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Effect of Genotype and Periodic Pruning on Storage Root Yield and Yield Components of Some Cassava Genotypes Under Rain-Fed Conditions In Ghana

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Abstract

Cassava (*Manihot esculenta* Crantz) is cultivated primarily for its starchy roots which provide a staple for millions of people in the tropics. The foliage contains high levels of protein which can be harvested for human and animal feed. Twenty five cassava genotypes were arranged in a randomized complete block design with three replications to investigate their tolerance to periodic pruning with respect to effect on root yield and yield components. The cassava plants were periodically pruned starting from three months after planting and at three months intervals until root harvest at 12 months after planting. Storage root yields ranged between 8.3-26.2 t/ha and 28.9-85.5 t/ha for the pruned plants and the control respectively. The pruned plants produced average root yield of 14.7 t/ha compared with 51.5 t/ha from the control. Though periodic pruning resulted in significant reduction in all components measured, genetic variations were observed in the level of reduction. Observed root yield reduction ranged between 40-80%. Five genotypes; 96/1642, *Afisiafi, Esambankye, Agbelifia* and *Bankyehemaa,* recorded storage root yield reduction of less than 50% of their respective controls. Starch content and mean storage root weight were also significantly reduced by periodic pruning. The significant genetic variations in the reduction of these traits indicated different levels of tolerance which can be exploited in further studies to identify ideal cassava genotypes for dual purpose utilization for foliage and root production.

Keywords: cassava, genotypes, periodic pruning, storage root yield, percentage yield reduction

1. Introduction

Breeding of cassava (*Manihot esculenta* Cranz) solely for root yield and its characteristics in Ghana has led to the neglect of genotypes that are good foliage producers. Though cultivation of cassava solely for foliage productivity is a common practice in Asia (Indonesia, China and Thailand) and some parts of Africa (Lockard et al., 1985; Bokanga, 1994; Limsila et al., 2007; Umuhozariho et al., 2011), the practice is not common in Ghana. Past breeding efforts have focused on developing early bulking, high storage root yield, pest and disease tolerant, low hydrocyanic acid content genotypes with good cooking quality (Annor-Frempong, 1994; Nweke et al., 1994). Boampong (2001) also stated that storage root yield and its characteristics as the most important goal of cassava breeding and ranks high in the choice of variety by farmers. Therefore agronomic practices such as foliage harvesting that will interfere with storage root yield are avoided by farmers.

Apart from the starchy storage roots, the fresh foliage is also utilized in several regions of the world for animal and human consumption (Chavez et al., 2005). Several researchers have reported the nutritive value of cassava foliage which makes it a major source of protein, minerals and vitamins in human diets in certain parts of Africa (Bokanga, 1994). The leaves which are rich in minerals and vitamins, contain 17-34% protein (dry weight basis) and are used for human consumption in certain parts of Africa (Bokanga, 1994; Kobawila et al., 2005; Adjebeng-Danquah & Safo-Kantanka, 2013). The leaves together with the unlignified upper part of the stem constitute a very good source of protein for both human and animal consumption (Ravindran and Rajaguru, 1988; Bokanga, 1994; Limsil*a* et al., 2007). In Rwanda, cassava leaves constitute a very important vegetable which is used in several food preparations (Umuhozariho et al., 2011). However in Ghana only the roots are of economic

importance and was ranked number one in Ghana after the country produced over 1.45 million metric tons in 2013 with over US$ 1.51 million in foreign exchange (FAO, 2014). The potential of the foliage has not been fully exploited due to its possible interference with root yield (Hunt et al., 1977; Fasae et al., 2009).

Tung et al. (2001) reported possible decline in storage root yield as a result of intensive pruning. This is because defoliation reduces root weight due to reduced carbohydrates synthesized per plant (Wright, 1962) and hence reduced quantity of photoassimilates available for storage. The choice of an ideal cassava variety for foliage production therefore depends on its ability to tolerate periodic pruning without a significant reduction in storage root yield. However there is scanty information on the genetic variation among different cassava cultivars in terms of tolerance to periodic pruning (Tung et al., 2001). Many factors such as the differences in maturity and plant architecture could account for this variation. Early bulking genotypes begin carbohydrate accumulation at an earlier stage than late bulking types and so they are likely to be less affected by pruning than late bulking types (Okogbenin & Fregene, 2002).

This study sought to evaluate some advanced cassava genotypes to (1) determine the extent of variation of among cassava genotypes in terms of storage root yield following periodic pruning (2) identify cassava genotypes that show least significant reduction in storage root yield with periodic pruning for possible dual-purpose utilization and (3) determine the effect of periodic pruning on storage root yield components in cassava.

2. Materials and Methods

2.1 Plant Materials

The study was conducted using 25 cassava genotypes obtained from different sources (Table 1). They were made up of eleven elite local accessions obtained from the Wenchi Agricultural Experimental Station of the Ministry of Food and Agriculture. These materials were being screened for early maturity and cooking quality. Five of the genotypes were also advanced breeding materials obtained from the International Institute of Tropical Agriculture (IITA). Nine already released varieties were also used in the study.

