permanent area of water. Closed beels are relatively "smaller" and well defined waterbodies (these may be above or below 8 ha in official area, but are generally not more than about 50 ha in the monsoon) with few outlets. Open Beels are relatively larger fisheries and are extensively used as capture fisheries for the purpose of subsistence fishing by a wide range of stakeholders.

Note 5. CBFM partner NGOs were assigned to work in particular waterbodies, they were not overlapped. Therefore, one particular NGO is treated as CBFM partner in a waterbody, while the same NGO is not treated as partner NGO in non-CBFM waterbodies. The number of waterbodies covered per NGO depends on their own implementation capacity and working areas.

Note 6. Principle Component Analysis (PCA) is based on the multiple correlation principle and can explain the variance of the dependent variables. The combination of the important variables with high factor loadings is used for constructing an index. The overall value of the index is calculated by percentage contribution of each selected highly loaded variables. This percentage contribution is used as weight for constructing an index of social capital.

Note 7. CBFM organised fishers failed to harvest their stocked fisheries in CB due to conflicts with landowners who influenced local administration, court cases resulted in delayed handover the water bodies to the CBFM, and due to flood occurs almost every year that destroyed their fish ponds in some of the CB sites.

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Nitrogen Release Pattern From Organic Manures Applied to an Acid Soil

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Abstract

Study of nitrogen release pattern from organic manures is very essential to ensure nitrogen supply in adequate quantity and at proper time to crop plants in an acid soil. A laboratory incubation experiment was conducted to determine the nitrogen release pattern from vermicompost (V.C), poultry manure (P.M), neem, inorganic fertilizer (I.O) and its combinations applied to an acid soil at two different rates. Cumulative nitrogen mineralization was significantly higher throughout the incubation period for I.O, V.C+P.M and I.O+V.C while it was two weeks after incubation for V.C and V.C+Neem due to the inhibitory effect of neem on nitrification. The results indicated a significant increase in the rate of N mineralization in the first one week in which the highest rate of 3.36 mg N day^{-1} was observed for inorganic fertilizer and thereafter it slowed down. V.C followed by V.C+Neem recorded higher rate of N mineralization of 0.24 and 0.23 mg day^{-1} respectively, from 48^{th} days after incubation. V.C+Neem at 120 kg N equivalent ha^{-1} recorded the highest ammonia content of 42.1% to total available N while V.C+P.M recorded higher nitrate content of 82.3% at the end of the incubation period. Positive correlation between initial nitrogen and total mineralized N from the manures and fertilizers ($R^2 = 0.563$) was observed. Furthermore, addition of organic manures resulted in increase in soil pH where as inorganic fertilizer showed a slight decrease (5.73) than control (5.78).

Keywords: N mineralization, organic manures, acid soil

1. Introduction

Ever since its introduction in the 1960's modern agricultural technologies involving the use of high yielding varieties, chemical fertilizers and pesticides among others have increased the food grain production tremendously across the world. Chemical fertilizers have played a predominant role in green revolution and initially the results were impressive. But, in the subsequent years unfavorable effects such as decreasing productivity, huge neglected areas of poor soils and water resources, and environmental impact have emerged. In addition the readily available forms of nutrients in inorganic fertilizers have pushed the traditional important sources of nutrients especially organic manures of different origin into a decline (Nagarajah, 1988). As agriculture became more settled, with a permanent land base, and as cultivation practices intensified, soil fertility became severely depleted. Ironically, this same period also marked the beginning of a massive global loss of soil organic carbon (SOC) associated with the rapid expansion of agriculture onto grassland and forest soils (Manna, Ghosh, & Acharya, 2003).

Organic matter plays a prominent role in increasing the level of soil fertility and sustaining the productivity of soils. Simultaneously rising costs of chemical fertilizers has further focused attention on recycling of plant nutrients through organic materials. Thus, the potential of organic manure use either alone or in combination with chemical fertilizers in crop production has received world wide attention. The importance of organic manure as a source of humus and plant nutrients to improve the soil fertility and soil health has been well established (Larson & Clapp, 1984; Doran & Parkin, 1994; Sudha & Chandini, 2003). In addition, the effect of different organic manures and its combinations on vegetable crops such as okra, tomato, onion etc., are widely studied (Masciandaro, Ceccanti, & Garcia, 1997; Buckerfield & Webster, 1998; Atiyeh et al., 2000; Bairwa, 2009; Islam et al., 2011; Kumar, Gowda, Shetty, & Karthik, 2011). In recent times, reports on organic manure as a source of plant nutrients for field crops particularly as an alternative to chemical fertilizers in rice cultivation is also increasing (Banik et al., 2006; Siavoshi, Nasiri, & Laware, 2011).

Undoubtedly organic manures have greater role to play in sustaining the agricultural production in any island agroecosystems due to its relative isolation and fragile ecosystem. In this context, it is imperative to study the effect of different organic manures on soil fertility of Andaman Islands, India. In recent times, inorganic input use is increasing particularly in vegetable production as these crops are more profitable. However due to the humid and high rainfall condition the nutrient loss from applied fertilizers is high leading to low input use efficiency and low productivity. The very limited scope available for area expansion under agriculture coupled with the low input use necessitates the importance of sustainable land management practices to increase the agricultural production. Such practices essentially include the use of different organic manures alone or in combination with inorganic fertilizers for which there is amble scope exists in these islands.

At the same time the suitability of organic materials as fertilizer depends to a great extent on its rapidity of mineralization and liberating the nutrients present in them (Weeraratna, 1979). Mineralization of nitrogen is the transformation of nitrogen from organic into inorganic form and the immobilization is the reversal of the process. These processes are biochemical in nature and are mediated through the activities of microorganisms (Bartholomow, 1965). The resulting effects of these two processes are expressed as net mineralization or net immobilization which decides the nitrogen supply to the growing crops. At the same time the mineralization immobilization turnover in soil is affected by soil properties such as temperature, soil moisture, pH, C/N ratio apart from total N and lignin content of the organic materials (Pathak & Sarkar, 1994). Besides, addition of nitrogen in proper dose at proper time is very essential for increasing the crop productivity. Thus understanding the process of mineralization and nitrogen availability in different organic manures, inorganic fertilizers and their combination at different doses are essential to avoid nutrient deficiency and successful crop production.

The investigations reported in this paper were carried out to examine the pattern of NH_4^+-N and NO_3^{2-} - N release from organic manures and in combination with inorganic fertilizers in an acid soil under humid tropical island climate. It is intended to evaluate the organic sources of nutrients supply as an alternate to inorganic fertilizers in a not so intensive cultivation system where soil acidity is also a problem.

2. Materials and Methods

2.1 Study Area

The tropical island of Andaman and Nicobar lie extending from north to south in the Bay of Bengal, at a distance of 1200 km from the Indian mainland. Out of the total geographical area of 8249 km^2, only 4.8% is cultivated whereas the rest of the area is under forest cover (DES, 2009). The Islands receive high rainfall of around 3100 mm from May to November as a result only rice is grown in valley areas and vegetables are mainly grown during the dry season starting from December to April. The soils are mostly acidic due to leaching of metal cations, medium to high in organic carbon content and the terrain is undulating (Ganeshamurthy, Dinesh, Ravisankar, Nair, & Ahlawat, 2002).

2.2 Experimental Design

An incubation study was conducted at Central Agricultural Research Institute, Port Blair, Andaman Islands, India to determine the nitrogen release pattern in a Typic Tropofluvents soil having pH 5.85 due to the addition of organic manures and inorganic fertilizers. Surface soil (0-30 cm) from cultivated land was collected, shade dried, processed and analyzed for the required soil fertility parameters following standard procedures (Jackson, 1973). The same soil was used for incubation experiment. The soil was found to be medium in organic carbon (0.65), low in available nitrogen (275 kg ha^{-1}), medium in phosphorus (16.1 kg ha^{-1}) and low in potassium (146 kg ha^{-1}).

Three major organic manures such as vermicompost (V.C), poultry manure (P.M) and neem cake were used in combination with inorganic fertilizers (I.O). The treatments consisted of control (T1), 120 kg N ha^{-1} through V.C (T2), 90 kg N ha^{-1} through V.C (T3), 60 kg N ha^{-1} through V.C (T4), 120 kg N ha^{-1} through V.C+P.M (T5), 90 kg N ha^{-1} through V.C+P.M (T6), 60 kg N ha^{-1} through V.C+P.M (T7), 120 kg N ha^{-1} through I.O (T8), 60 kg N ha^{-1} through I.O (T9), 120 kg N ha^{-1} through V.C+ I.O (T10), 90 kg N ha^{-1} through V.C+I.O (T11), 60 kg N ha^{-1} through V.C+I.O (T12), 120 kg N ha^{-1} through V.C+Neem and 60 kg N ha^{-1} through V.C+Neem. The V.C and P.M combinations were included to study its potential to supply the crop with adequate N during its early growth stages. The neem treatment was incorporated to assess its nitrification inhibiting effect.

The general recommended fertilizer dose of 120, 70, 60 kg ha^{-1} of NPK for the major vegetables grown in these islands viz. okra, brinjal, chilies and cole crops was taken as a reference value to estimate the quantity of organic manures required based on N equivalent basis. Inorganic nutrients were supplied through urea, single super phosphate and muriate of potash. The nutrient composition of the manures indicated that P.M has higher nutrient content than V.C. On an average P.M contains 2.2, 0.8 and 0.97% of N, P and K while V.C contains 1.3, 0.69 and

0.82% of N, P and K.

2.3 Incubation Leaching Study

Nitrogen mineralization was studied using specially fabricated polypropylene bottles. The bottom portion of the bottles were removed and placed in side the bottle in upside down position after making small holes to facilitate aeration and leaching. The mouth of the bottles was plugged with rubber cork attached with 15 cm long drainage pipes. A round piece of nylon cloth was placed in side the bottles above which a pad of glass wool of 2 cm thickness was placed. 100 gram soil and 50 gram acid washed quartz sand were moistened using distilled water and mixed thoroughly. This moistening treatment was given to get homogenous mixture and to prevent particle size segregation during transfer to leaching bottle. The soil quartz mixture was then transferred to the leaching bottles above which again some glass wool was placed to avoid water splash while adding chemicals or water. There were 42 bottles to accommodate 14 treatments and 3 replications. These bottles were placed on a specially fabricated stand and incubated at room temperature with field capacity moisture level. Leaching was performed by adding 100 ml of 0.01 M $CaCl_2$ solutions in 20 ml increment followed by 25 ml of N free nutrient solution ($CaSO_4.2H_2O$ 2 millimoles + $MgSO_4$ + $Ca(H_2PO_4)_2$ 5 millimoles +K_2SO_4 2.5 millimoles).

The soil column in the leaching bottles was leached after 0, 2, 4, 9, 16, 23, 34, 48, 62, 76, 90 and 104th days after incubation and the leachate was collected in a separate polyethylene bottles and the volume was determined using a measuring cylinder. Ammonia and nitrate nitrogen content in the leachate was determined using a nitrogen distillation system (Kelplus). The results were expressed as mg kg^{-1} (mg of nitrogen per kg soil). After every leaching field capacity moisture level was maintained until the next leaching. Similar set of leaching bottles were kept separately for the periodical monitoring of soil pH. The mean monthly minimum and maximum temperature during the experimental period was 22.1°C and 32.5°C, respectively. The relative humidity was high (82.3%) with less deviation (2.8%) typical to the tropical island ecosystem.

It is both conventional and expedient to divide the Method section into labeled subsections. These usually include a section with descriptions of the participants or subjects and a section describing the procedures used in the study. The latter section often includes description of (a) any experimental manipulations or interventions used and how they were delivered-for example, any mechanical apparatus used to deliver them; (b) sampling procedures and sample size and precision; (c) measurement approaches (including the psychometric properties of the instruments used); and (d) the research design. If the design of the study is complex or the stimuli require detailed description, additional subsections or subheadings to divide the subsections may be warranted to help readers find specific information.

Include in these subsections the information essential to comprehend and replicate the study. Insufficient detail leaves the reader with questions; too much detail burdens the reader with irrelevant information. Consider using appendices and/or a supplemental website for more detailed information.

2.4 Statistical Analysis

Analyses of variance were done on all soil parameters, the means of parameters were grouped for comparisons, and differences were separated by least significant difference (LSD) using SAS (SAS Institute, Inc. Cary, NC, USA 1990). Significant differences were determined at $P \leq 0.05$.

3. Results

3.1 Cumulative N Release

The NH_4^+, NO_3^{2-} and total available nitrogen content of the soil depend on the balance between the factors which influence the concentration of these nutrients. The nature of materials and condition of soil are the two most important factors affecting the N release. In the present study it was observed that manures and fertilizers had significantly increased the concentration of mineral nitrogen (NH_4^+ + NO_3^{2-} nitrogen) in soil due to net mineralization during the incubation period (Figure 1). Evidently the amount of N released in to the soil increased with increase in the rate of application of manures and fertilizers. However, amount of release of nitrogen from applied manures and fertilizers varied among the treatments and at different time periods. This was in agreement with the similar reports of Yadvinder Singh, Bijay Singh, and Khind (1992).

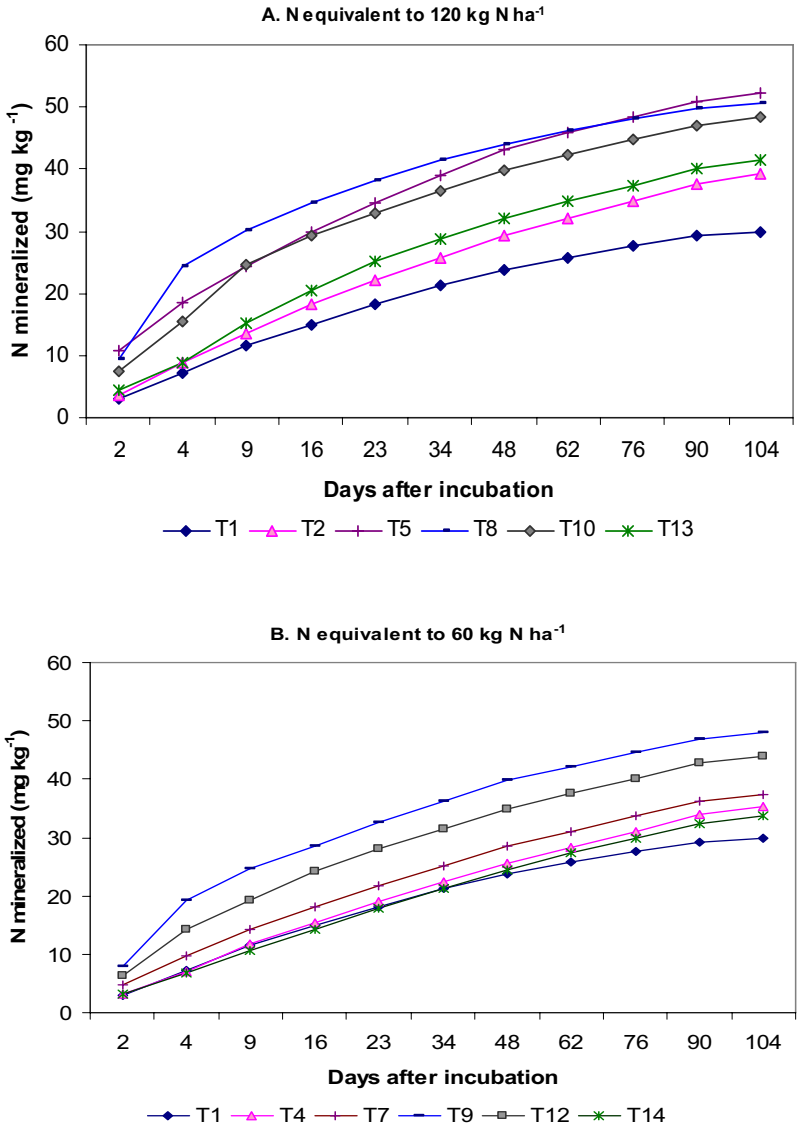

Figure 1. Cumulative N mineralization (mg kg^{-1}) during incubation

Significant increase in the cumulative N mineralization through out the incubation period was observed for inorganic fertilizers, V.C+P.M and I.O+V.C while V.C and V.C+Neem treatments showed significant increase over control only from 16th day onwards. Among the treatments which received 120 kg N equivalent ha^{-1}, V.C+P.M recorded the highest net N mineralization of 52.17 mg kg^{-1} which was at par with inorganic fertilizer and I.O+V.C at the same rate (Figure 1A). Though the net N mineralization in V.C+P.M was lower than the inorganic treatments in the initial 60 days, but it overtook to record the highest cumulative N mineralization at the end of the incubation period. Rayar (1984) also reported an increase in available N when poultry manure and FYM were applied to soil.

Similarly, manures and inorganic fertilizer treatments at 60 kg N equivalent ha-1 also significantly increased the net N mineralization. Among the treatments inorganic fertilizer application at 60 kg N ha^{-1} recorded significantly higher net N mineralization through out the incubation period (Figure 1B). This was followed by I.O+V.C, V.C+P.M and V.C at the same rate. At the same time V.C+Neem recorded slow rate of N mineralization till 34 DAI, there after it increased. But the cumulative N mineralized at the end of the incubation period was lower than inorganic and poultry manure combinations. This indicated the inhibitory effect of neem on nitrification as a consequence N was released slowly but for a longer period of time.

3.2 Rate of N Release

During the mineralization process ammonia is first released into the soil on which the nitrification bacteria acts and oxidizes into nitrate form. More ammonium tends to accumulate if the nitrification process is inhibited on the other hand ammonium content decreases with the increase in the nitrate form (Bartholomow, 1965; Power & Papendick, 1985). Application of manures and fertilizers had significantly increased the rate of N mineralization ($NH_4^+ + NO_3^{2-}$) in the first one week of incubation over the control. In general, with in a week after incubation it reached the peak thereafter it slowed down in all the treatments though at different rate. During the initial one week the highest rate of mineralization was observed for inorganic fertilizer (3.36 mg day^{-1}) followed by V.C+P.M and I.O+V.C at 120 kg N equivalent ha^{-1} (Figure 2A). Thereafter the rate of mineralization from inorganic treatments slowed down where as V.C+P.M maintained the higher rate of mineralization (0.91 mg day^{-1}) upto 48th DAI. Though V.C treatment recorded relatively low rate of mineralization than other treatments, interestingly from 48th DAI onwards it recorded higher rate of N mineralization (0.24 mg day^{-1}) followed by V.C+Neem (0.23 mg day^{-1}) till the end of the incubation period. Similar trend was observed at 60 kg N equivalent ha^{-1} as well (Figure 2B).

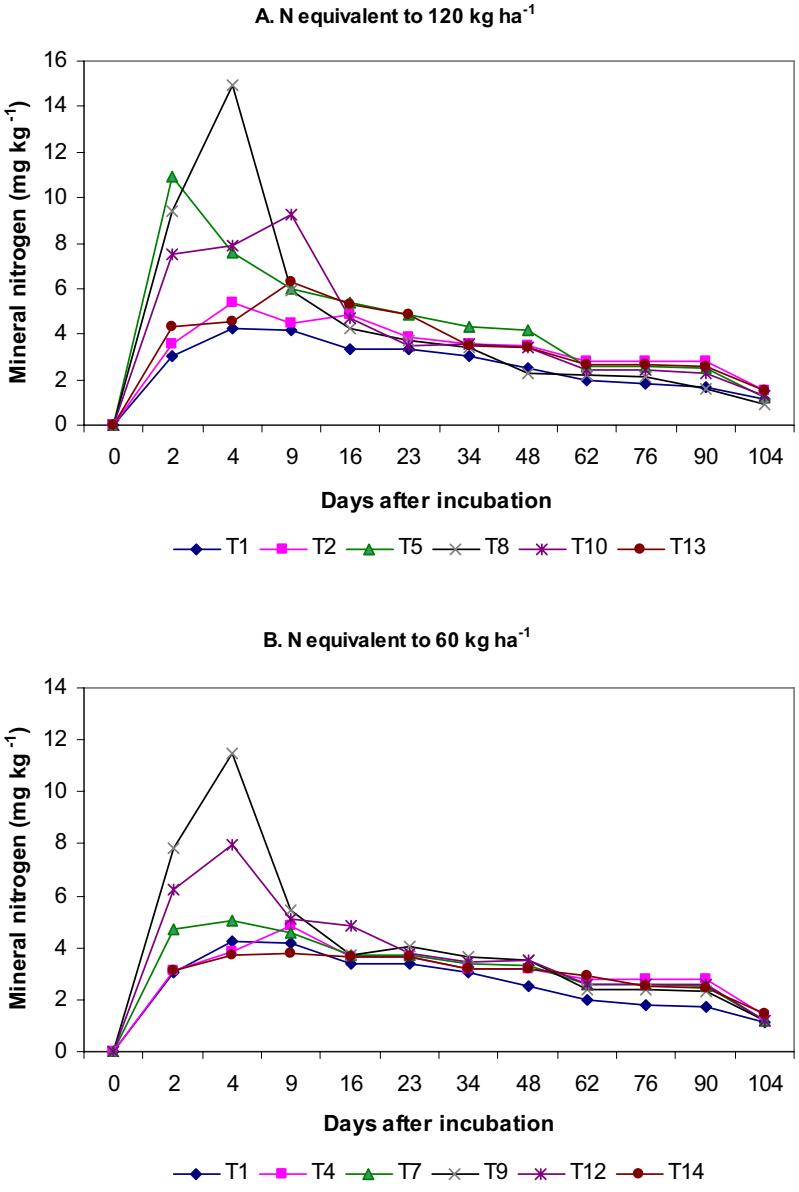

Figure 2. Rate of N mineralization in different treatments

With respect to the release of NH_4^+ N the results indicated that in all the treatments except V.C+Neem, it significantly increased over the control during the first two weeks. But, the rates of increase of NH_4^+ N in different treatments varied with the lowest recorded for V.C+Neem treatment. Similarly manures and inorganic fertilizer treatments significantly increased the rate of NO_3^{2-} N content during incubation indicating its higher availability to crops. The rate of nitrification was by and large followed the trend of release of ammonia from organic and inorganic fertilizers. Among the treatments inorganic fertilizers recorded the highest nitrification during the initial one week after incubation whereas V.C+Neem treatment was observed to be the lowest.