Table 1. List of cassava genotypes used for the study

Genotype	Background/origin	Genotype	Background/origin
96/0160	Exotic accession from IITA	DMA 004	Elite local accession
96/0603	Exotic accession from IITA.	*Dokuduade*	Released variety (IITA)
96/1565	Exotic accession from IITA	*Esambankye*	Released variety(IITA)
96/1569	Exotic accession from IITA	*Gblemoduade*	Released variety (IITA)
96/1642	Exotic accession from IITA	*IFAD*	Released variety (local)
Abasafitaa	Released variety (IITA)	*Kyempo*	Elite local accession.
ADI 001	Elite local accession.	*Nkabom*	Released variety (local)
ADI 002	Elite local accession	TANO 001	Elite local accession.
Adugyama	Elite local accession.	TANO 003	Elite local accession.
Afisiafi	Released variety (IITA)	TCH 001	Elite local accession.
Agbelifia	Released variety (IITA)	TCH 002	Elite local accession.
AWO 001	Elite local accession.	TCH 004	Elite local accession.
Bankyehemaa	Released variety (IITA)		

2.2 Field Experiment

The field experiment was laid out as a randomized complete block design (RCBD) experiment with three replications. The 25 cassava genotypes represented the treatments. Cuttings from the 25 cassava genotypes were planted on the flat using a spacing of 60 cm × 60 cm which is considered as the optimum spacing for foliage production (Limsila et al., 2007). Each plot consisted of four rows of plants with 10 plants in a row. The plot size was thus 2.4 m × 6 m resulting in a plant population of approximately 27,778 plants/ha. Cassava foliage (comprising the unlignified young stems, leaves and petioles) was hand harvested following Limsila et al. (2007).

Foliage harvesting commenced at three months after planting and was repeated at three months' intervals with the fourth harvest coinciding with root harvest (12 MAP). At root harvest, data were collected on storage root yield (t/ha), starch content (%), harvest index, number of storage roots per plant, mean storage root weight (g) and storage root dry matter (%). The starch content of the storage roots of each genotype was determined based on the specific gravity using the *Reimann* scale balance method as outlined by Prammanee et al. (2010). For dry matter content determination, random samples of storage roots of each of the 25 genotypes were taken and chopped into smaller pieces. These were then mixed thoroughly after which sub samples of 100g each were taken for oven drying at 80 ^{o}C for 48 hours. The dry matter content (%) was estimated based on the final dry weight as a percentage of the initial fresh weight taken.

2.3 Data Analysis

The data was subjected to analysis of variance (ANOVA) for randomized complete block design (RCBD) using the GenStat Release 12.1 for windows (GenStat, 2009). Genotypes and pruning were considered as factors to assess the genotypic response to pruning. Significant differences between genotypes under pruning and no pruning were tested using their standard errors of differences. Two sample t-test was performed to compare the mean performance of all genotypes under pruning and no pruning using GenStat (GenStat, 2009).

3. Results

3. 1 Analysis of Variance

Analyses of variance indicated highly significant (P<0.01) genotype effect for root yield (t/ha) and mean root weight (g) and very highly significant (P<0.001) genotype effect for starch content and storage root dry matter content (Table 2). The effect of pruning on all traits was also very highly significant (P<0.001). However the interaction between genotype and pruning effect on all traits were not significant (P>0.05).

Table 2. Mean squares for root yield and yield components of 25 cassava genotypes under pruning and no pruning

Source of variation	D.f.	Root yield (t/ha)	Mean root weight (g)	Starch content (%)	Harvest index	Dry matter content (%)
Rep	2	1877.70	0.08	2.76	0.081	3.12
Genotypes (G)	24	453.80**	0.02**	25.37***	0.034***	36.75***
Pruning (P)	1	45197.10***	2.66***	272.57***	1.193***	219.13***
GxP	24	263.40NS	0.12NS	2.72NS	0.010NS	3.65NS
Residual	98	231.10	0.11	3.03	0.009	5.73
Total	149					

, * = significant at P<0.01, and P<0.001 respectively. NS = Not significant (P>0.05).

3.2 Root Yield

Storage root yield was generally reduced when the plants were pruned as compared with the control (Table 3). Significant differences (P≤ 0.05) were established between the different genotypes in terms of this reduction. The pruned plants gave an average storage root yield of 14.7 t/ha as against 51.5 t/ha from the control. The reaction of the genotypes to the periodic pruning as given by their yield relative to their control could be grouped into classes of 10-20, 20-30, 30-40 up to 80-90 t/ha. Storage root yield from the pruned plants fell within the lower classes (Figure 1). Genotypes like TANO 003, DMA 004 and TCH 002 all had root yields below 10 t/ha. Most of the pruned plants recorded storage root yields that fell within the 10-20 t/ha and included 96/1565, *Dokuduade, Agbelifia*, 96/0603, etc. The highest storage root yield recorded in the pruned plants fell within the 20-30 t/ha class included *Afisiafi, Esambankye* and 96/1642. The highest yielding genotype under pruning was 96/1642 which gave 26.2 t/ha and this was significantly different (P≤ 0.05) from most of the other genotypes. The storage root yields from the control plants were quite high as they ranged between 20-30 t/ha and 80-90 t/ha. Only one genotype (*Bankyehemaa*) from the control produced a storage root yield within the 20-30 t/ha class which was the least. Majority of the genotypes including ADI 001, 96/1642, ADI 002, etc. recorded storage root yields which fell within the 40-50 and 60-70 t/ha classes. *Gblemoduade* gave the highest yield of 85.5 t/ha fell in the highest class of 80-90 t/ha category.