3.3 Available Nitrogen

The total available nitrogen (mineral N) includes initial mineral nitrogen and nitrogen mineralized during the incubation period of 104 days of both NH_4^+ and NO_3^{2-} form. Among these two forms of N, nearly 68% of the total available N was found to be in the form of nitrate indicating the faster nitrification process and aerobic condition. Among the treatments V.C+Neem at 120 kg N equivalent ha^{-1} recorded highest ammonia content of 42.1 % to total available N. Alternatively, higher nitrate content of 82.3% to total available N was observed for V.C+P.M treatment.

The total available N status decreased with the corresponding decrease in the rate of application of manures and fertilizers. I.O+V.C recorded the highest available N of 97 mg kg^{-1} followed by V.C+P.M and V.C+Neem at 120 kg N equivalent ha^{-1}. Significantly higher total available nitrogen was recorded in all the treatments except V.C and V.C+Neem at 60 kg N equivalent ha^{-1} due to slow rate of mineralization. It was also observed that the amount of available N from V.C combination with either P.M or neem has tremendously increased over the sole application of V.C alone at any rate. Amanullah (2007) also showed that poultry manure can increase the available N progressively and make it available to the plants for a longer period of time with other manures.

The results showed that initial N content of the manures and inorganic fertilizer had significant influence on the rate of N release as well as the total N mineralized though the magnitude varied among the treatments. There was a positive agreement between initial nitrogen content and available N from the manures and inorganic fertilizer (R^2 = 0.563). It was observed from Figure 3 that the total available nitrogen steadily increased with the increase in the initial N and reached the maximum at 45 mg kg^{-1} of initial N content. There after the total available N had decreased exhibiting a bell shape of the relationship curve. This was in agreement with the finding of Martin Burger and Venterea (2007).

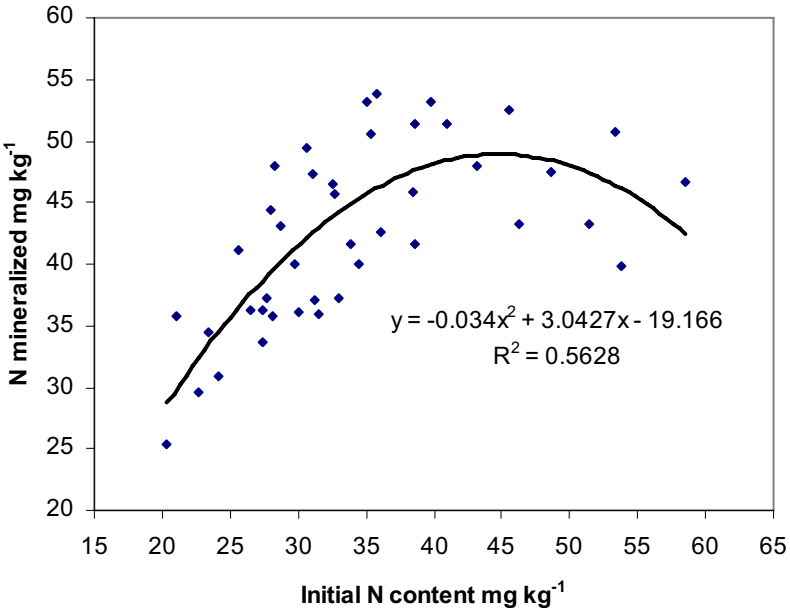

Figure 3. Relationship between initial nitrogen content and mineralization

The best fit equations for cumulative N mineralization in different treatments were derived and given in Table 2. The results indicated that 2nd order polynomial function fits better than logarithmic or linear model under island condition. In general, the model results had indicated that the cumulative mineralized N was the function of initial N content, type of materials and the presence of nitrification inhibitors. It was also seen that the R^2 value was high ($R^2 > 0.85$) for all the treatments. Odhiambo (2010) also reported similar such reports from his study on nitrogen release by green manure in different soil types.

Table 1. Effect of different treatments on mineral nitrogen content

Treatments	Initial N (mg kg^{-1})			N Mineralized at 104 DAI (mg kg^{-1})			Total Mineral N (mg kg^{-1})		
	NO_3^-	NH_4^+	Total	NO_3^-	NH_4^+	Total	NO_3^-	NH_4^+	Total
Control	19.76	3.74	23.50	17.44	12.91	30.35	37.20	16.7	53.85
120 kg N ha^{-1} through V.C	29.13	5.51	34.64	21.29	17.84	39.13	50.42	23.3	73.77
90 kg N ha^{-1} through V.C	27.16	3.64	30.80	22.09	14.74	36.83	49.25	18.4	67.63
60 kg N ha^{-1} through V.C	20.47	3.69	24.16	23.64	11.67	35.31	44.11	15.4	59.47
120 kg N ha^{-1} through V.C+P.M	36.06	5.64	41.70	41.19	10.98	52.17	77.26	16.6	93.87
90 kg N ha^{-1} through V.C+P.M	27.40	4.00	31.40	37.55	12.63	50.18	64.94	16.6	81.58
60 kg N ha^{-1} through V.C+P.M	27.17	3.33	30.50	24.42	13.02	37.44	51.59	16.4	67.94
120 kg N ha^{-1} through I.O	22.68	13.16	35.84	24.01	26.66	50.67	46.69	39.8	86.51
60 kg N ha^{-1} through I.O	24.64	7.28	31.92	23.97	24.07	48.04	48.61	31.4	79.96
120 kg N ha^{-1} through V.C+ I.O	39.47	9.93	49.40	25.66	22.61	48.27	65.13	32.5	97.67
90 kg N ha^{-1} through V.C+I.O	31.90	7.30	39.20	22.21	23.26	45.47	54.10	30.6	84.67
60 kg N ha^{-1} through V.C+I.O	23.37	5.33	28.70	23.47	20.49	43.96	46.84	25.8	72.66
120 kg N ha^{-1} through V.C+Neem	39.76	10.36	54.60	18.73	23.83	42.54	52.47	38.2	90.66
60 kg N ha^{-1} through V.C+Neem	22.14	5.12	27.26	15.24	18.53	33.77	37.38	23.7	61.03
CD (0.05)	4.56	1.46	5.99	4.56	4.56	6.25	7.64	5.58	11.36

Table 2. Best fit N mineralization prediction equations for different treatments

Treatments	Log equations	R^2	Polynomial equations	R^2
Control	y = 12.163Ln(x) - 0.0115	0.971	y = -0.1779x2 + 4.8626x - 1.6497	0.999
120 kg N ha^{-1} through V.C	y = 15.654Ln(x) - 0.8075	0.960	y = -0.1755x2 + 5.6606x - 1.7898	0.999
90 kg N ha^{-1} through V.C	y = 14.739Ln(x) - 1.1888	0.951	y = -0.137x2 + 5.0161x - 1.5327	0.999
60 kg N ha^{-1} through V.C	y = 14.235Ln(x) - 1.4769	0.948	y = -0.1273x2 + 4.7907x - 1.7167	0.999
120 kg N ha^{-1} through V.C+P.M	y = 18.333Ln(x) + 7.0207	0.978	y = -0.2981x2 + 7.6555x + 3.968	0.999
90 kg N ha^{-1} through V.C+P.M	y = 19.384Ln(x) + 2.7848	0.974	y = -0.342x2 + 8.4051x - 1.0724	0.997
60 kg N ha^{-1} through V.C+P.M	y = 14.422Ln(x) + 0.7812	0.961	y = -0.1622x2 + 5.2199x - 0.1287	0.999
120 kg N ha^{-1} through I.O	y = 16.896Ln(x) + 11.011	0.995	y = -0.4319x2 + 8.734x + 5.3589	0.972
60 kg N ha^{-1} through I.O	y = 16.882Ln(x) + 6.8329	0.993	y = -0.3159x2 + 7.4698x + 3.407	0.988
120 kg N ha^{-1} through V.C+ I.O	y = 17.67Ln(x) + 5.3845	0.993	y = -0.3585x2 + 8.1414x + 1.139	0.992
90 kg N ha^{-1} through V.C+I.O	y = 16.94Ln(x) + 3.4798	0.987	y = -0.2827x2 + 7.1316x + 0.647	0.995
60 kg N ha^{-1} through V.C+I.O	y = 16.316Ln(x) + 3.3776	0.982	y = -0.2503x2 + 6.6304x + 1.070	0.997
120 kg N ha^{-1} through V.C+Neem	y = 16.743Ln(x) - 0.3787	0.968	y = -0.2431x2 + 6.677x - 2.6197	0.998
60 kg N ha^{-1} through V.C+Neem	y = 13.627Ln(x) - 1.5433	0.941	y = -0.1104x2 + 4.4654x - 1.5724	0.999

3.4 Soil pH

In general, upon addition of organic manures a slight increase in soil pH was observed except inorganic fertilizer addition till 15th days after incubation (Figure 4) but it significantly differed only from 30th days onwards ($p<0.05$). Among the treatments V.C+P.M recorded higher pH (6.28) followed by V.C+Neem (6.24), V.C (6.20) and I.O+V.C (6.17) at the same time inorganic fertilizer resulted in low pH (5.73). The effect decreased after 90 days after incubation and at the end of 120 days soil pH did not differ significantly except for V.C+P.M (T5). In contrast to the effect of organic manures, inorganic fertilizer resulted in lowering of soil pH though it was non-significant at the end of incubation period.

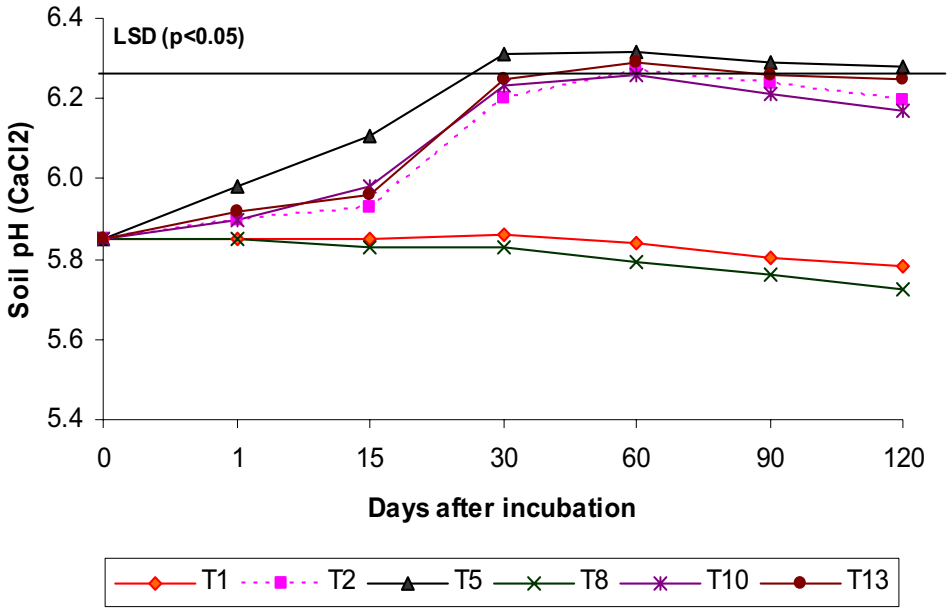

Figure 4. Changes in soil pH during mineralization

4. Discussion

Under the conditions of the investigations, immediately after the addition of manures and fertilizers an increase in the concentration of NH_4^+-N was observed. This rapid rise of NH_4^+-N content is attributable to the decomposition of the easily decomposable nitrogenous substances present in the organic materials. This is in corroboration with the results of Nagarajah (1988) and Gonzalex Priesto, Carballas, Villar, and Carballas (1995). This process is brought about by microorganisms present under various soil and climatic conditions (Power, 1962; Godlin & Olinevich, 1966) and also under anaerobic conditions (Morgham & Ayotade, 1968). Whereas the decrease in NH_4^+-N than control as observed in some of the treatments (T3, T4, and T7) indicated the utilization of NH_4^+ by soil microorganisms or by volatilization losses (Iritani & Arnold, 1960). Also, nitrification brings about a decrease in the concentration of NH_4^+-N. Similarly increase in the concentration of NO_3^{2-} N was observed in several treatments two days after the incubation as a result of the activity of nitrifying bacteria which converts NH_4^+-N into NO_3^{2-} N. As a result of rise in NH_4^+-N or NO_3^{2-} N or both the total available nitrogen has increased.

It was observed from the study that the amount of NH_4^+ and NO_3^{2-} N mineralized significantly differed among the treatments. At the end of the incubation period the highest total NH_4^+ nitrogen (39.8 mg kg^{-1}) was observed in inorganic fertilizer than V.C, P.M and neem treatments. This was due to the fact that application of inorganic fertilizer narrows down the C/N ratio of the soil resulting in higher net N mineralization (Patil, 1990). This indicated a lower rate of nitrification in soils not supplied with any organic materials which may results in ammonia volatilization losses. This was evidenced from the fact that higher NO_3^{2-} content of 82.3% to total available N observed for V.C+P.M treatment. This implies the presence of an easily mineralizable N pool in the manure usually related to less stable organic matter (Laos, Satti, Walter, & Mazzarino, 2000; Preusch, Adler, Sikora, & Tworkoski, 2002).

Neem acting as a nitrification inhibitor when mixed with inorganic fertilizers was widely reported (Santhi et al., 1986; Suganya et al., 2007; Gnanavelrajah, 2011). In the present study it was observed that the NO_3^{2-} N content remains significantly lower than the control in VC + Neem treatment during the initial 10 days indicating that nitrification has been inhibited. A large number of organic compounds are known to inhibit nitrification (Iritani & Arnold, 1960) and it is likely that one or more of these compounds were released during decomposition of neem cake. The NO_3^{2-} N production in V.C+Neem mixture has rapidly increased after the 5th week of incubation (Table 3) indicating that the nitrification inhibitory effect has disappeared after this stage probably due to the decomposition of the inhibitory compound or due to adaptation of nitrifying organisms to the inhibitor. Decomposition of nitrification inhibitors have been shown by Redemann, Martin, Wein, and Widofsky (1965), while Debona and Audus (1970) have reported adaptation of nitrifying organisms to nitrification inhibitors. Thus application of V.C mixed with neem resulted in slower availability of nitrogen but for longer duration and increased N use efficiency under island conditions.

Increased rate of application of N fertilizers and its equivalent organic manures resulted in increased N mineralization which was also reported by Woods, Cole, Porter and Coleman (1987) and Patil and Sarkar (1993). However, lower available N among different treatments receiving same rate of manures and inorganic fertilizer was due to the gaseous losses of nitrogen. The results further indicated that at 120 kg N equivalent ha^{-1} the total mineral N (initial + N mineralized) was highest in I.O+V.C followed by V.C+P.M > I.O > P.M > control. The low total mineral N of inorganic was possibly due to volatilization losses of N in the form of ammonia from inorganic fertilizer. On the other hand higher mineral N of IO+VC were due to favorable soil environment. It is to be noted that VC+PM can supply upto 208 kg ha^{-1} of mineral N which is one par with inorganic and IO+VC, if managed properly this could be best option of nutrient supply for vegetable crops.

The observed increase in soil pH could be due to proton (H^+) transfer from soil to the organic materials which is mainly governed by initial soil pH and the nature of organic materials (Marschner & Noble, 2000). The differences among organic amendments could be due to the release of base cations especially Ca and Mg which resulted in acid neutralization during microbial decarboxylation (Tang & Yu, 1999). In the present study the neutralizing effect of P.M was due to the presence of substantial quantity (2.49%) of calcium which resulted in significant improvement in soil pH. Thus, organic manures acted as an amendment to acid soils which might have improved the N mineralization.

5. Conclusions

The study indicated that manures and inorganic fertilizer increases the mineral nitrogen at different rate and the period of availability varies. Although the mineralization of nutrients from vermicompost, poultry manure is slower than the inorganic fertilizer, the period of availability is longer thereby the loss of nutrients into the environment can be reduced. Similarly V.C mixed with neem resulted in slower availability of nitrogen for longer duration. Thus the nutrient use efficiency of manures was increased and it can be a best organic source to supply nitrogen to the plant for a longer period of time.

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Influence of Soil Chemical Characteristics on the Nutritional Value of Morama (*Tylosema esculentum*) Bean Seed a Potential Crop in Botswana

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Abstract

Morama bean (*Tylosema esculentum*) is found wild in the Kgalagadi sands of Botswana, Namibia and some parts of South Africa. Morama plants were cultivated at Botswana College of Agriculture (BCA) in 2001 in a sandy loam soil which are producing seeds. The nutritional value of seed from a sandy loam soil (BCA) was compared to that of wild plants growing in a sandy soil and collected from Letlhakane in northern central region of Botswana. The effect of seed size on the nutritional value was also assessed. The results showed that ash, protein and potassium (K) contents were low by 12%, 9%, and 20%, respectively, for seeds from a sandy soil compared to those from a sandy loam soil. Calcium concentration was 40% high for seed from a sandy soil compared to a sandy loam soil. Phosphorus was not significantly affected by the soil type. Seed size significantly ($P<0.05$) affected fibre, ash, P, Ca and K contents while moisture, fat and protein were not affected. Soil chemical properties and seed size influenced the nutritional value of morama bean seed. Phosphorus, calcium and potassium fertilizer application to poor soils will improve nutritional value of morama bean seed.

Keywords: morama, seed size, nutritional value, sandy loam, sandy soil

1. Introduction

Morama plants (*Tylosema* esculentum) are found wild in Kgalagadi sands of Botswana and Namibia and some parts of South Africa. The seeds are known to contain 30-39% protein and 34-43% fat (Holse, Husted, & Hansen, 2010; National Academy of Sciences, 1979). Morama was reported to be a non-nodulating legume that depends on soil nitrogen to meet its requirements (Dakora, Lawlor, & Sibuga, 1999). Mitchell, Keys, Madgwick & Lawlor (2005) reported that morama bean has photosynthetic characteristics similar to other C_3 plants. In a field experiment conducted in Botswana, morama did not respond to phosphorus and nitrogen fertilization (Ramolemana, Machacha, Lebutswe, & Tsopito, 2003). The soils in areas where marama grows are relatively poor in mineral nutrients compared to soils where it is not found growing (Ramolemana Machacha, Lebutswe, Tsopito, Mosekiemang, & Tekane, 2007). The plant seems to have capacity to extract nutrients from poor soils to meet its requirement for shoot growth and seed development. The concentrations of N, P, K, Ca, and Mg in morama sprouts of both cultivated and wild plants were found to be the same (Ramolemana et al., 2003, 2007). The seed and young tubers collected from the wild are usually roasted and eaten as snack. Amaerteifio & Moholo (1998) analyzed morama seed collected from Botswana and found it to contain 3.7% ash; 33.5% crude fat; 34.1% crude protein; 4.4% crude fibre; 7760 mg/kg K; 3970 mg/kg P and 1520 mg/kg Ca. In that study, morama seed was found to contain more Ca and P than that of Bambara groundnut (*Vigna subterranean*), mung bean (*Vigna radiate*) and tepary bean (*Phaseolus acutifolius*). Holse et al. (2010) reported that in general morama seed from South Africa had higher content of protein and ash compared to beans from Botswan and Namibia and atttributed the differences to climate and soils. Maruatona, Duodu and Minnar (2010) reported high levels of tyrosine in morama bean flours. They also concluded that due to its high protein contents, morama bean flours could be used to increase the protein quality of cereal based foods to help alleviate protein-energy nutrition in sub-Sahara Africa.

Morama bean can be an important source of protein for the growing population of the world and efforts should be made to grow it commercially. The oil from the seed can also be evaluated for use in automobiles as one of the bio-fuels that can replace the fossil fuels that pollute our environment. In previous studies (Ramolemana et al.,

2003, 2007) plant materials for both planted and wild plants were evaluated and seed material was not evaluated. The objectives of this study were to evaluate the influence of soil type on the nutritional value of morama bean seed; and to evaluate the effect of seed size on the nutritional value of morama bean seed.

2. Materials and Methods

Seed of wild morama plants was collected from Letlhakane (21°34′ S; 25°42′ E) Botswana growing on a Kalahari sandy soil and the other seed was collected from cultivated morama plants at Sebele (24° 33′ S; 25° 54′; 994 m) Botswana growing on a sandy loam soil. The cultivated plants were established in the year 2000 and were fertilized with single super phosphate and urea between year 2000 and 2002 (Ramolemana et al., 2003). Some chemical characteristics of soils where the seeds were collected are given in Table 1 with the sandy loam soil and sandy soil described in Ramolemana et al. (2003) and Ramolemana et al. (2007), respectively. The seeds collected from the sandy soil and sandy loam soil, were a composite from many plants growing on those soil types. The seeds from the two soil types were sorted into three sizes; small (1.0-1.9 g), medium (2.0-2.9) and large (>3.0 g). For each seed size, three replicates of 50 seeds were shelled (de-hulled) and ground in a steel mill through a 2 mm sieve to make a composite sample. The composite samples were analyzed in triplicates. The samples were analyzed at the National Food Technology Research Centre in Kanye, Botswana using the Association of Analytical Chemists (AOAC) Method 2001.

Table1. Some soil chemical characteristics where morama bean seeds were collected

	pH ($CaCl_2$)	P (mg/kg)	Org. C (%Wt.)	Ca (cmol/kg)	Mg (cmol/kg)	K (cmol/kg)	Na (cmol/kg)	CEC (cmol/kg)
Sandy loam	4.72	5.2	0.40	1.29	0.65	0.76	0.17	4.60
Sandy	8.31	0.02	0.02	1.88	0.21	0.08	0.08	4.06

The parameters determined were as follows: moisture using the AOAC 934.01 method; crude fat by AOAC 960.39 method; crude fibre by AOAC 962.09 method (Crude fibre in animal feed and pet food), ash by AOAC 923.03 method; protein by Dumas combustion (AOAC 992.23 method: Crude protein in cereal grains and oilseeds), phosphorus by AOAC 995.11 method; calcium, potassium and iron were by AOAC 999.10 method.

The data was subjected to ANOVA using GLM Procedure of SAS (2004) progamme. Means which were significantly different were separated using LSD.

3. Results

3.1 Effect of Soil Type

The moisture content of seed was significantly ($P<0.0001$) affected by the soil type (Table 2). The moisture content of the seed from the sandy soil was 16% high compared to that from a sandy loam soil. The fat and fibre contents of the seeds were not significantly affected by the soil type.

Table 2. Effect of soil type on the nutritional value of morama bean seed

Soil Type	MC (%)	Fat (%)	Fibre (%)	Ash (%)	Protein (%)	P (mg/kg)	Ca (mg/kg)	K (mg/kg)	Fe (mg/kg)
Sandy[1]	5.80a	41.71a	2.19a	2.98b	37.90b	3427a	1765a	7508b	62.19a
Sandy loam[2]	4.99b	41.18a	1.99a	3.34a	41.26a	3672a	1260b	9033a	75.08a
P-Level	<0.0001	NS	NS	<0.0001	<0.05	NS	<0.0001	<0.0001	NS
LSD	0.28	1.01	0.45	0.05	2.53	491	92.9	472	17.25

Note: 1 = Seed from wild plants; 2 = Seed from cultivated plants; NS = not significant.

NB: Carbohydrates (%) are by computation.

The ash content of the seeds were significantly ($P<0.001$) different (Table 2). The ash content of seed from a sandy loam soil was 12% high compared to that from a sandy soil.

The protein content of the seeds was significantly (P<0.05) different with that from a sandy loam soil high compared to that from a sandy soil (Table 2). The protein content of seed from a sandy loam was 9% high compared to that from a sandy soil.

Phosphorus and iron contents of the seeds were not significantly affected by soil type (Table 2). Calcium and potassium were significantly (P<0.001) affected by the soil type (Table 2). The Ca in seeds from a sandy soil was 40% high compared to those from a sandy loam soil. The K in seeds from a sandy loam soil was 20% high compared to those from a sandy soil.

3.2 Effect of Seed Size

Moisture, fat and protein contents were not significantly affected by seed size (Table 3).

Table3. Effect of seed size on the nutritional value of morama bean seed

Seed size	MC (%)	Fat (%)	Fibre (%)	Ash (%)	Protein (%)	P (mg/kg)	Ca (mg/kg)	K (mg/kg)	Fe (mg/kg)
Small	5.48a	40.73a	2.18ab	3.22a	41.42a	3764a	1743	8059b	67.32ab
Medium	5.47a	41.78a	2.42a	3.15b	38.48a	3746a	1472	8072b	55.98b
Large	5.23a	41.82a	1.67b	3.12b	38.83a	3139b	1323	8680a	82.6a
P-Level	NS	NS	<0.05	<0.01	NS	<0.05	<0.0001	<0.05	NS
LSD	0.35	1.24	0.55	0.06	3.10	602	113.7	578	21.12

NS = not significant.

NB: Carbohydrates (%) are by computation.

Fibre of large seed was significantly (P<0.05) low compared to medium size seed (Table 3). The fibre for medium seed was 45% high compared to large seed.

Ash content for medium and large seed sizes were significantly (P<0.01) low compared to small seed (Table 3). Small seed ash was 3% high compared to large seed.

Phosphorus concentration of the seed was significantly (P<0.05) affected by seed size (Table 3). The P concentration of small seed was 20% high compared to that of large seed. The Ca concentration was significantly (P<0.0001) affected by seed size (Table 3). The seed sizes were significantly (P<0.05) different with the small seed having the highest Ca concentration and large seed the lowest concentration. The Ca concentration of the small seed was 32% high compared to that of large seed. The Ca concentration of the small seed was 18% high compared to that of medium seed.

Potassium concentration was significantly (P<0.05) affected by seed size (Table 3). The K concentration of large seed was 8% high compared to that of small seed.

4. Discussion

Curtin, Syers and Bolan, (1993) observed that the magnitude of the soil pH effect on desorption of nutrient elements depends on the composition of exchangeable cation suite. Specifically, P desorption is less sensitive to pH when cation exchange sites are occupied by Ca ($^{++}$) than when a monovalent cation ($^{+}$) is present. The soil chemical characteristics in this study show that the P concentration of the sandy loam soil was 260 times high compared to a sandy soil and yet there was no significant difference in the P accumulation of seed from the two soil types (Tables 1 and 2). However, the sandy soil had a relatively high Ca concentration compared to the sandy loam soil and this may have been responsible for desorption of P from the soil to meet the P requirements of the seed. The P seed accumulation under low soil P content may imply that morama plants have an efficient P uptake and seed P accumulation mechanism. Morama did not respond to P and N fertilization in a sandy loam soil (Ramolemana et al., 2003). The high P uptake has also been observed for some plants growing in low P soils (Ramolemana, Keltjens, Wessel, & Maphanyane, 2002). The sandy loam soil had a relatively high K (monovalent cation) concentration compared to the sandy soil (high Ca- divalent), and this may have hindered the P desorption from the soil to increase P uptake under the relatively high soil P content. The relatively poor P uptake in the sandy loam soil may be explained by the effect of the relatively high K (monovalent cation) content as reported by Curtin, et al. (1993). This may mean that liming acid soils to increase Ca may benefit morama bean seed and improve seed P accumulation. The seed Ca accumulation from a sandy soil was 40% high compared to that from a sandy loam soil, yet the soil Ca concentration of sandy soil was about 1.5 times that of a sandy loam. The sandy soil had 23.5

times Ca to K while sandy loam soil had 1.7 Ca to K. The high soil Ca concentration in sandy soil increased seed Ca and may have also benefited seed P accumulation which was as high as seed from soil with relatively high P concentration. This has implication on the nutritional value of food products developed from seed from soils high in Ca. The products such as morama milk and flour will be rich in Ca and P (Maruatona et al., 2010)

The sandy loam soil only increased the seed K accumulation by 20% but had 9.5 times high K concentration compared to a sandy soil. This may be an indication that morama plants have the capacity to absorb and accumulate in its seed mineral elements that have a low concentration in soils.

Seed Fe concentration would be expected to be high for sandy loam soil with a pH of 4.72 but there was no significant difference compared to a sandy soil with pH 8.31. Under low pH conditions Fe solubility and availability is usually high and solubility is low under high pH conditions. Therefore morama seed growing in high pH soils is able to accumulate Fe levels similar to those grown on low pH soils where Fe solubility is high. This makes morama seed a very good source of Fe for human beings and other animals. The Fe levels in this study were slightly higher than those reported by Amarteifio and Moholo (1998).

Small seed had significantly high P and Ca concentrations compared to large seed (Table 3). However, the K concentration of small seed was low compared to that of large seed. Small seed, with a high Ca and P concentrations, will make high quality morama food products. It should be noted that small seeds have reduced dry matter accumulation resulting in an increase in the concentration of mineral nutrients in seeds. This was observed in soya bean seed by Samarah, Mullen and Cianzio (2004). Small seed had significantly more fibre and ash than large seed (Table 3). The fibre was 31% high for small seed compared to large seed. Therefore small seed is more nutritious than large seed. Morama plants were found to have low seed rates and breeding for small seeds may improve the seed rate and nutritional value (Ramolemana et al., 2007; Hartley, Tshamekang, & Thomas, 2002).

5. Conclusions

Soil type had significant effect on the nutritional value of morama seed. Phosphorus, Ca and K containing fertilizers will improve the nutritional value of morama bean seed. Small seeds had more fibre, ash, P, and Ca and therefore more nutritious than large seed. Breeding or selection for small seed may improve the nutritional value of morama bean seed.

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Sunki Mandarin vs *Poncirus trifoliata* Hybrids as Rootstocks for Pera Sweet Orange

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Abstract

Obtaining new rootstocks that have resistance to biotic and abiotic factors is one of the main goals of breeding programs for citrus. This study evaluated the performance of 42 hybrids of Sunki mandarin (*Citrus sunki* Hort. ex Tanaka) vs. *Poncirus trifoliata* cv. Rubidoux, as rootstock for Pera sweet orange. The experiment was conducted in Colômbia, São Paulo, Brazil, in randomized blocks with three replications in a spacing of 6.0 m x 3.5 m. The trees were seven years old and the experiment was conducted without irrigation. We quantified the variables height, diameter and canopy volume, and production of plants, besides the physico-chemical analysis of fruits. After the natural period of drought in the region, the trees were evaluated for resistance to drought, with scale ranging from 1 to 3. Plants were also evaluated for symptoms of citrus sudden death and compatibility canopy/rootstock. Differences were observed in height, diameter and canopy volume of Pera sweet orange grafted on 42 hybrids of Sunki mandarin x *Poncirus trifoliata* (TSxPT). Differences were observed in the physico-chemical characteristics of fruits of Pera sweet orange grafted on different hybrids TSxPT. Different degrees of drought tolerance were observed and six hybrids were resistant. The hybrids TSxPT 245 and 254 showed incompatibility with variety of Pera sweet orange.

Keywords: citrandarin, *Citrus sinensis*, genetic breeding

1. Introduction

Hybrids from crosses of Sunki (*Citrus sunki* hort. ex Tanaka) or Cleopatra (*C. reshni* hort. ex Tanaka) mandarins with *Poncirus trifoliata* (L.) Raf. are called citrandarins, in which we want to gather the advantages presented by the mandarins, as lower susceptibility to citrus decline, to exocortis viroid and calcareous soils, with those of *P. trifoliata*, as resistance to citrus tristeza virus, to root rot caused by *Phytophthora*, frost, and induce the formation of smaller plants (Blumer & Pompeu Junior, 2005; Pompeu Junior & Blumer, 2009, 2011).

Today, with the emergence and spread of Huanglongbing (HLB) in the State of São Paulo, Brazil, smaller citrus plants are desirable because they have some advantages over larger ones. Smaller plants may increase the efficiency of inspection and hence the control of pests and diseases, reduce production costs, increase security at harvest, produce more fruit per cubic meter of canopy and allow high planting densities, enabling greater yield per area (Pompeu Junior, 2001; Pompeu Junior & Blumer, 2009).

In the State of São Paulo, most citrus orchards are not irrigated and flowering usually occurs between the months of August and September, a period of lower rainfall, making it necessary to use rootstocks tolerant to drought, an important characteristics of Rangpur lime (*C. limonia* Osbeck), conferring earliness and high yield to canopy varieties grafted on it (Pompeu Junior, 2005), however, the Rangpur lime is susceptible to citrus decline (Rodriguez et al., 1979) and citrus sudden death (Fernandes & Gimenes-Bassanezi, 2001).

Diversification in the use of rootstocks in citrus can overcome abiotic and biotic problems and result in gains in productivity. The use of limited number of canopy / rootstock generated great adversity of brazilian citriculture, such as root rot caused by *Phytophthora* (Alencar, 1941), the citrus tristeza virus (Bittancourt, 1940), the decline of citrus and citrus sudden death.

With so many adversities in citrus, obtaining new rootstocks for diversification in orchards, is one of the main objectives of the breeding programs of citrus. The Centro APTA Citros 'Sylvio Moreira'/Instituto Agronômico is developing a breeding program of rootstocks having as goal to maintain and extend the studies of selection of new rootstocks, either by obtaining hybrids or the use of existing accesses of the Active Germplasm Bank of citrus.

This study aimed to evaluate the performance of 42 hybrids of Sunki mandarin vs *Poncirus trifoliata* cv. Rubidoux as rootstock for Pera sweet orange [*C. sinensis* (L.) Osbeck].

2. Materials and Methods

The work was conducted at Muriti farm property of the Fischer group, in the municipality of Colômbia, São Paulo State, Brazil, geographic coordinates 24º 17' S and 48º 24' W, altitude of 480 meters and climate type aw, according to the Köppen classification (Ortolani, Júnior, & Alfonsi, 1991). The average annual rainfall is 1429.1 mm and the average annual temperature is 23.8°C, with an average maximum temperature equal to 30.7°C and minimum of 17.0°C (CEPAGRI, 2011).

The experiment was arranged in randomized complete block design. It was implemented in 2003, in a spacing of 6.0 m x 3.5 m, initially consisting of 111 hybrids of Sunki mandarin vs *P. trifoliata* cv. Rubidoux (TSxPT) grafted with Pera sweet orange with three replications and conducted without irrigation. Forty-two of these TSxPT hybrids were selected for purposes of evaluations in this work.

The height and diameter of the tree canopy were determined from measurements using a ruler graduated in centimeters. We calculated the volume of the canopy using the function (Mendel, 1956):

$$V = 2/3\ \pi R^2 H$$

Where,

V = canopy volume (m^3);

R = the canopy radius (m);

H = plant height (m).

We evaluated also the fruit yield (kg $tree^{-1}$) and calculated the values of productivity (kg m^{-3}), in the years 2007 and 2010.

For physical and chemical analysis, samples of five fruits were collected of each plant in the second week of October 2009 and sent to the Quality and Postharvest Laboratory of Centro APTA Citros 'Sylvio Moreira'/Instituto Agronômico, Cordeirópolis, SP. Measurements of height (H) and diameter (D) of the fruits were made by direct reading of each sample, with the help of ruler graduated in centimeters, and from these values, we calculated the relation H/D of the fruit. The total mass of the fruits was measured in grams, and the characteristics of juice were obtained as follows: the juice yield was determined after crushing of five fruits of each sample in extractor OIC model OTTO 1800 andcalculated using the relation between juice/fruit mass and expressed as a percentage, the soluble solids (^oBrix)content was obtained by direct reading using refractometer B & S model RFM 330, , the juice acidity was determined by titrating an aliquot of 25 mL of juice to pH 8.2, using a solution of sodium hydroxide (NaOH) with normality of 0.3125 N, and phenolphthalein as indicator. The ratio was calculated using the relation between soluble solids: acidity and the technological indexwas obtained according Di Giorgi et al. (1990) and expressed in Kg of total soluble solids (TSS) per box with 40.8 kg of fruit

In the years 2007 and 2010, after about 90 days of drought in the region of the experiment, the plants were evaluated for tolerance to drought, assigning scores ranging from 1 to 3, and score 1 to plant showing highly susceptibity, score 2 for moderately susceptible plant and score 3 to tolerant plants.

We also evaluated the compatibility between the scion variety (Pera) and each rootstock studied (TSxPT hybrids), identifying incompatible in combinations that had a line of gum or necrosis observed after removal of the bark of the trunk, in the region of grafting (Nauriyal, Shannon, & Frolich, 1958). It is known that Pera sweet orange is incompatible with trifoliate orange and some of its hybrids (Pompeu Junior, 2005).

The resistance of citrus sudden death was evaluated by withdrawing part of the cortex, evaluating the presence of symptoms characteristic of the disease, ie, yellow-orange color in the internal tissues of the rootstock below the graft region, contrasting with the color of the inner tissue of the bark of the canopy. These tissues yellowish correspond for the most part to the phloem vessels that are obstructed and degenerate (Gimenes-Fernandes & Bassanezi, 2001).

For statistical analysis of the studied variables, we used the parametric test Knott Scott (Scott & Knott, 1974), which separates the means by comparisons between groups of data, calculated by means of the statistical program SISVAR (Ferreira, 2008).

3. Results and Discussion

The results showed that the hybrids TSxPT 14, 26, 110 and 137 were those that produce less height (< 2.0 m), smaller diameter (< 2.2 m) and, consequently, lower canopy (≤ 5.0 m^3) for plants of Pera sweet orange evaluated at seven years after planting. These rootstocks can be used for high planting density. Moreover, some hybrids evaluated in this study (TSxPT 92, 132, 142 and 155) induced high vigor to Pera sweet orange plants, with trees exceeding 3.0 m in height, diameter above 3.2 m and canopy volume greater than 17.0 m^3 (Table 1 and Figure 1).

According Pompeu Junior (2005), rootstocks inducing dwarfism are usually selections or hybrids of *P. trifoliata*, like the Flying Dragon, which can be considered a genetically dwarfing rootstock, inducing the formation of mature plants with height less than 2.5 m, even under adverse weather and soil conditions, with or without irrigation. Pompeu Junior & Blumer (2009) reported the induction of dwarfism in Valencia sweet orange plants grafted on citrandarin (Cleopatra mandarin x *P. trifoliata* cv. Christian - 712), where they found plants with an average height of 1.90 m at 16 years.

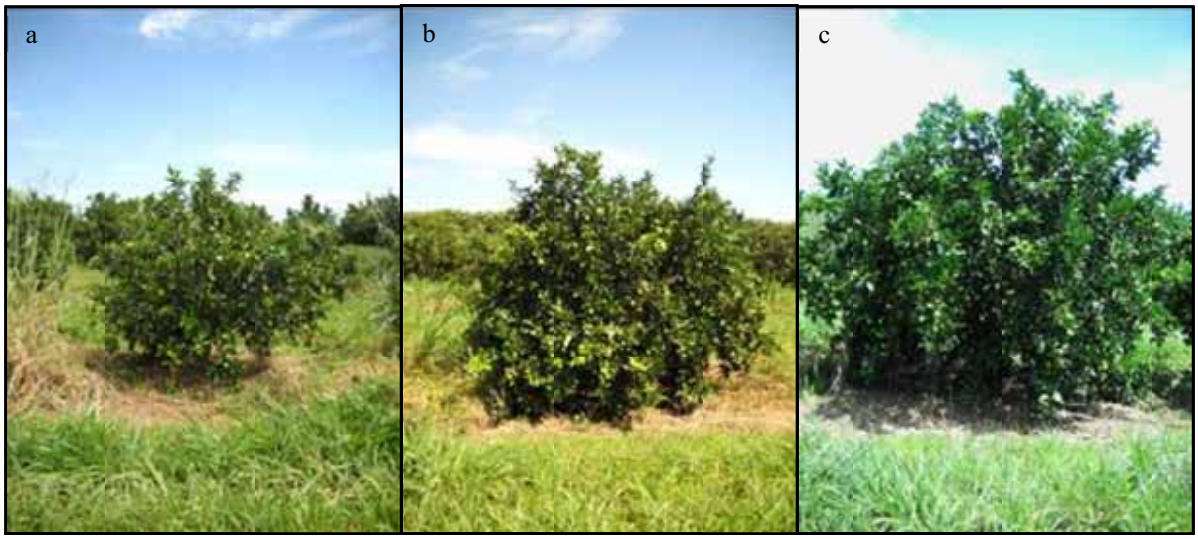

Figure 1. Pera sweet orange grafted on hybrids of Sunki mandarin x *Poncirus trifoliata* (TSxPT) presenting different scion sizesa) Hybrid TSxPT 137 - dwarf (1.97 m high), b) Hybrid TSxPT 106 - half-dwarf (2.50 m high), c) Hybrid TSxPT 108 - tall (2.90 m high) (Colômbia, SP, Brazil, 2010).