Storage root dry matter content (%) was also varied significantly ($P<0.05$) among the genotypes under pruning and the control (Table 3). Under pruning, dry matter content ranged between 32.1% and 41.3% for TANO 003 and IFAD respectively. The lowest storage root dry matter content from the control was obtained from 96/1565 (34.5%) whilst IFAD had the highest dry matter content (43.3%). Pruning resulted in significant reduction in dry matter content. Average root dry matter contents were 37.1% and 39.5% for pruned plants and the control respectively which were significantly different according to the t-test ($P<0.05$). However significant genetic variation was observed among the genotypes in the extent of reduction. The lowest reduction was recorded in 96/0160 (1.6%) with TANO 003 having the highest reduction in dry matter content (17.2%).

Table 3. Storage root yield and dry matter content of 25 cassava genotypes under pruning and no pruning

Genotypes	Storage root yield (t/ha)			Dry matter content (%)		
	Pruned plants	Control	% reduction	Pruned plants	Control	% reduction
96/0160	12.00	73.51	80.41	36.20	36.81	1.60
96/0603	17.90	64.42	72.61	35.20	36.40	2.91
96/1565	19.80	70.20	70.10	32.61	34.51	5.32
96/1569	17.20	41.30	58.10	38.70	40.40	2.41
96/1642	26.20	59.02	50.31	33.90	37.11	8.52
*Abasafitaa**	15.40	41.30	62.80	38.22	40.21	4.91
ADI 001	11.20	56.02	79.30	39.80	41.70	4.52
ADI 002	14.90	55.70	58.60	38.10	40.30	5.21
Adugyama	10.00	47.50	76.90	34.71	38.61	9.42
*Afisiafi**	20.90	52.90	55.51	36.20	40.71	10.90
*Agbelifia**	18.10	52.92	52.71	33.41	34.72	3.92
AWO 001	14.80	56.20	74.01	35.92	41.13	12.80
*Bankyehemaa**	14.60	28.90	40.10	38.01	42.31	10.32
DMA 004	8.30	37.81	69.71	38.20	39.82	3.60
*Dokuduade**	18.70	62.30	65.80	36.22	38.03	4.71
*Esambankye**	22.50	48.51	53.61	38.61	39.61	2.32
*Gblemoduade**	16.50	85.52	80.41	33.91	35.22	3.70
*IFAD**	14.40	45.31	65.61	41.30	43.31	4.63
Kyempo	8.90	41.90	78.00	41.22	41.90	1.71
*Nkabom**	11.80	30.81	63.22	38.70	40.83	5.12
TANO 001	12.30	44.71	70.60	40.51	41.31	2.03
TANO 003	9.60	35.01	51.41	32.22	38.82	17.22
TCH 001	10.01	41.42	75.90	38.91	40.20	3.21
TCH 002	9.60	71.50	68.62	40.10	43.31	7.42
TCH 004	12.50	41.90	68.21	37.42	41.62	10.15
Mean	14.70	51.46	65.70	37.13	39.56	5.94
SED	3.63	20.44	13.48	2.11	1.82	6.16
t (cal)(means)	12.35			3.29		
$t_{0.05(2),\ 48}$	2.011			2.011		

* Released varieties.