Table 1. Height, diameter and canopy volume of plants of Pera sweet orange grafted on hybrids of Sunki mandarin x *Poncirus trifoliata*. Colômbia, SP, Brazil, 2007-2010

Hybrids	Height (m)		Diameter (m)		Volume (m^3)	
	2007	2010	2007	2010	2007	2010
TSxPT 7	1.75 c	2.33 b	1.66 b	2.70 c	2.52 a	9.14 b
TSxPT 14	1.10 a	1.40 a	1.23 a	1.93 a	0.91 a	2.79 a
TSxPT 16	1.73 c	2.50 c	1.55 a	2.35 b	2.29 a	7.27 b
TSxPT 17	1.75 c	2.60 c	1.77 b	2.83 c	3.01 b	10.95 c
TSxPT 23	1.71 c	2.77 c	1.46 a	2.83 c	1.93 a	11.69 c
TSxPT 26	1.34 b	1.80 a	1.46 a	1.93 a	1.53 a	3.57 a
TSxPT 38	1.70 c	2.50 c	1.67 b	2.70 c	2.54 a	9.56 b
TSxPT 42	1.92 c	2.75 c	1.57 a	2.75 c	2.51 a	11.80 c
TSxPT 54	2.34 d	2.95 d	2.05 b	3.10 c	5.14 b	14.94 d
TSxPT 56	1.83 c	2.70 c	1.57 a	2.47 b	2.39 a	8.57 b
TSxPT 68	1.28 b	2.17 b	1.29 a	2.33 b	1.20 a	6.20 b
TSxPT 70	1.78 c	2.33 b	1.53 a	2.20 b	2.40 a	6.54 b
TSxPT 86	2.22 d	2.75 c	2.06 b	2.95 c	4.92 b	12.54 c
TSxPT 92	2.02 d	3.23 d	1.92 b	3.30 c	4.04 b	18.46 d
TSxPT 101	1.82 c	2.80 c	1.76 b	3.00 c	2.99 b	13.19 c
TSxPT 106	1.91 c	2.50 c	1.85 b	2.63 c	3.71 b	9.53 b
TSxPT 107	1.81 c	2.65 c	1.62 a	2.70 c	2.49 a	10.12 b
TSxPT 108	1.98 d	2.90 d	1.82 b	3.20 c	3.60 b	15.59 d
TSxPT 110	1.09 a	1.67 a	1.17 a	1.83 a	0.86 a	2.95 a
TSxPT 119	2.02 d	2.95 d	1.56 a	3.00 c	2.58 a	13.87 c
TSxPT 121	1.56 b	2.25 b	1.52 a	2.60 c	2.09 a	8.32 b
TSxPT 124	2.11 d	2.93 d	1.94 b	2.67 c	4.30 b	11.11 c
TSxPT 126	1.92 c	3.00 d	1.77 b	3.10 c	3.15 b	15.10 d
TSxPT 128	2.10 d	2.90 d	1.53 a	2.55 b	2.63 a	9.91 b
TSxPT 132	2.11 d	3.03 d	1.85 b	3.33 c	3.89 b	17.79 d
TSxPT 136	1.61 c	2.30 b	1.37 a	2.27 b	1.64 a	6.27 b
TSxPT 137	1.16 a	1.97 b	1.39 a	2.20 b	1.16 a	5.01 a
TSxPT 139	1.76 c	2.25 b	1.70 b	2.85 c	3.29 a	10.08 b
TSxPT 142	1.85 c	3.07 d	1.80 b	3.27 c	3.19 b	17.15 d
TSxPT 143	1.90 c	2.45 c	1.79 b	2.50 b	3.21 b	8.02 b
TSxPT 148	1.65 c	2.37 b	1.72 b	2.70 c	2.59 a	9.02 b
TSxPT 152	1.97 d	2.67 c	1.62 a	3.03 c	2.72 a	12.89 c
TSxPT 155	2.03 d	3.13 d	2.07 b	3.40 c	4.63 b	19.28 d
TSxPT 166	2.09 d	2.73 c	1.85 b	2.80 c	3.75 b	11.76 c
TSxPT 168	1.95 d	2.70 c	1.70 b	3.00 c	2.95 b	12.72 c
TSxPT 184	2.01 d	2.77 c	1.97 b	3.03 c	4.18 b	13.30 c
TSxPT 190	1.59 c	2.50 c	1.44 a	2.40 b	1.74 a	7.50 b
TSxPT 196	2.13 d	2.87 d	1.99 b	2.90 c	4.39 b	12.62 c
TSxPT 245	2.04 d	2.63 c	1.53 a	2.93 c	2.51 a	11.88 c
TSxPT 248	2.12 d	2.93 d	1.80 b	3.03 c	3.58 b	14.23 c
TSxPT 254	2.20 d	2.87 d	2.08 b	3.13 c	4.98 b	14.75 d
TSxPT 299	2.31 d	2.50 c	1.76 b	2.77 c	3.80 b	9.95 b
CV (%)	4.75	5.75	4.62	6.93	9.40	16.68

Means followed by same letter do not differ by Scott-Knott test ($P \leq 0.05$).

There were differences in the yield of the plants in both years of evaluation. The highest fruit yield of Pera sweet orange were observed in plants grafted on hybrid TSxPT 155, 142, 54, 92, which had the cumulative production above 135 kg tree^{-1} (Table 2). Cristofani-Yaly et al. (2007) also observed that the hybrid TSxPT 54 was one of the most productive. The correlation between the volume of plant canopy and fruit production in 2010 was 0.68 (P <0.05) (Figure 2), showing that the most productive plants also showed higher canopy volumes. Plant yield was calculated in kg of fruit per cubic meter of canopy and an average of two years of evaluation, the values ranged from 5.0 to 12.4 kg m^{-3} (Table 2).

Table 2. Production, productivity and drought resistance of rootstock hybrids Sunki mandarin x *Poncirus trifoliata* grafted with Pera sweet orange. Colômbia, SP, Brazil, 2007-2010

Hybrids	Production (kg tree^{-1})			Productivity (kg m^{-3})			Drought resistance		
	2007	2010	Total	2007	2010	Mean	2007	2010	Mean
TSxPT 7	25.3 a	42.7 b	68.0 b	10.0 a	4.7 a	7.4 a	1.7 a	2.0 b	1.8 b
TSxPT 14	15.8 a	10.7 a	26.5 a	18.2 b	3.8 a	11.0 b	1.0 a	1.0 a	1.0 a
TSxPT 16	32.0 b	51.8 c	83.8 c	16.3 b	7.4 a	11.8 b	2.5 b	2.0 b	2.3 c
TSxPT 17	36.0 b	57.5 c	93.5 c	12.8 b	5.4 a	9.1 b	2.0 a	1.3 a	1.7 b
TSxPT 23	25.3 a	49.7 c	75.0 b	13.7 b	4.5 a	9.1 b	1.3 a	1.7 a	1.5 b
TSxPT 26	17.2 a	24.0 b	41.2 a	13.9 b	6.6 a	10.3 b	1.0 a	1.0 a	1.0 a
TSxPT 38	17.1 a	27.6 b	44.7 a	7.2 a	2.8 a	5.0 a	1.5 a	1.0 a	1.3 a
TSxPT 42	27.0 a	27.6 b	54.6 b	10.4 a	2.5 a	6.5 a	2.0 a	2.0 b	2.0 b
TSxPT 54	66.0 b	77.7 c	143.7 c	13.0 b	5.3 a	9.1 b	3.0 b	1.7 a	2.3 c
TSxPT 56	27.3 a	39.0 b	66.4 b	11.9 a	4.5 a	8.2 a	2.0 a	1.3 a	1.7 b
TSxPT 68	18.7 a	30.3 b	48.9 b	19.7 b	4.8 a	12.2 b	2.7 b	1.0 a	1.8 b
TSxPT 70	35.8 b	39.0 b	74.8 b	17.2 b	7.7 a	12.4 b	3.0 b	2.3 b	2.7 c
TSxPT 86	42.0 b	41.2 b	83.2 c	8.6 a	3.4 a	6.0 a	2.5 b	2.0 b	2.3 c
TSxPT 92	52.7 b	82.4 c	135.1 c	14.4 b	4.5 a	9.5 b	3.0 b	2.3 b	2.7 c
TSxPT 101	33.4 b	68.7 c	102.2 c	12.0 a	5.3 a	8.6 b	2.7 b	2.0 b	2.3 c
TSxPT 106	39.3 b	69.5 c	108.8 c	11.4 a	7.0 a	9.2 b	2.3 b	1.7 a	2.0 b
TSxPT 107	25.4 a	35.8 b	62.2 b	10.2 a	3.6 a	6.9 a	1.3 a	2.0 b	1.7 b
TSxPT 108	45.9 b	72.3 c	118.2 c	13.2 b	4.7 a	8.9 b	3.0 b	2.0 b	2.5 c
TSxPT 110	14.6 a	9.8 a	24.4 a	16.2 b	3.2 a	9.7 b	1.3 a	1.3 a	1.3 a
TSxPT 119	28.3 a	76.9 c	105.2 c	11.0 a	5.5 a	8.3 b	3.0 b	2.0 b	2.5 c
TSxPT 121	16.5 a	22.8 b	39.3 a	9.6 a	2.6 a	6.1 a	1.7 a	1.0 a	1.3 a
TSxPT 124	43.3 b	78.6 c	121.9 c	11.0 a	7.3 a	9.2 b	2.3 b	2.7 b	2.5 c
TSxPT 126	35.0 b	66.7 c	101.7 c	11.3 a	4.4 a	7.9 a	3.0 b	2.0 b	2.5 c
TSxPT 128	32.3 b	71.0 c	103.3 c	12.8 b	7.2 a	10.0 b	3.0 b	2.7 b	2.8 c
TSxPT 132	30.4 a	90.7 c	121.1 c	8.0 a	5.2 a	6.6 a	2.7 b	3.0 b	2.8 c
TSxPT 136	14.4 a	24.6 b	38.9 a	12.2 a	4.1 a	8.1 a	1.7 a	1.0 a	1.3 a
TSxPT 137	12.8 a	16.0 a	28.8 a	10.7 a	3.2 a	7.0 a	1.0 a	1.0 a	1.0 a
TSxPT 139	37.2 b	73.7 c	110.9 c	13.3 b	7.2 a	10.3 b	1.7 a	2.0 b	1.8 b
TSxPT 142	50.3 b	95.2 c	145.4 c	17.3 b	5.5 a	11.4 b	3.0 b	3.0 b	3.0 c
TSxPT 143	29.5 a	37.9 b	67.4 b	9.7 a	4.8 a	7.3 a	2.0 a	1.7 a	1.8 b
TSxPT 148	19.8 a	37.4 b	57.2 b	7.4 a	4.2 a	5.8 a	1.7 a	1.0 a	1.3 a
TSxPT 152	24.9 a	78.6 c	103.5 c	9.3 a	6.1 a	7.7 a	2.7 b	2.7 b	2.7 c
TSxPT 155	52.5 b	119.6 c	172.1 c	11.2 a	6.3 a	8.7 b	3.0 b	2.7 b	2.8 c
TSxPT 166	30.0 a	60.0 c	90.0 c	8.1 a	5.3 a	6.7 a	2.7 b	2.3 b	2.5 c
TSxPT 168	26.3 a	58.5 c	84.8 b	9.0 a	4.7 a	6.9 a	3.0 b	2.3 b	2.7 c
TSxPT 184	42.7 b	71.0 c	113.8 c	10.5 a	5.3 a	7.9 a	2.7 b	2.3 b	2.5 c
TSxPT 190	29.2 a	46.9 b	76.1 b	16.8 b	6.2 a	11.5 b	2.3 b	1.7 a	2.0 b
TSxPT 196	36.6 b	61.2 c	97.8 c	8.4 a	4.8 a	6.6 a	2.7 b	1.7 a	2.2 c
TSxPT 245	38.2 b	62.9 c	101.1c	15.4 b	5.4 a	10.4 b	3.0 b	2.0 b	2.5 c
TSxPT 248	29.3 a	66.6 c	95.9 c	8.1 a	4.8 a	6.5 a	2.3 b	3.0 b	2.7 c
TSxPT 254	37.1 b	70.8 c	107.9 c	7.5 a	4.9 a	6.1 a	2.7 b	2.7 b	2.7 c
TSxPT 299	35.4 b	84.0 c	119.4 c	9.5 a	8.4 a	8.9 b	3.0 b	3.0 b	3.0 c
CV (%)	20.5	18.0		19.9	19.6		13.3	14.5	

Means followed by same letter do not differ by Scott-Knott test ($P \leq 0.05$).

Significant differences were observed for the variables mass (g), height (cm) and diameter (cm) of fruit, juice yield (%) and number of fruits per 40.8 kg box (Table 3). For the height/diameter of fruits no differences were observed. For fruit mass values were between 180 g (TSxPT 166) and 289 g (TSxPT 121). For juice yield values were between 42% (TSxPT 38) and 56% (TSxPT 299).

Table 3. Physical characteristics of the fruits of Pera sweet orange grafted on hybrids of Sunki mandarin x *Poncirus trifoliata*. Colômbia, São Paulo, Brazil, 2009

Hybrids	Mass (g)	Height (cm)	Diameter (cm)	Ratio H/D	Juice yield (%)	N° Fruits box^{-1} *
TSxPT 7	206.3 a	7.53 a	7.53 b	1.00 a	48.2 b	203 b
TSxPT 14	208.0 a	7.80 a	7.30 a	1.07 a	47.1 b	196 b
TSxPT 16	242.5 b	8.10 b	7.70 b	1.05 a	51.2 c	169 a
TSxPT 17	227.7 a	7.97 b	7.37 a	1.08 a	47.4 b	180 a
TSxPT 23	230.3 a	8.03 b	7.60 b	1.06 a	51.3 c	179 a
TSxPT 26	245.0 b	8.20 b	7.60 b	1.08 a	48.5 b	167 a
TSxPT 38	227.0 a	8.00 b	7.65 b	1.05 a	41.9 a	180 a
TSxPT 42	203.0 a	7.60 a	7.30 a	1.04 a	53.2 d	201 b
TSxPT 54	224.5 a	8.00 b	7.35 a	1.09 a	49.8 c	185 b
TSxPT 56	217.7 a	7.93 a	7.37 a	1.08 a	47.9 b	190 b
TSxPT 68	207.5 a	7.75 a	7.10 a	1.09 a	48.5 b	201 b
TSxPT 70	198.0 a	7.63 a	7.20 a	1.06 a	51.1 c	208 b
TSxPT 86	196.0 a	7.55 a	7.15 a	1.06 a	51.8 c	209 b
TSxPT 92	250.0 b	8.43 b	7.57 b	1.12 a	48.4 b	164 a
TSxPT 101	244.2 b	8.20 b	7.70 b	1.07 a	54.3 d	167 a
TSxPT 106	208.5 a	7.70 a	7.30 a	1.06 a	50.9 c	196 b
TSxPT 107	201.0 a	7.70 a	7.10 a	1.09 a	43.6 a	206 b
TSxPT 108	210.0 a	7.70 a	7.30 a	1.05 a	50.9 c	199 b
TSxPT 110	266.0 b	8.65 b	7.90 b	1.10 a	44.2 a	155 a
TSxPT 119	243.0 b	8.15 b	7.55 b	1.08 a	50.5 c	168 a
TSxPT 121	288.5 b	8.70 b	7.45 b	1.17 a	48.0 b	143 a
TSxPT 124	221.3 a	7.87 a	7.40 a	1.06 a	51.3 c	185 b
TSxPT 126	250.0 b	8.33 b	7.67 b	1.09 a	48.9 b	165 a
TSxPT 128	208.7 a	7.67 a	7.10 a	1.08 a	51.2 c	197 b
TSxPT 132	237.7 b	8.10 b	7.60 b	1.07 a	53.1 d	172 a
TSxPT 136	228.0 a	8.00 b	7.60 b	1.05 a	49.1 b	179 a
TSxPT 137	212.7 a	7.67 a	7.37 a	1.04 a	46.8 b	193 b
TSxPT 139	225.0 a	8.03 b	7.43 b	1.08 a	50.3 c	186 b
TSxPT 142	227.0 a	8.03 b	7.27 a	1.11 a	49.5 c	180 a
TSxPT 143	202.5 a	7.60 a	7.55 b	1.01 a	49.4 c	204 b
TSxPT 148	234.3 b	8.10 b	7.60 b	1.07 a	46.8 b	175 a
TSxPT 152	218.3 a	7.90 a	7.33 a	1.08 a	51.3 c	187 b
TSxPT 155	221.3 a	8.20 b	7.47 b	1.10 a	48.6 b	185 b
TSxPT 166	180.3 a	7.33 a	6.97 a	1.05 a	50.4 c	229 b
TSxPT 168	233.0 b	8.13 b	7.50 b	1.09 a	51.0 c	177 a
TSxPT 184	216.7 a	7.73 a	7.33 a	1.06 a	48.8 b	190 b
TSxPT 190	257.3 b	8.40 b	7.73 b	1.09 a	50.1 c	161 a
TSxPT 196	219.0 a	7.90 a	7.57 b	1.04 a	51.7 c	187 b
TSxPT 245	218.3 a	8.00 b	7.27 a	1.10 a	49.9 c	187 b
TSxPT 248	208.0 a	7.90 a	7.33 a	1.08 a	51.7 c	197 b
TSxPT 254	203.0 a	7.70 a	7.20 a	1.07 a	50.6 c	202 b
TSxPT 299	211.3 a	7.80 a	7.20 a	1.08 a	56.4 d	194 b
CV (%)	10.06	3.95	3.31	2.98	4.08	10.21

Means followed by same letter do not differ by Scott-Knott test ($P \leq 0.05$).

*Box of 40.8 kg of fruits.

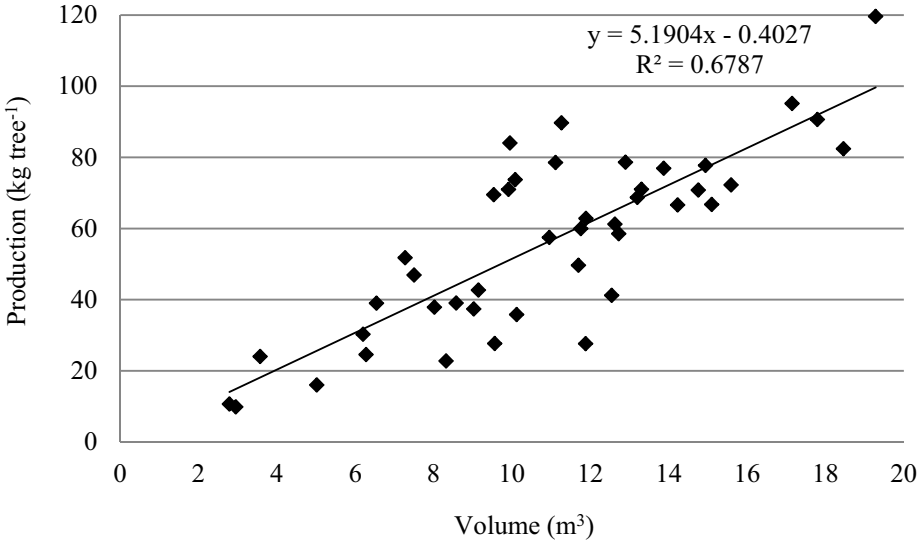

Figure 2. Correlation between production (kg tree^{-1}) and canopy volume (m^3) of Pera sweet orange plants grafted on 42 hybrids of Sunki mandarin x *Poncirus trifoliata* (Colômbia, SP, Brazil, 2010),

We observed significant differences among the rootstocks for acidity, Brix, ratio and total soluble solids per box with 40.8 kg of fruit (Table 4). Di Giorgi et al. (1994) report that for Pera sweet orange, the fruit must be harvested when normally meet acidity levels between 0.6 to 0.9%, achieving this stage largest concentrations of soluble solids and lower rates of degradation of fruit that causes the loss of their commercial value both for fresh fruit market or for processing in the industry. Thus, it was found that during assessment of internal qualities of the fruit (October/2009), the juice acidity ranged from 0.58 (TSxPT 121) to 1.29 (TSxPT 128), and most part of the canopy / rootstock combinations studied were within the standards set for harvest (Table 4).

For the values of ratio, Viégas (1991) reports that the range between 11 and 14 is ideal for industrialization and that these levels the acidity are still adequate to maintain the quality and conservation of juice after processing. In this study the majority of canopy / rootstock combination showed ratio values within the ideal range, however, some hybrids TSxPT (42, 121, 142, 152, 190 and 248) induced early ripening of the fruits of Pera sweet orange, with values above 15.5. Moreover, the hybrids TSxPT 68, 110, 128, 136 and 143 had values of ratio below 10, providing late maturation of the fruits of Pera sweet orange (Table 4).

Table 4. Chemical characteristics of the fruits of Pera sweet orange grafted on hybrids of Sunki mandarin x *Poncirus trifoliata*. Colômbia, São Paulo, Brazil, 2009

Hybrids	Acidity (g 100 mL^{-1})	°Brix	Ratio	TSS (kg box^{-1})
TSxPT 7	0.901 b	11.3 b	14.0 b	2.24 b
TSxPT 14	0.883 b	9.9 a	11.2 a	1.90 a
TSxPT 16	0.779 a	10.6 b	13.5 b	2.19 b
TSxPT 17	0.992 b	9.8 a	10.2 a	1.90 a
TSxPT 23	0.851 a	9.2 a	11.7 a	1.92 a
TSxPT 26	0.973 b	10.0 a	11.4 a	1.98 a
TSxPT 38	1.084 b	10.7 b	10.2 a	1.82 a
TSxPT 42	0.619 a	10.5 b	17.0 b	2.28 b
TSxPT 54	0.711 a	10.6 b	15.1 b	2.15 b
TSxPT 56	0.661 a	10.0 a	15.2 b	1.97 a
TSxPT 68	1.167 b	9.5 a	8.2 a	1.89 a
TSxPT 70	0.914 b	11.0 b	12.9 b	2.29 b
TSxPT 86	0.826 a	11.2 b	13.6 b	2.36 b
TSxPT 92	0.749 a	10.1 a	13.6 b	1.99 a
TSxPT 101	0.608 a	9.3 a	15.3 b	2.06 a
TSxPT 106	0.992 b	10.8 b	11.0 a	2.23 b
TSxPT 107	0.874 b	9.0 a	10.3 a	1.61 a
TSxPT 108	0.764 a	11.1 b	14.9 b	2.31 b
TSxPT 110	1.059 b	10.0 a	9.5 a	1.81 a
TSxPT 119	0.723 a	9.8 a	13.5 b	2.01 a
TSxPT 121	0.579 a	9.5 a	16.4 b	1.87 a
TSxPT 124	0.672 a	10.0 a	14.9 b	2.10 b
TSxPT 126	0.704 a	9.4 a	13.6 b	1.88 a
TSxPT 128	1.289 b	10.5 b	9.4 a	2.21 b
TSxPT 132	0.745 a	10.9 b	14.7 b	2.36 b
TSxPT 136	1.124 b	9.9 a	8.8 a	1.99 a
TSxPT 137	0.875 b	10.0 b	12.8 b	1.90 a
TSxPT 139	0.968 b	9.9 a	11.4 a	2.02 a
TSxPT 142	0.711 a	11.1 b	15.8 b	2.25 b
TSxPT 143	1.231 b	11.1 b	9.8 a	2.23 b
TSxPT 148	0.639 a	9.6 a	15.4 b	1.85 a
TSxPT 152	0.677 a	11.0 b	16.6 b	2.29 b
TSxPT 155	0.764 a	9.8 a	13.3 b	1.96 a
TSxPT 166	0.940 b	10.3 a	12.1 a	2.12 b
TSxPT 168	0.744 a	11.2 b	15.1 b	2.33 b
TSxPT 184	0.870 b	10.0 a	12.5 a	2.00 a
TSxPT 190	0.656 a	9.9 a	15.5 b	2.02 a
TSxPT 196	0.774 a	11.7 b	15.1 b	2.46 b
TSxPT 245	0.736 a	11.1 b	15.2 b	2.27 b
TSxPT 248	0.635 a	10.1 a	16.6 b	2.14 b
TSxPT 254	0.744 a	11.1 b	14.9 b	2.28 b
TSxPT 299	0.970 b	10.4 a	11.7 a	2.38 b
CV (%)	25.32	7.32	20.09	9.74

Means followed by same letter do not differ by Scott-Knott test ($P \leq 0.05$).

For drought tolerance, among the 42 hybrids TSxPT evaluated in the years 2007 and 2010, eight were highly susceptible, with average scores ranging from 1.0 to 1.3, 11 had moderately susceptibility (1.5 to 2.1), and 23 hybrids were tolerante to drought, with scores varying from 2.2 to 3.0 (Table 2). The Figure 3 illustrated two hybrids, one highly susceptible (TSxPT 26) and the other, (TSxPT 299), that is very tolerant to drought. As in 2010 there was a very marked dry season in the region of the experiment, the plants showed low production (Table 2). The correlation between the scores for drought tolerance and the production of plants of Pera sweet orange grafted on hybrids TSxPT was 0.61 ($P < 0.05$) (Figure 4). The more susceptible to drought was the rootstock, less productive was the canopy, Pera sweet orange, due to fruit dropping. In the present study, we found a moderate correlation between plant vigor and tolerance to drought. The correlations between the scores for drought tolerance and plant height or canopy volume were 0.43 and 0.45, respectively. However, the smaller canopy showed the lowest tolerance to drought (Tables 1 and 2). According to Pires et al. (2005), the duration and intensity of drought, initially reduces the size and number of cells and organs, leading to the closure of stomata, reduction of photosynthesis, wilting and drop of leaves and fruit and consequent decrease in production. The architecture of the root system and the ability to explore soil moisture in deep soil layers, along with the relation between scion/rootstock may be an important mechanism of escape to water deficits (Medina et al., 2005). Ford (1954) reports that drought tolerance of citrus grafted on Rough lemon was associated with deeper roots.

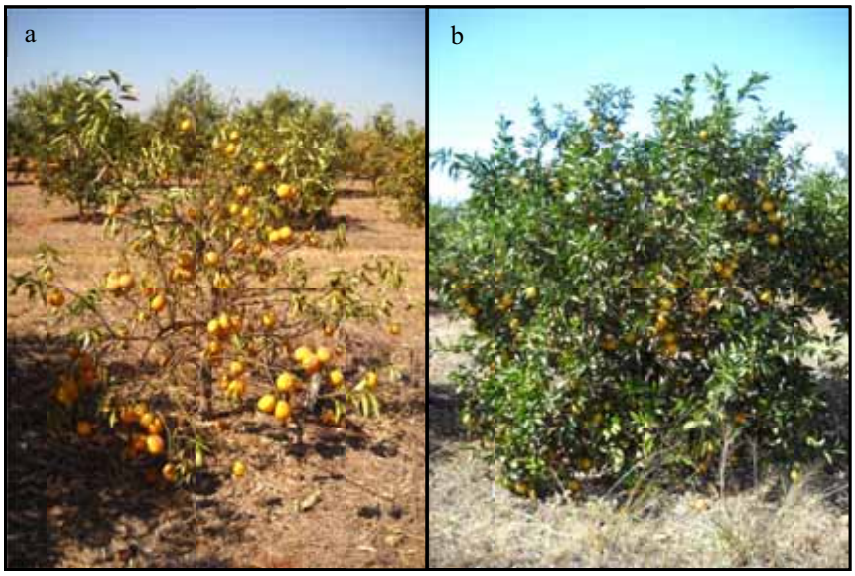

Figure 3. Pera sweet orange grafted on hybrids of Sunki mandarin x *Poncirus trifoliata* showing different levels of susceptibility to drought, after 90 days of a dry season. a) TSxPT 26 - high susceptibility (score 1), b) TSxPT 299 - resistant (score 3). (Colômbia, SP, Brazil, 2010)

It is known that Pera sweet orange presents incompatibility with various rootstocks as *Poncirus trifoliata* and its hybrids, Volkamerian lemon (*C. volkameriana* V. Tennore & Pasquale) and several selections of Rough lemon (*C. jambhiri* Lushington) (Pompeu Junior, 2005). Therefore, this experiment evaluated the compatibility of Pera sweet orange with 42 hybrids of Sunki mandarin x *Poncirus trifoliata* and it was found that two of them (TSxPT 245 and 254) showed the typical symptom of incompatibility (Figure 5), ie, we observed the gum line or necrosis in the region of grafting, similar to that reported by Nauriyal et al. (1958). Cristofani-Yaly et al. (2007) had already observed incompatibility of hybrid TSxPT 245 and 254 with Pera when the plants were four years old.

The characteristic symptom of citrus sudden death, the development of a yellow stain in the phloem part of the rootstock in the region of grafting, observed after removal of part of the cortex (Gimenes-Bassanezi & Fernandes, 2001), was not observed in any of the hybrids TSxPT evaluated in 2010, when the plants were seven years old. In 2007, when the plants were four years old, symptoms of citrus sudden death were also not observed in evaluations by Cristofani-Yaly et al. (2007).

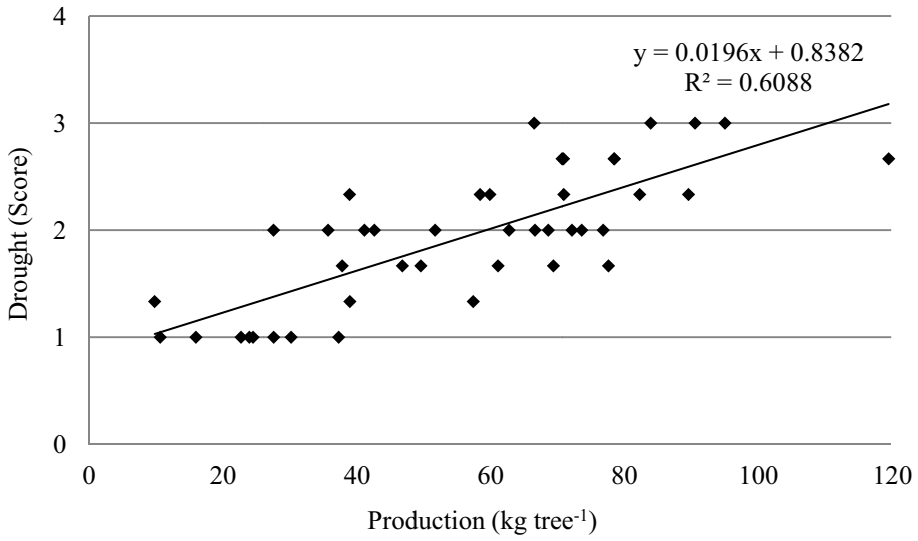

Figure 4. Correlation between the scores of drought and production (kg tree^{-1}) of plants Pera sweet orange grafted on 42 hybrids of Sunki mandarin x *Poncirus trifoliata* (Colômbia, SP, Brazil, 2010)

Figure 5. Symptom of incompatibility in the region of grafting between Pera sweet orange with hybrids of Sunki mandarin x *Poncirus trifoliata* 245 (a) and 254 (b) (Colômbia, SP, Brazil, 2010)

4. Conclusions

There were differences in height, diameter and volume of Pera sweet orange grafted on 42 hybrids of Sunki mandarin x *Poncirus trifoliata* and the hybrids TSxPT 14, 26, 110 and 137 were considered to induce dwarfing to the canopy.

The hybrids TSxPT 155, 142, 54, 92 induced higher production of Pera sweet orange.

Differences were observed in physico-chemical characteristics of the fruit of Pera sweet orange grafted on different TSxPT hybrids.

Different degrees of drought tolerance were observed among the hybrids and TSxPT 245 and 254 showed to be incompatible with Pera sweet orange.

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The Application of Multigamma Radiation as a Physical Mutagen for Breeding of Local Soybean

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Abstract

The general effect of multigamma radiation causes mutation on the all species of plant. The largest effect of multigamma radiation was occurred on genetics factor and chromosome, specific on structure and composition of chromosome and DNA. This case can be used for breeding of several importent plants in the world. The objective of this research is the breeding of local soybean (*Glycine max* L) from Bajawa Flores NTT Indoneisa with aplicaton of multigamma radiation to obtain the primer seed of local soybean with high production and tolerant to dry condition. There were ten (10) varieties of primer seed or superior seed was obtained from mutation by multigamma radiation treatment. The production was revolved between 3.78 tons/ha up to 4.92 tons/ha, with mean production 4.41 tons/ha. The mean production of initial soybean (control) is 2.54 tons/ha, just as the production was increased significantly by 42.40 percent.

Keywords: multigamma, radiation, breeding, mutation, soybean

1. Introduction

Mutation is a changing process on genetics matter of an organism, which spontaneously occurred (called spontaneous mutation) and random, and also through induce mutation (Soedjono, 2003). Those process produce genetics variation as the basic of plant selection (natural or breeding), in order that, the breeder is easy to select of genotive apropriate to purpose of breeding (Gepts & Hancock, 2006; Carsono, 2008). Induce mutation on the plants can be done to reproduction organ of plant like as: seed, cutting stalk, pollen, rhizome, etc. On general, physical mutagen is radioactive source which high energy that produced by nuclear reaction.

There are two categories about theory of radiation effect (Hollaender, 2002): 1) *Target theory* or direct action theory. The biologists qualitatively and quantitatively investigate and explain the cell multiplication and mutation on the organism, and around vital structure or molecular structure. Biologists begin their investigation on outher morphology effect of organism, continuously to sensitive spot and vital structure (Handayani, 2006). Continuously research on citology and genetics obtained the changing in molecular structure; 2) *Indirect action theory.* This theory comes from chemicalists whose begin their investigation about radiation effect on act aspect of molecular and continued to macromolecular which cell composing, like as DNA, RNA, protein, etc for easy explaining of organism mutation. Another effects of nuclear radiation:

1.1 Radiation Effect Ionizes Nucleic-Acid and Nucleo-Protein.

Nucleic-acid and nucleo-protein are important component in chromosome which support characterisric of generation (Hollaender, 2002). Ionization energy disturbs of cell fission and mutation that causes chromosome aberation. Multigamma radiation produces depolarization and viscosity descent on thymonucleic acid (TNA), impedes syntesis of deoxyribo nucleic acid (DNA).

1.2 Radiation Effect to Protein

Multigamma radiation leads the changing of struture and composition of chromosome and DNA on several species of plant. This process leads several forms of mutation on the plant generation which characteristics differenced to initial plant. The small doses of radiation as big as 10 mSv up to 100 mSv cause 1% speed of DNA naturally broken. The several approaches of physics and biology have been done for illustrating of doses limit and speed of

low doses. According to microdosimetry aspect, low doses is smaller than 1 mGy, radiobiology: low doses is 20 mGy, epidemiology: low doses 200 mGy (UNCEAR, 2005).

1.3 Radiation Effect on DNA

DNA structure formed of double helicks which composed from bundle between phosphate group and dioxiribo sugar that form of strand DNA, and bundle between nitrogen bases which connect to two strands DNA. A large parts broken of DNA are broken on bases, bases lost, the bundle between bases has broken, and the bundle of sugar and phosphate has broken, in order that, occurred broken on one strand is called single strand break (*ssb*). This damage can be quickly reconstructed without mistake by enzymatic repairs process with using strand DNA that is not break as mold. Cell can do the contruction process to the broken of DNA in a few hours, but can be not perfect, mainly to the broken of DNA is called double strends breaks (*dsb*) (Brenner, 2006). The reconstruction process with mistaking causes mutaion of abnormality genetics and chromosom. Figure 1, illustrates the broken on DNA as consequnce of radiation.

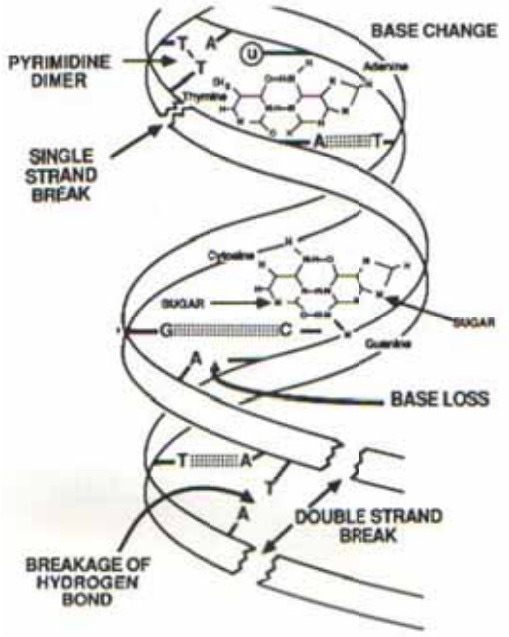

Figure 1. The broken of DNA as consequnce of radiation (Brenner, 2006)

1.4 Radiation Effect on Chromosome

Multgamma radaition causes changing of chromosome structure. Normally, chromosome comprises of upper-arm and fore-arm connected by a centromer. Multigamma radiation causes the forming of: 1) assentric fragment (formed of chromosome fragment without centromer); 2) disentric chromosome (chromosome has two centromers); 3) ring chromosome; 4) translocation (removal of genetic matter betwen chromosome arm) (UNCEAR, 2005).

Mutation on the plant is spontaneously changes of genetic matters in cells caused by (IAEA, 2008): 1) rearrangement occurred on chromosome structure; 2) changing in genetics; 3) segments duplication of chromosome loss. Physical mutagen is mutagen which uses ionizing radiation, like as: alpha ray, beta ray, gamma/multigamma ray, X-ray, neutron, proton, acceletaor particle, etc. Radiation technique has several superiorities among others: 1) radiation technique is easy to do and practical; 2) the change of genotive a few only, but causes much changes of characteristics on generation species; 3) the generation species obtain in the short time.

Dosage standard of gamma/multigamma radiation is used on breeding of plants (IAEA, 2003, 2004, 2006): 1). Mutation on plants: 100 rads up to 3,000 rads; 2). Mutation in seeds plant: 1,000 rads up to 4,000 rads; 3.) Growth stimulation of seeds plant: 250 rads up to 1,000 rads; 4). Growth obstruction on root: 5,000 up to 10,000 rads (NNEA, 2005).

This research focused on development of local soybean from Bajawa Flores with using multigamma radiation technique. This method obtain enable several variations of superior generation variety, in order that is easy to select superior variety. The general characteristics of superior seed of soybean variety are: 1) high production; 2) the age of plant is shorter; 3) tolerant to germ specially viruses (Radiyanto & dkk, 2011); 4) tolerant to plant disease like as agromyza, phaedonia inclusa, lamprosema litura, riptortus linearis, etiella zinkkenella, and nezara viridula (Sunarto, 2003; DP2TP, 2006); 5) tolerant to abiotic conditions like as dry condition (Hartati, 2000); 6) the quality of seed increase (content of protein and fat) (Irwan, 2006; NNEA, 2008). The soybean is the first impotent logumes in Indonesia, which high content of nutrient as a protein sources of concerning plants and low cholesterol, and also the price is reached by the all societies. The soybean requirements on every year in National scale always increase (Amaliyah, 2009). In 2004, the production of soybean in the country only 1,878,898 tons, while requirement of soybean in National scale on this time achieve 2,955,000 tons (Indrawan, 2009). The mean production of soybean in the world this time achieve 1.9 tons per hectare, while the mean production on National scale achieve 1.2 tons per hectare Amaliyah (2009). The last time, soybean production decrease. It's consequence is the goverment to import the soybean as many as 300 thousand tons every year (Arsyad & Syam, 2004).

As the fasting 2018, estimetad that request projection of soybean achieve 6.11 million tons, while in 2003, the production of soybean 672,000 tons only, in 1992, the production of soybean achieve 1.87 million tons (Hilman et al., 2004; Atman, 2006). The commodity production of soybean per hectare in Indonesia is not achieve maximum product. That is influenced by soil factor which damaged and poor of micro-elements, growing hormone, dry conditions, germ, climate, and the using of superior seed (DP2TP, 2006). The soybean as the part of food self-sufficient in Indonesia is a commodity which still low production in the farmer level (Indrawan, 2009).

These research aimed: to develop local soybean variety from Bajawa Flores through breeding with aplication of multigamma radiation method and carefully selection to obtain superior seed with high production, and tolerant to dry condition.

1.5 Several Researches have been Done by Researcher

On 2009, successed to develop local sweet yellow corn and sweet white corn, which tolerant to dry conditions, high calsium and salt. The increase of mean production is 46.20% (from 3-7 tons/ha go up to 11-15 tons/ha) for sweet yellow corn and 40.00% (from 3-6 tons/ha go up to 9-10 tons/ha) for sweet white corn (Pasangka and Jaelani, 2010).

On 2010, researcher successed to develop erect local penaut and creep local peanut from East Sumba with using multigamma radiation. The increase of mean production 43.86% for creep local peanut and 42.22% for erect local peanut, or mean production 5.7 tons/ha (from 3.2 tons/ha up to 5.7 tons/ha) for creep local peanut, production potential 5.9 tons/ha, and 4.5 tons/ha (from 2.6 tons/ha- 4.5 tons/ha) for erect local peanut, production potential 4.7 tons/ha (Pasangka and Jaelani, 2011).

On 2011, continuous research, of perifying obtained production to be revolved between 4.75 tons/ha up to 6.84 tons/ha for creep local peanut, and between 3.95 tons/ha up to 5.45 tons/ha for erect local peanut (Pasangka and Jaelani, 2011).

2. Material Studied

The main instruments used in this research consist of: 1) Multigamma radiation source; 2) counter of radiation doses; 3) protein analyzer; 4) tractor; 5) digital balance; 6) other equipments. The sample for breeding is local soybean from Bajawa, Flores Island Indonesia. There are two groups: control sample and treatment sample.

3. Area Description

The research area located in Kupang West Timor Island Indonesia, at five areas (the name of locations are: Fukdale, Oesao, Baumata, Tabenu, and Bolok). The 5th location is different condition like as high salt and calcium, dry condition, soil struture, and illumination.

4. Methods

The methods of research comprise of: obsrvation/ surveying, sampling, radiation, selection, comparison, and interpretation. Collecting and data analysis are done with observation, measurement, protein analysis on initial soybean and also on generation soybean (mutan). Quality control is done to compare between analysis results of control (initial soybean) and analysis results of generations soybean (mutan). The superiority of this methods is obtained superior seed in short time and many variations of superior seed varieties (is easy to select generation variety or the best superior seed). The development of local soybean in these research, uses multigamma radiation

method. Multigamma radiation method lead of gecetic effect like as the changes of structure and composition of chromosome, and molecule of deoxiribo nucleat acid (DNA) on several species of food plant.

4.1 Procedures of Research

The simple procedures of this research comprise of : 1) to determine research location and choose sample of local soybean; 2) to design example garden; 3) to irradiate samples of soybean seeds; 4) to plant sample of soybean in the area has been prepared; 5) watering if it is necessary; 6) weeding and cultivating; 7) observation to tenacity of germ, growth in dry area, high calsium and salt, and physical characteristics which is needed as a standard comparison, and also select plants. On the resemble harvest, is done selection, measurement of high plant, in time after harvest is measured of mass for a group of 1,000 soybean seeds; 8) to analize protein (service analysis model); 9) drying and selection; 10) For quality control is done comparison between analysis results of control (initial local soybean) and analysis results of generation varieties (mutan); 11) The last procedure is to put insecticide sufficient to superior seeds of soybean and storage for continuously development. The first selection of soybean plant is done since the age of plant is one month, the second selection since the age of plant two months, M_n selection since near to harvest, and the third selection after harvest.

4.2 Observes and Measures

The amount of physical characteristics of soybean (Control sample and mutan) during growth and after harvest was observed and measured like as adaptation, tenacy of germ, grow time, age to be flowered, high of plant, and weight per 1,000 seeds, protein content, potential production, and mean production.

4.3 Research Design

The Soybean sampling was chosen from local soybean. There were two categories of sampling, i.e control sampling (initial soybean) and treatment sampling. The treatment sampling (seeds of soybean) was irradiated by multigamma sources on radiation dosage 3.500 rads. The both sampling was planted in the same time and location. During growth was done observation and three selections (First, second, and third), and after harvest was done one selection. Figure 1, show detail of research design. The first selection of soybean plant was done since the age of plant was one month, the second selection since the age of plant was two months, M_n selection since near to harvest, and the third selection after harvest.