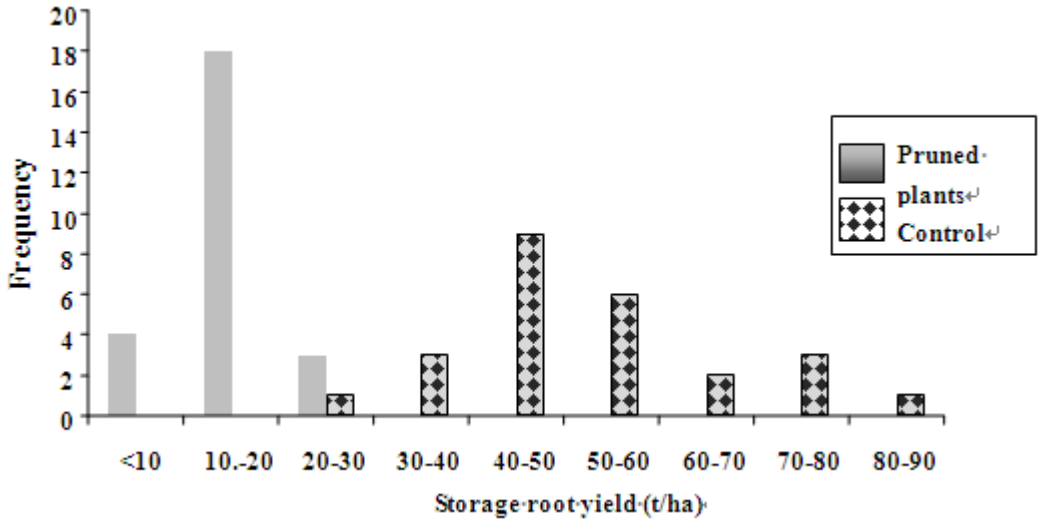

Figure 1. Range of storage root yield from the pruned plants and the control

There were also significant differences (P≤ 0.05) among the different genotypes in terms of storage root yield reduction as a percentage of the yield from the control plants (Table 3). The percentage (%) reduction was also used as an index of tolerance of the different genotypes to pruning. In other words genotypes that recorded the least reduction in storage root yield were considered tolerant to pruning and as against susceptible genotypes which recorded relatively high percentage reduction. The average percentage reduction for the different genotypes which ranged between 80.4% and 40.1% can again be categorized into classes of 40-50, 50-60, up to 80-90% reduction (Figure 2). Majority (8) of the genotypes like ADI 001, *Kyempo*, TCH 001, TANO 001, etc. recorded reductions that fell within the 70-80% category with equal number of seven each being recorded in the 50-60 and 60-70% categories. Only one genotype (*Bankyehemaa*) recorded a reduction within the 40-50% category. Two genotypes, *Gblemoduade* and 96/0160 suffered yield reductions which fell within the 80-90% category.

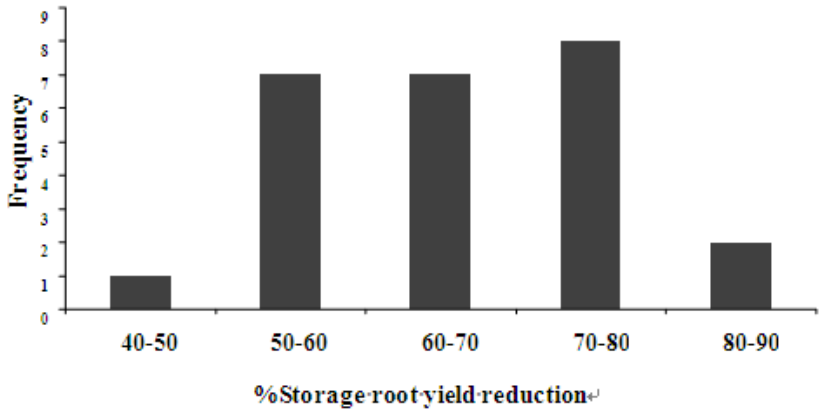

Figure 2. Frequency distribution of the range of % storage root yield reduction

3.2 Mean Storage Root Weight and Starch Content as Affected by Pruning

Storage root yield in cassava is determined by the number and the size of storage roots. Periodic pruning had a significant effect (P≤ 0.05) on mean storage root weight (Table 4). Generally smaller storage roots were produced by the periodically pruned plants as compared with the control plants that produced relatively bigger

storage roots. The average mean storage root weight of the pruned plants was 180 g which was significantly ($P \leq 0.05$) lower than those of the control plants which produced an average mean storage root weight of 450 g. Mean storage root weights from the pruned plants ranged between 120 g for *Kyempo* and 250 g for 96/1642. Genotypes like AWO 001, 96/1569, *IFAD*, *Agbelifia*, *Esambankye*, *Gblemoduade* and 96/1642 all produced mean storage root weights that were above the average values recorded in the pruned plants. The mean storage root weight from the control also ranged between 290g and 600g for DMA 004 and AWO 001 respectively. Genotypic variation was observed in the reduction in mean root weight in the pruned plants compared with the control as indicated by the percentage reduction which varied from a low of 41.9% for *Agbelifia* and 73.4% for *Kyempo* (Table 4).

Pruning also had a significant effect on the starch content of the different cassava genotypes being especially higher in the control than the pruned plants (Table 4). The different genotypes however responded differently to the starch content reduction. *IFAD* had the highest starch content of 19.7% which was significantly different ($P \leq 0.05$) from the most of the other genotypes when pruned. The starch content was however lower than the starch content obtained from the control (22.8%). Genotypes 96/1565 and 961642 recorded the least starch contents of 12.1% and 12.2% respectively. In the case of the control, TCH 004 recorded the highest starch content of 23.2% with 96/1565 recording the least starch content (12.7%). *Nkabom, Dokuduade, Bankyehemaa* and *IFAD* were the released varieties that maintained relatively high starch content when pruned. Average starch content for all genotypes under pruning was also significantly lower under pruning than the control according to the two-sample t-test as well as the difference in starch contents expressed as a percentage of the control. The least percentage reduction was recorded in 96/1565 (3.0%) with 96/1642 having the highest percentage reduction in starch content (26.3%) in the pruned plants compared with the control.