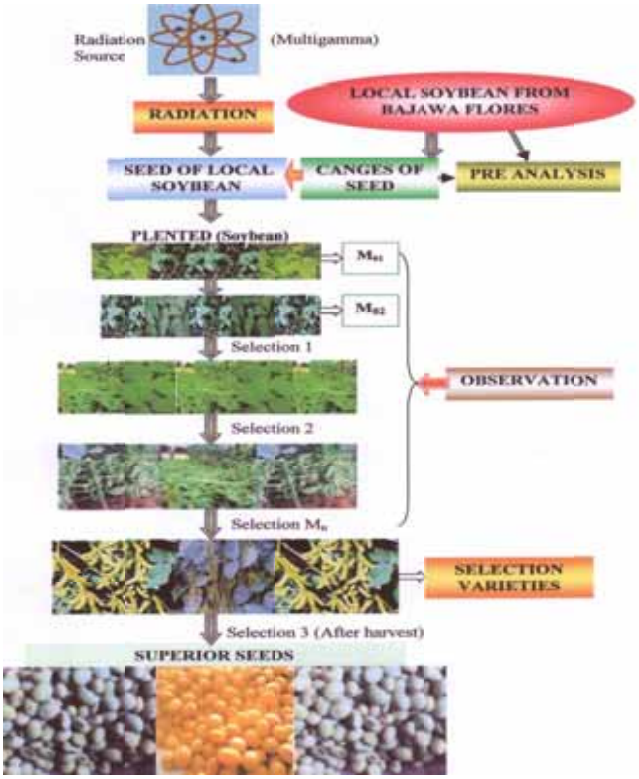

Figure 1. Research design

4.4 Statistical and Data Analysis

Statistical formula was needed to calculated grow percentage and percentage of increasing of mean production. For testing of grow percentage, we choose five sample groups at random on control and treatment sample. The number of test sample is 100 seeds on every group. The number of seeds sample was not grown to be observed. The grow percentage was calculated by equation:

$$GP = \left(\frac{T_{SG} - N_{SN}}{T_{SG}} \right) \times 100\,\% \quad (1)$$

Where: GP is grow percentage (%), T_{SG} is total number of seeds to be planted (prepared sample), N_{SN} is the number of seeds was not grown. The percentage of increasing of mean production was calculated by formula:

$$P_i = \left(\frac{M_P - M_C}{M_P} \right) \times 100\,\% \quad (2)$$

Where: P_i is percentage of increasing of mean production, M_P is mean production of mutan, and M_C is mean production of control. Mean production was calculated by equation:

$$M_P = \frac{P_1 + P_2 + P_3 + P_4 + P_5}{5} \quad (3)$$

Where: P_i, P_2, P_3, P_4, P_5 are production at 5 locations.

4.5 Calculations

Based on equation (1), grow percentage of seeds on control sample and mutan can be determined. Grow percentage of control sample $GP = \left(\frac{T_{SG} - N_{SN}}{T_{SG}} \right) \times 100\,\% = \left(\frac{100 - 18}{100} \right) \times 100\,\% = 82\,\%$.

Grow percentage of mutan $GP = \left(\frac{T_{SG} - N_{SN}}{T_{SG}} \right) \times 100\,\% = \left(\frac{100 - 2}{100} \right) \times 100\,\% = 98\,\%$.

The percentage of increasing of mean production was determined by equation (2), i.e:

$$P_i = \left(\frac{M_P - M_C}{M_P} \right) \times 100\,\% = \left(\frac{4.41 - 2.54}{4.41} \right) \times 100\,\% = 42.40\,\%$$

Mean production of control sample: $M_P = \frac{P_1 + P_2 + P_3 + P_4 + P_5}{5} = \frac{2.78 + 2.47 + 2.41 + 2.49 + 2.65}{5} = 2.54\ tons/ha$.

Mean production of mutan: $M_P = \frac{P_1 + P_2 + P_3 + P_4 + P_5}{5} = \frac{4.92 + 4.87 + 3.96 + 3.78 + 4.53}{5} = 4.41\ tons/ha$

5. Results

5.1 Observes and Measures

The important physical and chemical characteristics were observed and measured of local soybean on control sample and treatment sample (Mutan) included in Table 1, and the production level at the several planting locations of local soybean on control and treatment sample incude in Table 2.

Table 1. The important physical and chemical characteristics were observed, measured, and calculated

No	Description	Control (Initial Variety)	Mutan (Generation Variety)
1	Grow time	7 days after planted (dap)	4 dap
2	Flowered age	62 dap	44 dap
3	The mean High of plant	19.25 cm	24.65 cm
4	Tenacity of germ	not tenacity	Tenacity
5	Adaptation	Adaptation to area with high calcium and salt.	Adaptation to area with high calcium and salt, dry condition
6	Grow percentage	82%	98%
7	Protein content	12.40%	13.92%
8	Mass per 1,000 seeds	172.69 grams	273.77 grams
9	Maximum production potential	3.10 tons/ha	4.92 tons/ha
10	Mean production	2.54 tons/ha	4.41 tons/ha

Table 2. Production level at several planting locations of local soybean (Control and treatment sample)

No	Planting Location (1 hectare)	Control		Mutan	
		Production level (tons/ha)	Mean production (tons/ha)	Production level (tons/ha)	Mean production (tons/ha)
1	Oesao (P_1)	2.78		4.92	
2	Fukdale (P_2)	2.47		4.87	
3	Tabenu (P_3)	2.41	2.54	3.96	4.41
4	Bakunase (P_4)	2.39		3.78	
5	Bolok (P_5)	2.65		4.53	

5.2 Explanation of Figures

The physical growth of control (initial local soybean from Bajawa Flores Indonesia) and mutan (variety of soybean was obtained from mutation by multigamma radiation treatment) were shown on Figure 2 and Figure 3.

Figure 2, shows two examples of control (initial local soybean variety from Bajawa Flores), which are good to grow at area with high calcium, although fruits of this plants are a little. While, Figure 3, shows two examples of mutan (soybean was obtained from mutation by multigamma radiation treatment) , which has meny fruits. On Figure 2 and Figure 3, were show clearly that the fruits of control sample and the fruits of mutan (generation soybean variety) is significantly differenced.

Figure 4, shows one example of control sample (seeds of soybean without treatment), and Figure 5a up to Figure 5e, show 5 examples of mutan (mutan seeds of soybean was obtained from mutation by multigamma radiation (there were 10 varieties). Mutan-1 up to mutan-5 on Figure 5, were obtained by selection. Selection is done appropriate to several important physical and chemical characteristics like as production level, age of plant, mass of seed, seed measuring, high of plant, and flowered age, and other characteristics.

Figure 2. The physical growth of control sample (initial soybean from Bajawa Flores Indonesia)

Figure 3. Physical growth mutan (soybean was obtained from mutation by multigamma radiation treatment)

Figure 4. The seeds of control sample (seeds of initial soybean from Bajawa Flores Indonesia)

Figure 5a. Mutan-1 variety

Figure 5b. Mutan-2 variety

Figure 5c. Mutan -3 variety

Figure 5d. Mutan-4 variety

Figure 5e. Mutan -5 variety

Figure 5. Five examples of mutan seeds varieties were obtained from muation by multigamma radiation

The number of seeds was not grown on the control sample and mutan to be included in Table 3.

Table 3. Data for evaluating of grow percentage (the number of seeds was not grown on the control sample and mutan).

Sample Group	Total number of seeds every group	Control Sample	Mutan
		Number of seeds was not grown	Number of seeds was not grown
I	100	14	3
II	100	17	1
III	100	23	2
IV	100	19	3
V	100	17	1
Mean	**100**	**18**	**2**

6. Discussion

6.1 Mean Production and Another Characteristics

Grow time of control sample (initial soybean) is 7 days after planted, and mutan (generation varieties) is 4 days after planted. Flowered age of control sample is 62 days after planted (dap), and mutan vaieties is 44 days after planted (dap). The range of plant high of control sample is 15.32 cm up to 25.78 cm (mean is 19.25 cm) and mutan varieties is 15.38 cm up to 32.29 cm (mean is 24.65 cm). The control was adapted to area with high calcium dan salt, and the mutan varieties of local soybean was adapted to area with high calcium, salt, and dry condition. The grow percentage of control sample (initial soybean) is 82% and the mutan (generation varieties) is 98%. Protein content of control sample is 12.40% and the mutan is 13.92%. The mean mass of 1,000 seeds of control sample: 172.69 grams, and mutan of soybean: 273.77 grams. The maximum production potential of control sample is 3.10 tons/ha, with mean production 2.54 tons/ha, and generation verieties is 4.92 tons/ha with mean production 4.41 tons/ha. Those data show that physical and chemical characteristics of mutan is superior than control sample (initial soybean). The all characterictics was different clearly. There were ten (10) varieties of primer seed or superior seed were obtained from mutation by multigamma radiation. The improved traits stably inherited in the later generation of those varieties of soybean (10 varietes) will be tested on continuosly research. In other research (Pasangka & Jaelani, 2009) on corn breeding, we found that the generation of corn has the traits stably inherited up to 7 generations, and after that the production to be more decreased.

Figure 5a-5e, show five examples of mutan seeds. Soybean seeds on: Figure 5a tolerant to germ, dry condition and area is high calcium and high salt, Figure 5b tolerant to germ, dry condition, and area is high salt, Figure 5c tolerant to germ and dry condition. Agricultural location in Kupang Timor Island Indonesia was dominated by soil with high calcium, high salt, and dry condition. So, necessary develop plants that can be adapted to those conditions.

6.2 Age of Soybean

On the observation result can be shown that seeds of soybean was irradiated by multigamma radiation grow faster than seeds of soybean without irradiation (Control sample), i.e 4 days after planted, and since the age is 7 days, the growth of sprout is 98%. The plants begin to be flowered since 68 days after planted (dap), and the age of harvest is 94 dap. This case show that production of mutan was obtained faster than initial soybean (control sample).

6.3 Tolerant to Germ

Based on observations from first growth until to harvest time was shown clearly that growth of superior local soybean (mutan) tolerant to germ. This case is shown by soft leaf since growth of soybean.

6.4 The Increase of Mean Production

Based on data in Table 1 and Table 2, can be suggested any arguments that mutan is more quickly powered, more vertile, mass per 1,000 seeds is higher, grow prcentage is higher, adapted to: germ, dry condition, the main production is higher. The mean production of control sample (initial variety) is 2.54 tons/ha, and mean production of mutan (generation variety) is 4.41 tons/ha. This result shows that mean production of mutan of local soybean from Bajawa Flores Indonesia (was obtained from mutation by multigamma radiation treatment) was increased significantly. The increase of mean production is 42.40 percent.

7. Conclusion

From figures and analysis upon can be proposed that development of local soybean from Bajawa Flores Indonesia by multigamma radiation and carefully selection was obtained primer seeds or superior seeds of soybean with physical and chemical characteristics is superior. The production of superior seeds was obtained from mutation by multigamma radiation treatment is 3.78 tons/ha up to 4.92 tons/ha, mean production 4.41 tons/ha, and the increase of mean production is 42.40 percent.

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Influence of Water Regimes and Potassium Chlorate on Floral Induction, Leaf Photosynthesis and Leaf Water Potential in Longan

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Abstract

This study verifies the influence of water regimes and potassium chlorate ($KC1O_3$) on photosynthetic rate, flower emergence and media moisture content of longan trees. The trees were grown in 150 liters lysimeter tanks filled with fine sand. The experimental design was a 2x2 factorial in completely randomized design (CRD) with 2 factors; 1) two levels of water regimes (well-watered and water deficit) and 2) two levels of $KClO_3$ at 10 and 0 g. The results revealed that the well-watered treatment produced faster days of terminal bud break than that of the water deficit treatment. The 10 g $KClO_3$ treatment induced 91 % flower emergence at 35 days after commencing the treatment, while the 0 g $KClO_3$ treatment had 82 % leaf flushing and had no flower emergence. Water deficit or $KClO_3$ treatments reduced the net carbondioxide (CO_2) exchange, transpiration and stomatal conductance rates. Moreover, the combination of well-watered and 0 g $KClO_3$ treatments gave the greatest values of the parameters. The well-watered treatment had higher volumetric water content in the growing medium and leaf water potential than the water deficit treatment, while for the 10 g and 0 g $KClO_3$ treatments had similar the media moisture content.

Keywords: potassium chlorate, water deficit, the net CO_2 exchange rate

1. Introduction

Longan (*Dimocarpus longan* Lour.) is one of the most popular fruit crops of northern Thailand. Longan flowers from late December to late February and is harvested from late June to August. At the present, the main region of longan cultivation is in the upper northern part of the country, such as 'Daw', 'Haeo', 'Bieo Khieo' and 'Si Chomphu'. Floral induction is an important step of flowering and fruiting. Due to the fact that the soil moisture levels and levels of $KClO_3$ used vary from place to place, induction of flowering at certain times is necessary in longan production. There are many factors that control flowering in longan, such as temperature, tree health, cultivar, water stress and potassium chlorate. (Subhadrabandhu, 1990; Manochai et al., 2004; Davenport & Stern, 2005; Sritontip et al., 2005).

Water constitutes a major part of the tissue mass and is required for growth and development. Plant water status is a good indicator of plant health and how well adapted the plant is to its environment. Plant water status can provide information on potential crop yield or be used for irrigation strategy. The water potential of a plant governs transport across cell membranes. Water potential can be used to evaluate the water status of a plant and provides a relative index of water stress. Technological advancements have increased the relative ease and number of variations to measure water potential in plant or plant leaves. Water is the most important factor among the environmental factors affecting growth; it may reduce the growth rate, metabolic activities and leaf area

(Metheneg et al., 1994). Soil water content should be correlated with physiological responses; growth and fruit production in fruit crops, to determine the appropriate amount of water to apply to the crop. Irrigation intervals and water supply for the vegetative growth reduction have counter relationships (Blum, 1996). However, a few studies have indicated that water limitation can have economically beneficial consequences on fruit production (Caspari et al., 1994; Chalmers et al., 1981; Shackel et al., 2000). In Asian pear, water stress reduces vegetative growth but increases return bloom and decreases flesh to dry weight ratio (Caspari et al., 1994; Shackel et al., 2000). A water deficit condition reduces plant growth and affects photosynthesis and that, in turn, reduces leaf area, enhances stomata closure, decreases water status in the leaf tissue, reduces the rate of CO_2 assimilated, causes ultra structural changes in chloroplasts, affects electron transport and CO_2 assimilation reaction and alters the level of photosynthesis in tissues (Dubey, 1997; Fitter, 1987). In 1998, Thai longan growers have begun to apply potassium chlorate ($KClO_3$) to induce off-season flowering, the methods applied potassium chlorate by soil drench, foliar spray and stem injection (Sritontip et al., 2005; Davenport & Stern, 2005; Manochai et al., 2005). However, the Chlorate group chemical is a strong oxidizing agent and considered phytotoxic to all the green parts of plant cell (Stecher et al., 1960; Thomson, 1993). The $KClO_3$ is dissociated into potassium ion (K^+) and chlorate ion (ClO_3^-) when dissolves in water. Chlorate or chlorine is chemically analog of nitrate (NO_3^-) and used widely as a herbicide. The reduction products, chlorite ($C1O_2^-$) and hypochlorite ($C1O^-$) have been shown to be rapidly acting toxins that poisoned all cell types. Root growth is severally inhibited and leaf is yellow, withered and die (LaBrie et al., 1991). It was previously shown that the low temperature and $KClO_3$ applications decreased leaf photosynthesis in longan tree (Sritontip et al., 2010). Although, $KClO_3$ has been extensively used to promote flowering in longan production, the mode of action and mechanism of flower induction is not yet clear. However, the effect of water deficit and/or $KClO_3$ on flower induction of longan is still lacking. Thus, this research studies what effects water deficit and ($KClO_3$) applications have on leaf photosynthesis change and flower induction in longan.

2. Method

2.1 Plant Material and Lysimeter Tank Facility

The sixteen of two-years-old air layered longan trees cv. Daw were grown in lysimeters with a capacity of 150 liters consisting of special containers filled with sand and connected by a tube to a 30 liter plastic container with nutrient solution as indicated in Figure 1.

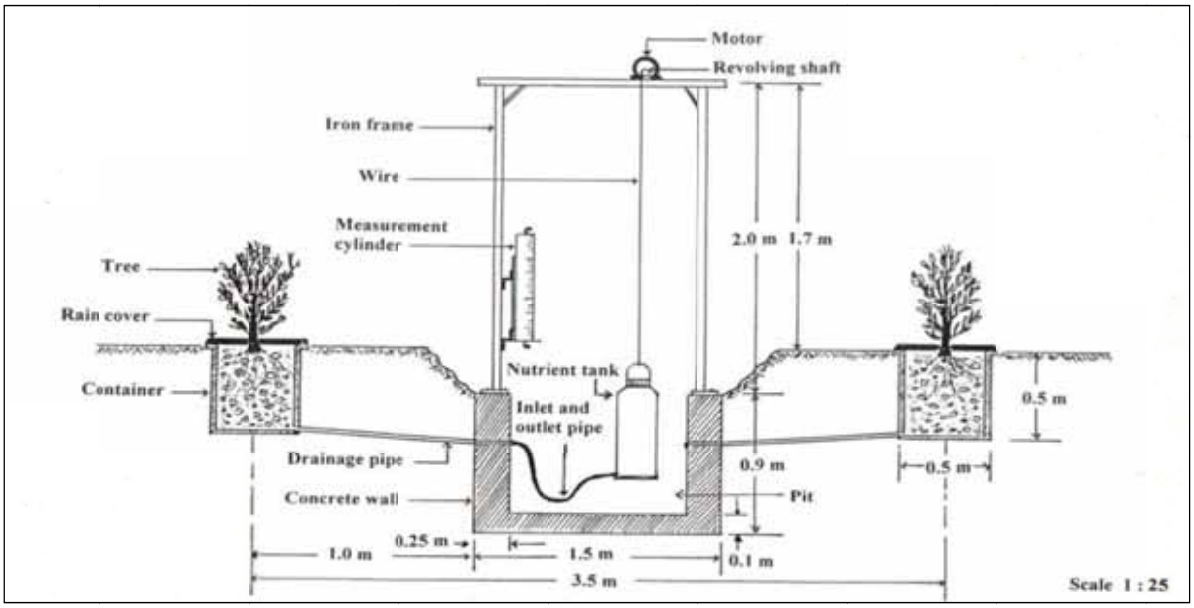

Figure 1. Schematic cross-section diagram of the lysimeter facility

A lysimeter facility was built at the Agricultural Technology Research Institute, Rajamagala University of Technology Lanna, Lampang in 1999 to study the water relationships of fruit crops. For precise measurement of water use by fruit crops in a lysimeter the soil-water status can be controlled more accurately than in the field. The ion concentration of the applied nutrient solution is indicated in Tab 1.

Table 1. Composition of the standard nutrient solution* used for longan trees growing in lysimeters

Cations	meq/l	Anion	meq/l
Ca^{2+}	7.0	NO_3^-	7.0
K^+	4.0	PO_4-	3.0
Mg^{2+}	4.0	SO_4^-	8.0
NH_4^+	1.0		
H^+	2.0		
Total	18.0	Total	18.0

*Micronutrients were added according to [11], the nutrient solution pH was adjusted weekly to 6.5 by addition of H_2SO_4.

2.2 Experiment Treatment

The experimental design was a 2x2 factorial in completely randomized design (CRD) with 4 replications, total of 16 longan tree pots, with water regimes of well-watered (WW) or water deficit (WD) and $KClO_3$ at 10 and 0 g. pot^{-1}. The WW treated plants were supplied daily with a constant volume of water of 30 liters throughout the experimental period. The WD treated plants received a starting amount of water of 15 liters (in the 30-liter capacity container) and were supplied daily until the water container was empty. Then, the container was refilled to the starting volume of 15 liters. The nutrient solution was replaced every 15 day. The longan trees at the fully mature leaf stage were treated with 10 g pot^{-1} $KClO_3$ mixed into the nutrient solution containers. The study was conducted in the lysimeters at the Agricultural Technology Research Institute, Rajamangala University of Technology Lanna, Lampang, Thailand from November 1, 2008 to January 31, 2009.

2.3 Data Collection

1. Percentage of flowering, the sixteenth longan trees were sampled for data. These data were the percentage and days to visible active buds. The data collection lasted 49 days after treatment.

2. Leaf photosynthesis and chlorophyll fluorescence were measured immediately after $KClO_3$ application and then monitored at 1, 4, 7, 10, 13, 17, 21, 28 and 35 days after $KClO_3$ application (4 leaves per tree were sampled). The measurements were made on the 3rd or 4th leaf position of the fully expanded mature compound leaves at 09.00 to 10.00 a.m. Chlorophyll fluorescence (*Fv/Fm*) was measured at the leaf using a plant efficiency analyzer (Model PEA, Hansatech Instruments, UK.). On the same leaf, net CO_2 exchange, transpiration, and stomatal conductance rates were measured using a portable steady-state leaf photosynthesis system at an irradiance of 1,200 μ mol m^{-2} s^{-1} PAR (Model LCA-4 with the PLC-4 leaf chamber; Analytical Development Company Ltd. (ADC), UK).

3. The moisture content in each growing media was measured with a moisture meter (Type HH2, Delta-T Devices Ltd., UK) and profile probe (Type PR2-UM-2.0, Delta-T Devices Ltd., UK) at 1, 4, 7, 10, 13, 17, 21, 28 and 35 days after $KClO_3$ application.

4. Longan trees were measured for leaf water potential by a pressure bomb (Plant Water Status Console-Model 3005, Soilmoisture Equipment Corp, USA)) at 1, 4, 7, 10, 13, 17, 21, 28 and 35 days after $KClO_3$ application.

2.4 Data Analysis

The data was analyzed for statistical significance by using the Statistic 8 analytical software package (SXW Tallahassee, FL). The Least Significant Difference (LSD) was used to compare treatment differences with ANOVA ($P<0.05$).

3. Results

3.1 The Effects of Water Regimes and $KClO_3$ on the Physiology of the Trees

The longan trees that were treated with well watered produced terminal bud break significantly earlier than that of water deficit. Different water regimes had no differences on percentage of floral emergence or leaf flushing measurements after commencement of treatment. Those treated with 10 g $KClO_3$ had greater floral emergence and lower leaf flushing. However, the 10 g and 0 g $KClO_3$ were similar in the days of terminal bud break. There was no interaction between the water regimes and $KClO_3$ treatments on days of terminal bud break, percentage of flower emergence or leaf flushing after treatment (Table 2).