Table 4. Storage root yield components as affected by pruning

Genotypes	Mean storage root weight (g)			Starch content (%)		
	Pruned plants	Control	% reduction	Pruned plants	Control	% reduction
96/1642	250.00	420.00	42.60	12.20	16.70	26.30
*Esambankye**	240.00	490.00	43.90	16.70	20.50	17.60
*Afisiafi**	210.00	390.00	43.00	16.00	18.50	13.70
96/1565	140.00	430.00	64.90	12.10	12.70	3.00
*Dokuduade**	180.00	550.00	64.40	17.10	18.80	9.90
*Agbelifia**	200.00	380.00	41.90	14.30	15.10	5.10
96/0603	230.00	450.00	50.20	14.70	16.40	10.10
96/1569	200.00	350.00	42.30	17.20	20.30	15.00
*Gblemoduade**	200.00	570.00	65.50	13.10	15.70	16.00
*Abasafitaa**	180.00	450.00	59.40	16.00	18.90	14.70
ADI 002	160.00	510.00	65.00	15.10	19.30	21.40
AWO 001	240.00	600.00	59.90	15.50	20.30	23.50
*Bankyehemaa**	130.00	290.00	56.20	16.90	21.10	20.40
*IFAD**	210.00	490.00	57.90	19.70	22.80	12.40
TCH 004	150.00	420.00	62.80	18.00	23.20	22.30
TANO 001	150.00	470.00	65.30	16.90	19.20	12.40
96/0160	170.00	480.00	63.80	17.30	19.80	12.20
*Nkabom**	180.00	430.00	49.30	18.90	20.30	6.90
ADI 001	150.00	510.00	69.70	17.70	18.60	4.70
Adugyama	140.00	410.00	65.20	14.30	18.80	23.60
TCH 001	120.00	430.00	71.30	17.50	19.50	10.30
TCH 002	140.00	470.00	52.10	15.30	17.20	15.90
TANO 003	160.00	480.00	64.60	18.20	21.80	11.30
Kyempo	120.00	460.00	73.40	17.70	18.50	4.40
DMA 004	130.00	280.00	49.50	16.10	18.10	9.10
Mean	180.00	450.00	57.8	16.20	18.90	13.70
SED	41.35	82.20	11.33	1.52	1.32	4.21
t (cal)(means)	15.99			4.40		
$t_{0.05(2),\ 48}$	2.011			2.011		

* Released varieties.

3.4 Effect of Pruning on Harvest Index (HI)

Significant differences ($P \leq 0.05$) were observed for harvest index in both the pruned and the control plants (Table 5). Genotype 96/1642 recorded the highest HI of 0.54 in the pruned plants with the least being produced by TANO 003 (0.22). Generally the pruned plants produced lower HI as compared to the control. *Agbelifia, Abasafitaa*, 96/0160, 96/1565 and 96/0603 had quite high HI in the control plots. The comparison from the control also reveals that most of the genotypes with high HI in the control plants also had lower HI in the pruned plants except *Agbelifia, Esambankye* and 96/0603 which produced quite high HI both in the control and the pruned plants. Significant ($P \leq 0.05$) percentage (%) reduction in harvest index in the pruned plants relative to the control was also observed. *Esambankye* had the least percentage reduction in HI (3.85%) which was significantly lower than most of the other genotypes. The highest percentage reduction was observed in *Kyempo* (59.32%).

Table 5. Harvest index (HI) from pruned plants and the control

Genotypes	Harvest index		% reduction
	Pruned plants	Control	
96/1569	0.54	0.63	14.29
*Agbelifia**	0.53	0.68	22.06
*Esambankye**	0.50	0.52	3.85
96/0603	0.48	0.61	21.31
96/1642	0.44	0.51	13.73
*Abasafitaa**	0.44	0.68	35.29
*IFAD**	0.43	0.58	25.86
96/1565	0.42	0.63	33.33
*Afisiafi**	0.40	0.58	31.03
*Nkabom**	0.37	0.54	31.48
*Bankyehemaa**	0.37	0.39	5.13
AWO 001	0.36	0.56	35.71
*Gblemoduade**	0.35	0.61	42.62
TCH 001	0.35	0.56	37.50
Adugyama	0.34	0.58	41.38
96/0160	0.34	0.60	43.33
TCH 002	0.34	0.42	19.05
*Dokuduade**	0.31	0.57	45.61
ADI 002	0.31	0.48	35.42
ADI 001	0.30	0.57	47.37
TANO 001	0.29	0.47	38.30
TCH 004	0.27	0.47	42.55
DMA 004	0.27	0.45	40.00
Kyempo	0.24	0.59	59.32
TANO 003	0.22	0.37	40.54
Mean	0.37	0.55	32.53
SED	0.08	0.07	14.29
t (cal)(means)	7.37		
$t_{0.05(2),\ 48}$	2.011		

* Released varieties.