Table 2. Changes in terminal bud break, floral emergence and leaf flushing after start of the treatments

Factors	Days of terminal bud break	Percentage of floral emergence after treatment	Percentage of leaf flushing after treatment
Water (A)			
WW	26 b	47.82	45.89
WD	35 a	43.34	44.95
$KClO_3$ (B)			
10 g	32	91.00 a	8.84 b
0 g.	29	0 b	82.00 a
A	*	NS	NS
B	NS	*	*
A x B	NS	NS	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

3.2 Changes in the Leaf Photosynthesis Characteristics Caused by Water Regimes and $KClO_3$

The water deficit longan trees had efficiency of photosystem II lower at 7, 10, and 28 days after commencement treatment. Moreover, the 10 g $KClO_3$ reduced the efficiency of photosystem II at 4, 7, 10 and 28 days after application (Table 3). The interaction effect showed that the well water with 0 g $KClO_3$ had the highest value for the efficiency of photosystem II (Table 4).

Table 3. Chlorophyll fluorescence (Fv/Fm) changes of 'Daw' longan trees after $KClO_3$ and water regime treatments

Factors	Time after application (days)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	0.69	0.70	0.70 a	0.68 a	0.63	0.69	0.69	0.65 a	0.62
WD	0.66	0.66	0.61 b	0.58 b	0.59	0.63	0.64	0.56 b	0.54
$KClO_3$ (B)									
10 g	0.66	0.64 b	0.61 b	0.59 b	0.58	0.64	0.64	0.56 b	0.55
0 g.	0.69	0.72 a	0.70 a	0.67 a	0.64	0.68	0.69	0.64 a	0.61
A	NS	NS	*	*	NS	NS	NS	*	*
B	NS	*	*	*	NS	NS	NS	*	NS
A x B	NS	NS	*	*	NS	*	*	*	*

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

Table 4. Interaction effect of $KClO_3$ and water regime on chlorophyll fluorescence (Fv/Fm) of 'Daw' longan trees after treatment

Treatments	Time after application (days)								
	1	4	7	10	13	17	21	28	35
WW+ $KClO_3$	0.66	0.66	0.64 ab	0.62 b	0.60	0.65 ab	0.68 ab	0.60 b	0.60 ab
WW-$KClO_3$	0.71	0.75	0.76 a	0.74 a	0.66	0.72 a	0.71 a	0.69a	0.65 a
WD+ $KClO_3$	0.65	0.62	0.57 b	0.55 b	0.56	0.62 b	0.61 b	0.52 b	0.51 b
WD- $KClO_3$	0.67	0.69	0.65 ab	0.60 b	0.62	0.64 ab	0.67 ab	0.59 b	0.57 a
F-test	NS	NS	*	*	NS	*	*	*	*

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

The water deficit treatments decreased the net CO_2 exchange rate during 1 to 35 days after treatment and the 10 g $KClO_3$ had a net CO_2 exchange rate that was lower than the 0 g $KClO_3$ at 4, 7, 10, 13, and 21 days after treatment (Table 5). The interaction effect between water regimes and $KClO_3$ rates showed that the well water with 0 g $KClO_3$ had the greatest effect on the net CO_2 exchange rate of those treatments (Table 6).

Table 5. Effects of water regimes and $KClO_3$ rates on the net CO_2 exchange rate (μ mol M^{-2} S^{-1}) after treatment

Factors	Time after application (days)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	5.58 a	3.95 a	3.90 a	5.12 a	4.75	7.36 a	5.61 a	4.12 a	4.84 a
WD	2.99 b	2.62 b	2.05 b	2.68 b	1.91	3.71 b	3.96 b	2.80 b	3.08 b
$KClO_3$ (B)									
10 g	3.87	2.17 b	1.89 b	2.20 b	2.26 b	4.32	3.82 b	3.06	3.67
0 g.	4.70	4.40 a	4.07 a	5.60 a	4.41 a	6.75	5.75 a	3.85	4.25
A	*	*	*	*	*	*	*	*	*
B	NS	*	*	*	*	NS	*	NS	NS
A x B	NS	*	*	*	*	*	NS	NS	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

Table 6. Interaction effect of water regimes and $KClO_3$ rates on the net CO_2 exchange rate (μ mol M^{-2} S^{-1}) after treatment

Treatments	Time after application (days)								
	1	4	7	10	13	17	21	28	35
WW + $KClO_3$	4.81	2.10 b	2.43 b	3.06 b	3.90 b	5.63 ab	4.40	3.83	4.40
WW - $KClO_3$	6.34	5.80 a	5.38 a	7.18 a	5.60 a	9.08 a	6.82	4.40	5.28
WD + $KClO_3$	2.94	2.24 b	1.36 c	1.34 b	0.62 c	3.00 b	3.25	2.30	2.94
WD - $KClO_3$	3.05	3.00 b	2.75 b	4.01 b	3.21 b	4.41 b	4.68	3.30	3.23
F-test	NS	*	*	*	*	*	NS	NS	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

For changes in transpiration rate, the water deficit had lower transpiration rate than the well-watered at 7 and 10 days after treatment and the 10 g $KClO_3$ treatment depressed the transpiration rate at 4-17 days after treatment (Table 7). The interaction effect between water regimes and $KClO_3$ resulted that the well-watered with 10 g $KClO_3$, the water deficit with 10 g $KClO_3$ and water deficit with 0 g $KClO_3$ treatments decreased the transpiration rate when compared with the well-watered with 0 g $KClO_3$ treatment (Table 8).

Table 7. Effects of water regimes and $KClO_3$ rates on the transpiration rate (m mol M^{-2} S^{-1}) after treatment

Factors	Time after application (days)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	1.09	1.08	0.78 a	1.24 a	1.07	0.97	0.98	0.86	1.04
WD	0.95	0.82	0.43 b	0.90 b	0.97	0.82	0.99	0.63	0.95
$KClO_3$ (B)									
10 g	0.92	0.79 b	0.47 b	0.75 b	0.84 b	0.65 b	0.90	0.66	0.93
0 g.	1.10	1.08 a	0.72 a	1.33 a	1.16 a	1.08 a	1.05	0.82	1.04
A	NS	NS	*	*	NS	NS	NS	NS	NS
B	NS	*	*	*	*	*	NS	NS	NS
A x B	NS	NS	*	*	*	*	NS	*	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

Table 8. Interaction effect of water regimes and $KClO_3$ rates on the transpiration rate (m mol M^{-2} S^{-1}) after treatment

Treatments	Time after application (days)								
	1	4	7	10	13	17	21	28	35
WW + $KClO_3$	1.02	0.90	0.54 b	0.81 ab	0.89 b	0.72 b	0.88	0.62 b	0.94
WW -$KClO_3$	1.17	1.26	1.02 a	1.67 a	1.26 a	1.22 a	1.08	1.10 a	1.15
WD + $KClO_3$	0.82	0.67	0.39 b	0.68 c	0.79 b	0.59 b	0.93	0.70 b	0.93
WD -$KClO_3$	1.09	0.97	0.48 b	1.12 ab	1.14 ab	1.04 a	1.06	0.57 b	0.97
F-test	NS	NS	*	*	*	*	NS	*	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

The water deficit reduced the stomatal conductance at 7, 13, 17 and 28 days after treatment. A similar result was obtained with the 10 g $KClO_3$ where the stomatal conductance declined at 4, 7, 10, 13, 17 and 28 days after treatment (Table 9). The interaction effect between water regimes and $KClO_3$ rates showed that the well-watered with 0 g $KClO_3$ treatment had the greatest stomatal conductance after the beginning of the experiment (Table 10).

Table 9. Effects of water regimes and $KClO_3$ rates on the stomatal conductance rate (mol M^{-2} S^{-1}) after treatment

Factors	Time after application (days)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	0.06	0.04	0.05 a	0.05 a	0.05 a	0.05	0.06	0.03 a	0.05
WD	0.04	0.03	0.02 b	0.03 b	0.04 b	0.04	0.06	0.02 b	0.05
$KClO_3$ (B)									
10 g	0.04	0.02 b	0.02 b	0.03 b	0.03 b	0.04 b	0.05	0.02 b	0.05
0 g.	0.06	0.04 a	0.04 a	0.05 a	0.05 a	0.06 a	0.06	0.03 a	0.05
A	NS	NS	*	*	*	NS	NS	*	NS
B	NS	*	*	*	*	*	NS	*	NS
A x B	NS	NS	*	*	*	*	NS	*	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

Table 10. Interaction effect of water regimes and $KClO_3$ rates and on the stomatal conductance rate (mol M^{-2} S^{-1}) after treatment

Treatments	Time after application (days)								
	1	4	7	10	13	17	21	28	35
WW + $KClO_3$	0.03	0.03 b	0.03 b	0.03 b	0.03 b	0.04 bc	0.05	0.02 b	0.05
WW - $KClO_3$	0.05	0.07 a	0.07 a	0.07 a	0.07 a	0.07 a	0.06	0.04 a	0.06
WD + $KClO_3$	0.02	0.02 b	0.02 b	0.02 b	0.03 b	0.03 c	0.05	0.02 b	0.05
WD - $KClO_3$	0.03	0.03 b	0.04 b	0.04 b	0.04 b	0.06 ab	0.06	0.02 b	0.05
F-test	NS	*	*	*	*	*	NS	*	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

3.3 Changes of Volumetric Water Content and Leaf Water Potential

The volumetric water content in the growing media after treatment showed that the water deficit reduced the moisture value while there was no effect between 10 g and 0 g $KClO_3$ treatment. In addition, there was no interaction among all the treatments (Table 11).

The leaf water potential after the start of a treatment showed that the well-watered had higher leaf water potential than that of water deficit at 10 to 35 days after treatment while there was no effect on that from 10 g and 0 g $KClO_3$. However, there was no interaction effect among treatments (Table 12).

Table 11. Effects of $KClO_3$ and water regime on the volumetric water content of growing medium after treatments

Factors	Volumetric water content (%)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	10.76 a	9.80 a	10.41 a	10.30 a	10.53 a	10.28 a	10.08 a	10.00 a	9.93 a
WD	6.81 b	5.83 b	5.46 b	5.09 b	4.96 b	4.39 b	4.11 b	4.70 b	4.44 b
$KClO_3$ (B)									
10 g	8.70	7.81	7.69	7.20	7.36	6.75	6.38	6.73	6.53
0 g.	8.88	7.81	8.19	8.19	8.13	7.91	7.81	7.98	7.84
A	*	*	*	*	*	*	*	*	*
B	NS	NS	NS	NS	NS	NS	NS	NS	NS
A x B	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

Table 12. Effects of $KClO_3$ and water regime on the leaf water potential of longan trees after treatment

Factors	Leaf water potential (MPa)								
	1	4	7	10	13	17	21	28	35
Water (A)									
WW	-1.22	-1.28	-1.25	-1.04 a	-1.02 a	-1.02 a	-0.95a	-1.01 a	-0.92 a
WD	-1.35	-1.46	-1.50	-1.93 b	-2.21 b	-2.20 b	-2.35 b	-2.26 b	-2.28 b
$KClO_3$ (B)									
10 g	-1.38	-1.38	-1.41	-1.52	-1.65	-1.75	-1.81	-1.77	-1.74
0 g.	-1.23	-1.35	-1.33	-1.45	-1.58	-1.47	-1.49	-1.50	-1.46
A	NS	NS	NS	*	*	*	*	*	*
B	NS	NS	NS	NS	NS	NS	NS	NS	NS
A x B	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Means within the column followed by the same letter were not significantly different at p=0.05 by LSD. NS = Non-significant.

4. Discussion

The full irrigation had faster the days of terminal bud break about 9 days. The well-watered and water deficit treatments gave approximately 43.34-47.82% of all buds flowered and 44.95-45.89% of leaf flushing. The reduction of the water amount had effect on time of terminal bud brake due to water deficiency was a factor that usually causes the limitation of growth and metabolic activity rates of the plant (Boland et al., 1993). Borchert (1994) reported that water deficit inhibited bud break and shoot growth in tropical tree. In the present study, the 91% of all buds flowered at 25–27 days after the application of $KClO_3$. The off-season flowering in longan trees

was induced by $KClO_3$ (Sritontip et al., 2005; Hegele et al., 2008; Davenport & Stern, 2005; Manochai et al., 2005). The $KClO_3$ application induced floral emergence and reduced leaf flushing, whereas the treatments without $KClO_3$ application did not induce flowering. The efficiency of photosystem II (Fv/Fm), leaf net CO_2 assimilation, transpiration and stomatal conductance rates were reduced in water deficit and $KClO_3$ application, except for the application of combination of full irrigation and without $KClO_3$ treatments. In longan trees treated with water deficit, $KClO_3$, and a combination of water deficit and $KClO_3$ leaf photosynthesis decreased because water deficit caused closure of the stomata and reduced CO_2 assimilation and stem extension, leaf expansion, and fruit growth (Flore & Lakso, 1989; Menzel, 2005). The high photosynthesis rate indicated the optimal irrigation management for longan and low value was led to drought stress. The photosynthetic rate of apricot trees daily irrigated to 25% of field capacity was lowered by 55% compared to control trees (100% field capacity), while a 75% reduction in photosynthesis was observed in the rest of water deficit stressed treatments (Ruiz-Sanchez et al., 2000). Diurnal changes in leaf gas exchange in well-water and drought were studied in Tai So litchi trees. Stomata conductance and net CO_2 assimilation reached maximum values at 0700-0800 h, and were lower in drought trees than in the controls for most of the day (Menzel & Simpson, 1994; Menzel, 2005). Water stress decreased the net CO_2 assimilate in papaya (Marler et al., 1994). Induced reduction in net CO_2 assimilated and stomata conductance were also observed in Valencia orange trees (Syvertsen & Lloyd, 1994). In Kensington mango trees effective stomatal closure was reached at -1.2 and -3.0 MPa (Schaffer et al., 1994; Pongsomboon, 1991). The water deficit had a lower leaf water potential in longan trees at 10 days after treatment due to decreasing of moisture content in growing media. The reduction of leaf water potential decreased leaf photosynthesis characteristics in longan tree because water deficit led to decreasing turgor pressure (Akinci & Lösel, 2012). Moreover, Drought conditions are usually associated with a decrease in plant productivity and the course of growth leads to the increase of abscisic acid (ABA) and decrease of indole-3-acetic acid (IAA) and cytokinins (CKs), which may result in the early stoppage of branch growth in comparison with its natural trend (Bradford & Hsiao, 1982; Ferguson et al., 1992)

The treatment with $KClO_3$ induced the longan flowering process and could be used for off-season longan production. The mechanism of how $KClO_3$ induces the flowering process in longan is not entirely understood. Some researches claimed that in plants, the chlorate (ClO_3^-) ion competitively inhibited the nitrate reductase enzyme and is reduced to chlorite (ClO_2^-) and hypochlorite (ClO^-) (Duke, 1985; King, 1974). Furthermore, the reduction products chlorite ($C1O_2^-$) and hypochlorite ($C1O^-$) were shown to be rapidly acting toxins that poisoned all plant cell types (Aberg, 1947). It was previously shown that $KClO_3$ application also decreased chlorophyll fluorescence and leaf gas exchange (Sritontip et al., 2010), the leaf photosynthesis considerably decreased up to 6 days after $KClO_3$ application and remained rather low compared to the control up to 11 days (Hegele et al., 2008), consequently, the detrimental effects of $KClO_3$ on the plant's photosynthetic system could be caused by the phytotoxic effect of ClO_2^- and ClO^-. Thus, water deficit and $KClO_3$ treatments seemed to be the inhibiting factors of the photosynthetic efficiency.

Floral initiation in longan is dependent on cool temperature and some chemicals treatment. However, the leaf photosynthesis was reduced during flower induction stage by low temperature and $KClO_3$, whereas the longan trees can induce flowering after treatments, which probably account for the depression in leaf photosynthesis rate reposed during the floral initiation in subtropical tree species (Sritontip et al., 2010; Hegele et al., 2008).There is considerable evidence for the regulatory role of plant hormones controlling floral induction, particularly in perennial fruit trees. It has been reported for trees that an increase of CKs stimulates flower induction, while high levels of gibberellic acids (GAs) and IAA result in inhibition (Bangerth, 2009). Furthermore, it was found that CKs concentrations in terminal buds of longan increased, whereas IAA concentrations reduced during the first fourteen days after $KClO_3$ application and GAs also decreased at twenty days after treatment (Hegele et al., 2008).

Although, water deficit and $KClO_3$ decreased leaf photosynthesis, water deficit treatment reduced media volumetric water content and leaf water potential, while both $KClO_3$ concentrations did not significantly affect these parameters.

5. Conclusion

The water deficit delayed time of terminal bud break by 9 days, while, the full irrigation and water deficit treatments gave similar flowering and leaf flushing percentages. Whereas, the longan trees in treatments without $KClO_3$ application did not have floral emergence. The efficiency of photosystem II (Fv/Fm), leaf net CO_2 assimilation and transpiration rates, and stomata conductance were reduced in water deficit and with $KClO_3$ treatments. The volumetric water content and leaf water potential declined with the water deficit treatment, while there was no difference between 10 g and 0 g $KClO_3$.

Although water deficit impacted on the efficiency of photosystem II (Fv/Fm), leaf net CO_2 assimilation and transpiration rates, and stomata conductance; it did not affect off–season flower induction by $KClO_3$. Therefore off-season production during dry season or under controlled deficit irrigation seems to be feasible, at least, in terms of how effective $KClO_3$ is as a flower inducing agent. Irrigation management during further fruit development still requires detailed investigation in order to optimize yield and quality of off-season longan fruit.

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Loan Repayment Performance of Public Agricultural Credit Agencies: Evidence from Jordan

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Abstract

The agricultural production process in many developing countries has been negatively impacted by poor loan repayment. Most of public credit agencies in such countries suffer from this critical problem. This study aimed at evaluating the loan-repayment performance of public agricultural credit agencies. The Agricultural Credit Cooperation (ACC) in Jordan was chosen to be investigated. To achieve its goals, the study delved into the determinants of loan-repayment performance among ACC borrowers. Data from ACC sources for the period of Financial Year 1960 to Financial Year 2011 (52 years) were analyzed. Simple descriptive statistics tests and regression techniques were conducted. Factors related to the accessibility of farmers to credits, to the collection performance of ACC, and to the administration performance of ACC were included in the analysis. The results of the study revealed that the repayment rate of the investigated public credit agency (ACC) was 0.92, indicating a high level of repayment performance and a low default rate (0.08). The positive effects of the volume of loans borrowed, volume of loans repaid number of borrowers, number of credit agency staff, and borrower experience were the most important factors related to this result. Sufficient and strict controls as well as monitoring are required. Outreach to beneficiaries should be improved to enhance repayment performance. To avoid the burden of provisioning on agricultural credit agencies, legal actions and guarantees should be taken against loans defaulters. Introducing a reward system for those individuals who pay on time will be helpful in enhancing repayment performance.

Keywords: public agricultural credit, loan, repayment performance, repayment rate, default rate

1. Introduction

Depending on its production, in a developing country such as Jordan the agricultural sector assumes greater importance. The public agricultural credit activities are a major factor in determining the trends of this production. It is more useful to examine the validity and viability of public agricultural credit agencies as agricultural development instruments.

A critical problem most public credit-lending agencies face is poor loan repayment. This problem has negatively affected agricultural producers who need to obtain capital for their operations (Njoku & Obasi, 2001). Several studies investigated the importance of the credit facilities in less-developed countries. These studies concentrate on the effects of providing a large amount of money in the form of agricultural loans on the agricultural sector growth (FAO, 1996; Adams & Graham, 1981; Gonzalez, 1977; Pischke, 1980). An efficient utilization of agricultural credit is necessary to enhance the agricultural sector's productivity and, hence, the national economy (Yasir et al., 2012).

Public agricultural credit activities in many developing countries suffer from the problem of a high incidence of default rate among borrowers. Many of these credit agencies are inefficient or heavily subsidized to remain in business. In order to understand the reasons behind this problem, it is crucial to evaluate the agricultural credit agencies based on borrowers' repayment performance. It is important to accomplish this evaluation because a low repayment rate will reduce the volume of loanable funds to offer other borrowers, create a longer time for loan recovery and lower profitability (Timothy & Olatomide, 2010). Loan-repayment performance is largely affected

by factors related to the borrower, the firm itself, the loan, and the lender. Among these factors, many studies concentrate on the borrower as the core of the problem. Most of these studies stated that, when the loan is not paid, it might be a result of the borrowers' unwillingness and/or inability to repay (Greenbaum & Thakor, 1995; Hoque, 2000; Colye, 2000; Ozdemir & Boran, 2004).

Unstable prices or agricultural inputs and outputs, interest rates, and the borrowers' social relations and responsibilities may influence the credit repayment-performance of the lending agencies. The negative effect of these factors may lead to the failure of these agencies (Mohammed, 2005). According to this situation, lending agencies should categorize the borrowers as good borrowers and bad borrowers. Monitoring the borrowers will aid in making sure that they are using the loans for the right purposes meaning that they can pay back their loans (Stiglitz & Weiss, 1981). Looking at the borrowers' past record is another criterion to determine if the borrower is likely to repay the loan or not (Greenbaum & Thakor, 1995). Borrowers with no training related to their agribusiness have a higher possibility to default (Roslan & Zaini, 2009). The lending firm characteristics may also affect their repayment performance. (Oke et al., 2007; Nannyonga, 2000; Arene, 1992). A firm's Poor management procedures may contribute to most of the default. The design of the loan, access methods, screening methods, and incentives to repay may largely affect the lending agencies repayment performance (Hulme & Mosley, 1996). The loan volume may be another issue to discuss. Awunyo (2012) stated that the larger the loan size, the lower the probability of repayment default. A Poorly designed lending program and improper implementation may lead to defaults (Copisarow, 2000). To minimize the loan default in the process loan repayment, both the borrowers and the institutional characteristics are important and should be taken into account (Derban et al., 2005).