4. Discussion

4.1 Effect of Pruning on Storage Root Yield and Dry Matter Content

Even though periodic pruning at 2-3 month intervals have been reported to result in marginal reduction in storage root yield (Dahniya et al., 1981; Lockard et al., 1985) the results from this study suggested otherwise. Periodic pruning generally had a significant negative effect on storage root yield but there were genotypic differences in the extent of reduction. For example the reduction in yield as a percentage of the control varied from as high as 80% for *Gblemoduade* to as low as 40% for *Bankyehemaa*. Similar observed variations in cassava genotypes in terms of their ability to tolerate periodic pruning have been reported (Ravindran & Rajaguru, 1988). Therefore careful varietal selection would be very critical in choosing cassava for foliage production. The decline in storage root yield resulting from pruning could be expected since the foliage is the

photosynthetic apparatus and therefore responsible for photosynthesis. The foliage removal therefore led to reduction in the amount of photosynthates produced and stored in the roots. Hunt et al. (1977) asserted that deposition of starch in storage roots can be reduced if the supply from the top is interrupted as occurs when much of the leaf and stem materials are removed. Genotypic differences were however observed in the extent of reduction in root yield resulting from pruning. Many factors such as variation in maturity and plant architecture could account for these genetic differences. Early bulking genotypes begin carbohydrate accumulation at an earlier stage than late bulking types and so they are likely to be less affected by pruning than late bulking types. Okogbenin and Fregene (2002) observed that late bulking cassava genotypes have the foliage as the active growing sinks for the first seven months after planting whilst in fast or early bulking genotypes, rapid increase in storage root development occur in the first six months of growth. In this case the fast bulking genotypes might have accumulated some amount of photosynthates at the time the pruning was initiated compared with the late bulking genotypes. Another trait that could affect a variety's tolerance to pruning in terms of root yield is the plant's architecture (whether erect or profuse branching at an early stage of growth). Erect non-branching genotypes are more likely to be severely affected as compared with profuse branching genotypes owing to their rapid initial vegetative growth at the expense of storage root bulking. In a study involving two cassava varieties with different growth habits, Dahniya et al. (1981) reported a greater storage root yield reduction in an erect local cassava variety *Isunikakiyan* than TMS 30211, a branching type when they were both pruned. From the results of this study, it can be inferred that 96/1642, *Afisiafi, Esambankye, Agbelifia* and *Bankyehemaa* are relatively tolerant to periodic pruning as their storage root yield reduction was just around 50%. Storage root dry matter content was also significantly reduced when the plants were pruned though genetic variability was observed among the genotypes in terms of the reduction. Similar declines in dry matter content among pruned plants were observed in Thailand by Chantaprasarn and Wanapat (2003) in a study involving a local cassava variety, Rayong 60. The reduction in dry matter content of the pruned plants could be attributed to the need to remobilize the stored carbohydrates for regrowth of new shoots any time the plants were pruned but this was not the case in the control plots where the foliage were left intact (Sagrilo et al., 2003). These periodic disturbances might have interfered with the accumulation of carbohydrates leading to the reduction as reported by other studies (Oliveira et al., 2010; Andrade et al., 2011). The significant genetic variation in the extent of reduction observed among the cassava genotypes provides opportunity for selection in the genetic improvement of cassava for tolerance to periodic pruning.

4.2 Pruning and Root Yield Components

Though periodic pruning significantly reduced the storage root yield and its components, there were differences among the different genotypes on the extent of reduction. Ayoola and Agboola (2004) observed reduction in yield and mean storage root weight when cassava plants were pruned irrespective of the pruning method. This was corroborated by the results of this study. There were however differences in the degree of reduction. The reduction in storage root sizes in the pruned plants could be explained by the fact that as the plants were pruned, photosynthates that should have been used for storage root bulking were used for regrowth of new shoots. This therefore resulted in smaller storage roots due to reduced carbohydrates available for storage root bulking possibly due to reduced carbohydrates synthesized per plant and hence reduced quantity of food available for storage (Wright, 1962).

4.3 Starch Content and Pruning

Pruning generally reduced the starch content of the storage roots compared to the control but there were varietal differences. For example the average starch content of the pruned plants was 16.2% compared to the 18.9% recorded by the control. Genotypes 96/1565 was most affected by pruning giving a starch content of 12% compared to its corresponding starch content of 16.7% in the control. The reduction in starch content when the plants were pruned could be explained by the fact that as the plants were pruned, the stored starch was converted to sugar and translocated to the buds for regrowth. According to Mitchell (1970) carbohydrate reserves are used for regrowth of new leaves until the new leaves can photosynthesise before further starch accumulation can begin sometimes to the original level. As the supply from the top part was curtailed following the repeated pruning, deposition of starch in the roots was reduced (Hunt et al., 1977) resulting in the smaller storage roots compared with the control which was left undisturbed.

4.4 Harvest Index

According to Alves (2002) harvest index is a measure of dry matter distribution to the economically important part and it represents the efficiency of storage root bulking (Fregene & Puonti-Kaerlas, 2002). Harvest index was significantly affected by pruning. This was to be expected since the top growth was periodically removed, more

vegetative growth was promoted at the expense of root production. There were however genetic differences considering the wide range of values obtained for the different genotypes under pruning and the control. For example the harvest indices from the control plots ranged between 0.37-0.68 as against 0.24-0.54 for the pruned plants. Significant genetic differences in cassava genotypes in terms of the harvest index have been reported (Alves, 2002). This therefore provides opportunity for selection for high yield potential under pruning and no pruning regimes (Kawano et al., 1998).