In Jordan, the Agricultural Credit Cooperation is a major formal source of agricultural credits. Farmers are its main target group. The total value of the loans provided to farmers through this cooperation by the end of 2011 was nearly 500 million Jordan Dinars (JD), which is around 700 million US Dollars (USD); (1 JD = 1.4 USD) and benefited nearly 215,000 farmers (ACC, 2011). The amount of the loans introduced to the farmers by ACC has increased in recent years, but the number of loans, on the other hand, has decreased despite the increased values (Rashrash, 2004).

The present study is an attempt to assess the repayment performance of the public agricultural credit agencies as well as the repayment performance of the borrowing farmers who has received agricultural loans from these agencies. Jordan's Agricultural Credit Cooperation (ACC) is the agency studied. The study also tries to investigate some important factors related to loan repayment performance. Drawing lessons from the ACC's experience and making recommendations are other objectives for the study.

2. Materials and Methods

2.1 Data

To achieve the goals of this study assessing the repayment performance of public agricultural credit agencies as well as the repayment performance of the loanee farmers, secondary sources of information were the main data sources. Due to the availability and accessibility of required data from their reliable sources, these secondary sources were preferred. It is difficult to collect primary data from all borrowers who received loans from Jordan's Agricultural Credit Cooperation (ACC) over a long time of more than 50 years (since 1960). Hence, secondary data based on annual reports from the Agricultural Credit Cooperation (ACC) were the main source of data applied in this study. Meanwhile, other secondary sources such as the Department of Statistics (DoS), the Ministry of Agriculture (MoA), and the Agricultural Directorates (ADs) in the governorates were helpful. These available sources confirmed data for the period of Financial Year 1960 to Financial Year 2011 (52 years). Because a broad range of farm sizes and enterprises was included, the data gave a good representation of the borrowers' characteristics throughout Jordan. Table 1 shows the main credit related items of the ACC since 1960. Loans are in Jordanian Dinars (1 JD = 1.4 USD).

Table 1. Main credit-related items of the ACC since 1960

Year	Volume of loan borrowed (JDs)	Volume of loan repaid (JDs)	Number of borrowers	Number of staff
1960	457680	80500	4242	115
1961	470196	150718	2000	127
1962	1036306	150028	2688	158
1963	902802	473896	2938	197
1964	833471	707835	3333	215
1965	1206990	869414	2049	214
1966	1293935	636221	2147	213
1967	675440	695608	1331	182
1968	747458	573021	1350	217
1969	467328	922153	833	217
1970	437705	500556	886	215
1971	710019	606248	1616	206
1972	1439351	1031623	1213	207
1973	1843519	1078099	2448	207
1974	2139375	1509987	1617	214
1975	3190479	2351309	1633	216
1976	2792022	2074318	1029	223
1977	2366753	2267215	815	221
1978	3224883	2794577	705	221
1979	3466360	2707104	1450	213
1980	4855184	3473554	740	211
1981	6793872	4241738	889	218
1982	6287454	4426914	937	240
1983	5605485	5235767	956	215
1984	5467559	5718727	1438	224
1985	7930299	5916410	1910	234
1986	5682638	5508608	1453	229
1987	5145023	4298174	1913	226
1988	4750785	3942975	1865	227
1989	4954498	5375840	1687	230
1990	7404847	6216038	4745	237
1991	10527668	6511604	3133	255
1992	32388507	7505174	7952	264
1993	18909314	12010890	8759	302
1994	14307265	12337558	4989	350
1995	19345227	15236697	6391	379
1996	21188319	18242230	7627	400
1997	16797331	19876409	6174	402
1998	19680900	18726206	7248	419
1999	27368944	22978324	10003	424
2000	20891375	24667207	8260	423
2001	13376951	20737899	4166	417
2002	17199522	19452318	4669	442
2003	13206155	22151404	3854	443
2004	10991096	23196712	3761	443
2005	16266946	26258937	4462	455
2006	19387065	27800000	4809	474
2007	20777039	27123474	5038	479
2008	25488470	32168543	5130	487
2009	24930487	26385876	4748	501
2010	24082721	27600000	5268	480
2011	28613085	30800000	5321	480
Total	501204103	518133836	176618	

Source: ACC, 2011.

2.2 Analytical Framework

Simple descriptive statistics, correlation determinations, and regression techniques were used in the data analysis for this study. To test the differences between the mean volume of credit borrowed and the mean volume repaid, Students t-test was used. The Ordinary Least Square (OLS) method of regression was used in estimating the relationship between the repayment rate and the predictor (explanatory) variables. The predictor variables were chosen according to their importance to the repayment process. Results of many studies investigating repayment performance considered these factors critical in determining the credit agency's repayment performance level (Yasir et al., 2012; Greenbaum & Thakor, 1995; Hulme & Mosley, 1996; Derban et al., 2005). The variables considered in our study include the volume of loans borrowed from the ACC (X_1); the volume of loans repaid to the ACC including ACC profits (X_2); the number of borrowers (X_3); the number of ACC staff members (X_4), the borrower's age (X_5), and the borrowers' farming experience (X_6). The volume of loans borrowed from the ACC and the number of borrowers are variables related to farmers accessibility to credits offered by the ACC. The volume of loans repaid to the ACC is a variable related to ACC collection performance. The dependent variable is the repayment rate (volume of repaid loans divided by the volume of loans given by the ACC). The volume of loans per ACC staff member and the number of borrowers per ACC staff member are variables derived from X_1, X_3, and X_4. These two variables are related to the ACC's administrative performance and they were not included in the regression model to avoid a multicollinearity problem that is mostly the result of including a variable that is computed from other investigated variables.

The regression model is specified explicitly as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots.. + \beta_6 X_6 + e$$

Y = repayment rate (volume of repaid loans divided by the volume of loans given by the ACC).

X_1 = volume of loans borrowed from ACC.

X_2 = volume of loans repaid to ACC.

X_3 = number of borrowers.

X_4 = number of ACC staff members.

X_5 = borrower's age.

X_6 = borrower's farming experience of borrower.(the word borrower`s should be omitted).

e = error term.

β_o, β_1, β_2, …, β_6 are regression parameters to be estimated.

3. Results and Discussion

In general, agricultural credit has an indirect effect on production through providing the financial resources to overcome a lack of inputs so that farmers can effectively engage in the agricultural production process. The process of offering loans to farmers, along with the repayment of these loans, is actually subject to many factors that may prevent agricultural credit agencies from being efficient in providing the needed financial resources to farmers. The repayment performance of any agricultural credit agency may be negatively affected by these factors, meaning that the agency will not be able to perform its job efficiently as part of the process of agricultural development. This study investigated some factors that are strongly related to the repayment performance of a public agricultural credit agency in Jordan (the ACC). The effects of factors related to the farmers' accessibility to credits offered by agricultural credit agencies, the collection performance of agricultural credit agencies, and the administrative performance of agricultural credit agencies were investigated.

3.1 Main Credit-Related Items

Figure 1 shows the development of loans offered to borrowers (dark line) and the loans repaid to the ACC since 1960. Table 2 shows the repayment rate, or the percentages of repaid loans to borrowed loans (R/B), since 1960 for the ACC. Figure 2 shows the development of the repayment rate since 1960.

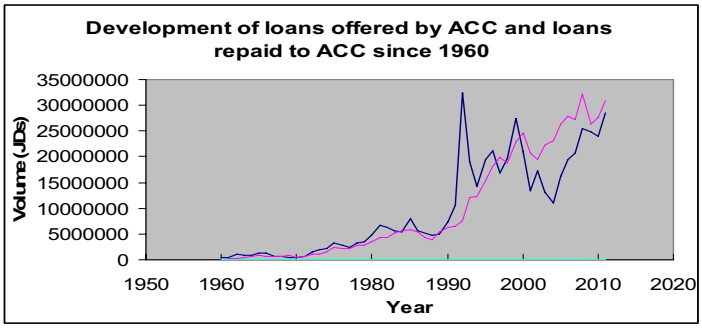

Figure 1. Development of loans offered by the ACC and loans repaid to the ACC since 1960

Source: Based on data from the ACC's annual reports.

Table 2. Repayment rate (R/B) since 1960

Year	R/B (%)	Year	R/B (%)	Year	R/B (%)	Year	R/B (%)	Year	R/B (%)
1960	18	1971	85	1982	70	1993	64	2004	211
1961	32	1972	72	1983	93	1994	86	2005	161
1962	14	1973	58	1984	105	1995	79	2006	143
1963	52	1974	71	1985	75	1996	86	2007	131
1964	85	1975	74	1986	97	1997	118	2008	126
1965	72	1976	74	1987	84	1998	95	2009	106
1966	49	1977	96	1988	83	1999	84	2010	115
1967	103	1978	87	1989	109	2000	118	2011	108
1968	77	1979	78	1990	84	2001	155	Average	0.92
1969	197	1980	72	1991	62	2002	113		
1970	114	1981	62	1992	23	2003	168		

Source: Calculated by the researchers using data from the ACC's annual reports.

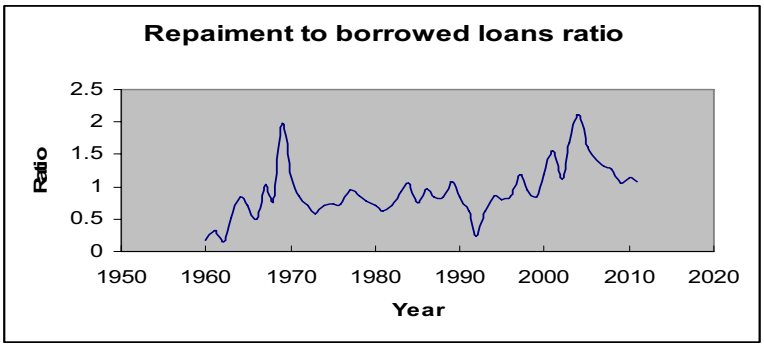

Figure 2. Development of the repayment rate since 1960

Source: Based on data from the ACC's annual reports.

As shown in Figure 1 (see Table 1), the volume of loans offered by the ACC to the borrowers increased from 457,680 JDs (1 JD =1.4 USD) borrowed by 4,242 farmers in 1960 to 28,613,085 JDs borrowed by 5,321 farmers in 2011. The increase in the offered loans is nearly 62% while the increase in borrowers for the same period is nearly 25%, indicating that the average volume per beneficiary is higher in the later operational years of the ACC compared to the earlier years. The small increase in borrowers compared to the loans' volume increase is a crucial factor in determining the repayment performance of an agricultural credit agency. This situation seems to be good

in the ACC's case according to the results presented in Table 2. This goodness may be attributed to the experienced staff, good logistic facilities, and better efficiency with respect to both coordination and performance. The repayment rate will surely go up under such circumstances.

On the other hand, the volume of repaid loans increased from 80,500 JDs in 1960 to 30,800,000 JDs in 2011. The highest volumes of repaid loans were recorded in the last 20 years because most ACC loans were long-run loans and needed more than 20 years to be paid. Political and Middle East area related factors might be reasons for low repayment rate in certain years (1967 and 1973).

The results presented in Table 2 show that the average ACC repayment rate is 0.92 (1960-2011), indicating good repayment performance. Considering the fact that no agricultural credit agency can have a 100% repayment rate, as this rate is approaching 1, the agency is doing well. Because the rate is away from 1 for some years, the agency has a poor repayment performance. The ACC repayment rate from 1960-1966 is low when compared with the other periods. The repayment rate in other periods is higher. (Above 100% from 2000-2011). This high value of the repayment rate could be attributed to the lack of consistency in the growth performance of Jordan's agricultural sector from 1960-1966 as well as to instability and inconsistencies in agricultural policies, policy implication, and poor monitoring and management during other periods, except those after the year 2000.

Concerning the ACC's administrative performance, on average, the volume of loans per staff member was 28,108.83 JDs, and the number of borrowers per staff member was 11. These low figures compared to agricultural credit agencies in other developing countries indicate that the ACC's administrative performance is good. The lower the two indicators are, the better the administrative performance that, in turn, reflects on the agency's repayment performance. Loan defaults arose from poor management or administrative procedures.

3.2 T-test

T-test could be used to compare statistical differences. It assesses whether the means of two groups of data are statistically different from each other. This analysis is appropriate whenever a comparison of the means of two groups is wanted.

The statistical difference between the mean values of loans obtained (X_1) and the mean amount of loans repaid (X_2) by borrowers is presented in Table 3.

Table 3. Repayment rate (R/B) since 1960

Variable	Mean	Standard Deviation	Degrees of Freedom	t-value	Sig. (2-tailed)
Volume borrowed	9813540.4	9248949.8	51	7.651	.000
Volume repaid	9967358.4	10354951.4	51	6.941	.000

Source: Based on data from the ACC's annual reports.

The results presented in Table 3 reveal that further analysis using a student's t-test at the 5% level of significance showed no significant difference between the mean volume of loans borrowed and the mean volume of loans repaid by borrowers. The implication of these results is that farmers exhibited high loan-repayment performance that will be positively reflected in the credit agency's repayment performance. These results confirm the information presented in Table 2.

3.3 Regression Model

The results for the multiple regression of factors that influence the ACC's loan repayment performance are presented below (Table 4). An evaluation of the model for loan repayment performance showed that the R^2 value was 0.796 (80%) while the adjusted R^2 value was 0.763 (77%). This result means that nearly 80% of the variation in the repayment rate (the dependent variable) was due to the joint effects of the explanatory variables. Regression estimates showed that the volume of loans repaid, the number of staff members, and the borrowers' farming experience prove to be significant at the 95% confidence level. The remaining three variables were not significant at that level. This result shows the importance of considering these variables when planning to analyze the repayment performance of agricultural credit agencies. The signs of the variables in the model were previously expected. Comparisons related to the signs were conducted after model estimation. The positive sign of the borrowed loans coefficient is not in line with the prior expectations for this variable. It was expected to have a negative sign. The positive sign for the coefficient of the volume of the repaid loan, the number of borrowers, the numbers of staff members, and borrowers' farming experience as well as the negative relationship for the

coefficient of the borrower's age with the repayment rate are in line with prior expectations. The signs for the coefficient of independent variables and the significance of these variables are used to determine the impact of each independent variable on the dependent variable. The results presented in Table 4 reveal that all the explanatory variables in the model have a positive effect on the repayment rate, and hence repayment performance, except the borrower's age which had a negative effect. Each 1% increase for the volume of loans borrowed from the ACC; the volume of loans repaid to the ACC, including ACC profits; the number of borrowers; the number of ACC staff members; and the borrowers' farming experience causes an increase of 0.256%, 0.574%, 0.079%, 0.613%, and 0.071% in the repayment rate, respectively. A 1% increase for the farmer's age will cause a 0.047% decrease for the repayment rate.

Table 4. Output of the multiple linear regression model

Variables	Coefficient (B)	t -value	Sig.
Constant	0.922	16.77	0.000
Loan borrowed (X_1)	0.256	1.874	0.337
Loan repaid (X_2)	0.574	4.954	0.000
No. of borrowers (X_3)	0.079	0.559	0.579
No. of staff members (X_4)	0.613	5.481	0.000
Borrower's age (X_5)	- 0.047	- 0.331	0.742
Borrower's Experience (X_6)	0.071	0.505	0.015

Source: Conducted by researchers based on data from ACC annual reports.

As shown in Table 4, the volume of the borrowed loans has a positive influence on the repayment performance. It was hypothesized to have a negative relationship with the repayment rate. The regression result disagreed with this hypothesis. This result could be attributed to the explanation that higher loans make larger investments with potentially higher returns possible. In other words, larger loan sizes would enhance the beneficiary farmer's access to basic inputs and improved farm-management opportunities, which would lead to higher productivity, reduced per unit cost, and increased income.

The volume of the repaid loans is a very important factor in determining the agricultural credit agencies' repayment performance. The results of this study revealed that the average repayment rate to the ACC was 92%. This high repayment rate may be attributed to the sound lending policy adopted by the ACC that was believed to result in low probabilities of loan default.

The relationship between the number of borrowers and the repayment rate, as indicated by the figures in Table 4, is a positive one. The number of borrowers could be a useful indicator for the credit agency's outreach. The coefficient of the variable was positive, suggesting that the greater the number of people covered, the greater the repayment rate.

The borrower's age is a very important factor in agriculture operations because youths and young adults who are full of vigor are required for production. The average age of the ACC beneficiaries was 48 years. The majority of borrowers were between 30 and 50 years old, an age in which they are considered highly productive and active to practice farm work. In spite of this fact, the older borrowers cannot have a meaningful impact on agricultural production even if they are adequately motivated with the needed credit facilities. The results of this study showed that there is a negative effect for aging on the repayment rate, which is acceptable and true. Based on this result, the agricultural credit agencies should be able to consider the age of borrower when offering loans. Older farmers tend to be conservative and less vulnerable to the winds of change.

As with the borrower's age variable, it is very important to consider the borrower's experience when offering loans to beneficiaries. The average experience of the ACC beneficiaries is 20 years. This experience is reflected in the high repayment rate. Borrowers who have a lot of farming experience exhibit a willingness to adopt new technologies. The result is that there is higher productivity, more revenues, and higher abilities to repay loans.

The administrative performance of agricultural credit agencies is largely affected by the number of staff members introducing services to clients. As shown in Table 1, the ACC has a well-trained and experienced staff. This staff is a major factor for the ACC achieving its high repayment rate. A sufficiently trained staff will result in proper

monitoring and supervision of the credit agencies. Insufficient staff numbers cause lack of supervision and monitoring services. Hence, farmers may transfer their agricultural credit to non-farm purposes.

4. Conclusions

According to the results of this study, the repayment rate of the investigated public credit agency (ACC) is 0.92, indicating a high level of repayment performance and a low default rate (0.08). This result may be attributed to many exogenous and endogenous factors. The volume of loans borrowed, the volume of loans repaid, the number of borrowers, the number of credit agency staff members, the borrower's age, and the borrower's experience were the most important factors related to the credit agencies' repayment performance. All these factors had a positive effect on the repayment performance of the investigated agency, except the age of the borrower that had a negative effect, contrasting the prior expectations. Sufficient and strict controls along with monitoring are required, and outreach to beneficiaries should be improved to enhance the repayment performance. To avoid the burden of provisioning on agricultural credit agencies, legal actions and guarantees should be taken against loans defaulters. Introducing a reward system for those individuals who paid on time will be helpful in enhancing the repayment performance.

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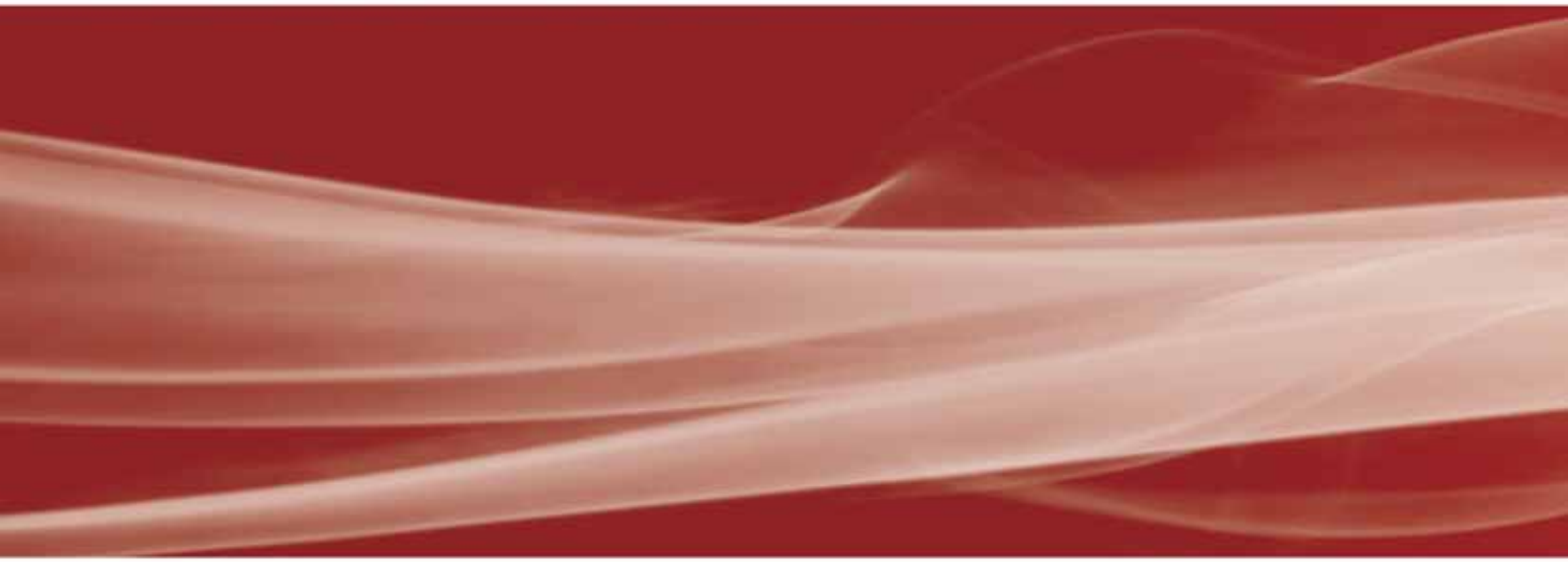

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