5. Conclusion

Cassava research in Ghana has been based mostly on developing improved varieties that are high yielding with high dry matter and also tolerant to common diseases and pests and meet farmers' expectation. Evaluation for tolerance to periodic pruning is seldom considered. This study has opened a new chapter in the area of identifying cassava genotypes that are tolerant to periodic pruning and for that matter suitable for dual purpose production. The identification of genotypic variation in tolerance to periodic pruning will afford researchers the opportunity to explore the cassava gene pool for the ideal genotype that will produce adequate amount of foliage without significant reduction in storage root yield.

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Studies on Influence of Bagging of Fruits at Marble Stage on Quality of Mango cv. Alphonso

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Abstract

Preharvest fruit bagging has emerged as a novel technology in practice, which is simple, grower friendly, safe and beneficial for production of quality fruits. An investigation was undertaken in 2013 and 2014 for two consecutive fruiting seasons entitled studies on influence of bagging of fruits at marble stage on quality of mango cv. Alphonso. The fruits were bagged at marble stage (30 days from fruit set) with different types of bags which constituted the various treatments *viz*: T_1: Newspaper bag; T_2: Brown paper bag; T_3: Scurting bag; T_4: Polythene bag; T_5: Butter paper bag; T_6: Muslin cloth bag; T_7: Brown paper bag with polythene coating; T_8: control (no bagging). The experiment was conducted in Randomised Block Design with eight treatments replicated three times. The preharvest bagging modified fruit retention, period required for harvesting after bagging, physico-chemical composition of mature and ripe fruit, shelf life, occurrence of spongy tissue and pest incidence. Bagging with newspaper bag and brown paper bag improved fruit retention, weight of fruit, diameter of fruit, pulp weight, total soluble solids and reducing sugars at ripe stage and produced spongy tissue free fruits. The brown paper bag with polythene coating improved fruit retention, weight of fruit, pulp weight and decreased occurrence of spongy tissue and incidence of mealy bag. The butter paper bag, muslin cloth bag and scurting bag improved fruit retention, reduced occurrence of spongy tissue and incidence of mealy bag. Preharvest bagging with different types of bag did not change the sensory qualities of ripe fruits mango cv. Alphonso.

Keywords: mango (*Mangifera indica* L.), 'Alphonso', marble stage, bagging, fruit retention, physico-chemical composition, spongy tissue

1. Introduction

Mango (*Mangifera indica* L.) is the 'National Fruit' of India. Among the different varieties dominantly cultivated in India, Alphonso is the choicest variety. It is especially preferred for its exemplary flavour, attractive golden yellow fruit colour, orange flesh colour, good keeping quality and excellent processing properties. The Konkan region of Maharashtra is one of the major mango growing belts in India. Mango is established in Konkan on 1.85 lakh hectares of which about 90 per cent is occupied by 'Alphonso' (Haldankar et al., 2013). 'Alphonso' is the major source of economy and livelihood in this region. An attractive, spotless and pest free fruits of this variety fetch premium rate in the market. In recent years, the climatic aberrations such as sudden rise in the temperature and humidity, abnormal rains especially during fruit development are often experienced. It had not only affected the external appearance of the fruit but also aggravated the pest such as mealy bugs and physiological disorder like spongy tissue which further added in the losses. The affected fruits gain poor price in the market and such fruits are also rejected for processing. It causes serious economic loss to mango growers. Recently, the pre harvest bagging technique of fruits has shown promise in the fruits like banana, litchi and apple (Sharma, Reddy, & Jhalegar, 2014). It provides physical barrier over fruit and prevent mechanical damage and bruises to fruit, protect the fruit from pest and diseases and also help for appropriate fruit development (Sharma et al., 2014). Several types of locally available materials can be used for bagging. However, the technique is seldom attempted in mango in India and specifically in Alphonso under Konkan agro climatic conditions. Hence, an experiment was undertaken to study the influence of bagging of fruits at marble stage on quality of mango cv. Alphonso.

2. Materials and Methods

The trial was conducted in the mango orchard of cv. Alphonso at Department of Horticulture, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri (MS) India, 415712 from 2013 and 2014 for consecutive two years during February to May. The soil of experimental plot was red lateritic with uniform depth and good drainage conditions. Uniformly grown 18 year old Alphonso mango grafted trees were selected. The experiment was conducted in Randomised Block Design with eight treatments replicated three times with a unit of 40 fruits per treatment per replication. Different types of bags constituted the treatments *viz*.: T_1: News paper bag; T_2: Brown paper bag; T_3: Scurting bag; T_4: Polythene bag; T_5: Butter paper bag; T_6: Muslin cloth bag; T_7: Brown paper bag with polythene coating; T_8: control (no bagging). Uniformly grown fruits at marble stage (30 days after fruit set) were selected for bagging. The size of bags was 25 × 20 cm. Before bagging six perforations (≤ 4 mm diameter) were made for proper ventilation at the bottom of all bags except for scurting and muslin cloth bags. The particular bags were stapled properly at the stalk of each fruit of respective treatments so that it would not be fall down as well as there would not be open space. The scurting and muslin cloth bags were tied with the help of thread. The observations *viz.* fruit retention (%) and day's require for harvesting after bagging were recorded. Four fruits were randomly selected per treatment per replication to record various physical and chemical observations. The physical and chemical composition was estimated by the following procedures

2.1 Length and Diameter of Fruit (cm)

The length from stalk end to the apex of fruit and diameter was measured with the help of digital Vernier calliper and expressed in centimeters (cm).

2.2 Fruit Weight and Pulp Weight (g)

The weight of fruit was recorded by using monopan electronic balance and expressed in grams (g). Then the pulp weight was meseared by same method.

2.3 Total Soluble Solid (TSS)

5g pulp of was crushed in mortar and pestle which was transferred to 100 ml beaker and diluted in 1:2 proportions with distilled water. Total soluble solids were found out by using Erma Hand Refractometer (0 to 32°Brix) and expressed in ^{o}Brix (A.O.A.C., 1980).

2.4 Citric Acid (%)

5g pulp of was crushed in mortar and pestle and transferred to 100 ml volumetric flask. Distilled water was added to make volume up to 100 ml. Then the sample was filtered and 25 ml filtrate was taken in the beaker and was titrated against 0.1N NaOH using phenolphthalein as an indicator. The results were expressed in percent of citric acid (Ranganna, 1997).

2.5 Reducing Sugars

5 g of pulp was crushed in mortar and pestle. It was transferred to 250 ml volumetric flask. To this, 100 ml of distilled water was added and the contents were neutralized by 1N Sodium Hydroxide. Then, 2 ml of 45 per cent lead acetate was added to it. The contents were mixed well and kept for 10 minutes. Appropriate quantity (2.5 ml) of 22 per cent potassium oxalate was added to it to precipitate the excess of lead. The volume was made to 250 ml with distilled water and solution was filtered through Whatman No.40 filter paper. Determination of reducing sugars was done by the method of Lane and Eynon (1923) as described by Ranganna (1997). The results were expressed on per cent basis.

2.6 Total Sugars

In 100 ml volumetric flask, 50 ml of diluted sample prepared for reducing sugar estimation was taken. To this, 5 ml HCl (1:1) was added and allowed to stand at room temperature for 24 hours. The flask was then kept in thermostatic water bath at 70 ºC to 80 ºC temperature for 30 minutes. The hydrolysed sample was neutralized by adding pinch of Sodium Carbonate till formation of effervescence stopped. After cooling, the volume was adjusted to 100 ml with distilled water. This sample was used for determination of total sugars by the method of Lane and Eynon (1923) as described by Ranganna (1997).

2.7 Ascorbic Acid (mg/100g of Fruit Pulp)

Determination of ascorbic acid was done by 2, 6 - dichlorophenol indophenol dye method of Johnson (1948) as described by Ranganna (1997). 5 ml of sample was blended with 3 per cent metaphosphoric acid (HPO_3) to make the final volume of 100 ml and then filtered. 25 ml quantity of aliquot was titrated against 0.025 per cent 2,

6 - dichlorophenol indophenol dye to a pink colour end point. The ascorbic acid content of the sample was calculated taking into consideration the dye factor and expressed as mg ascorbic acid per 100 g fruit pulp.

2.8 β - carotene (µg/100 g of Pulp)

Total carotenoid pigments (expressed as β–carotene) were determined as per the method described by Roy (1973) as described by Ranganna (1997). The results were expressed in terms of β– carotene as µg/100 g sample.

2.9 Shelf Life of Fruits (Days)

The end of shelf life was noted when the fruits were spoiled.

The mature fruits were harvested at 80 - 85 percent maturity. Twenty harvested mature fruits of each treatment were ripened at ambient temperature by using traditional paddy straw as ripening material. In this method plastic crates with perforation were used. At the bottom, 2.5 cm layer of paddy straw was made on which fruit were arranged. Simultaneously, two more layers were kept on the first layer. After ripening the various observations *viz*. shelf life (days) and incidence of spongy tissue (%) were recorded. The end of shelf life was noted when the fruits were spoiled. The chemical compositions *viz*. TSS (^{o} Brix), acidity (%), reducing sugars (%) and total sugars (%) were estimated by the above given procedures. The observations on incidence of mealy bug (%) were recorded. The ripe fruits were also examined for their sensory qualities for assessing colour, flavour and texture by panel of five judges with nine point Hedonic Scale *viz*.1-Dislike extremely, 2-Dislike very much, 3-Dislike moderately, 4-Dislike slightly, 6-Like slightly, 7-Like moderately, 8-Like very much and 9-Like extremely (Amerine, Pangborn, & Rocssler, 1965).

3. Statistical Analysis

The statistical analysis was performed as per the ANOVA suggested by Panse and Sukhatme (1997). The P values of data were estimated by students paired T-Test. SD was computed as per the procedure advocated by Rangaswamy (1995).

4. Results and Discussion

Fruit retention was significantly improved by pre-harvest bagging with newspaper bag (71.25%), brown paper bag (71.67%) and scurting bag (71.67%) over control (Table 1). The fruit retention found in butter paper bag (68.75%), muslin cloth bag (68.58%) and brown paper bag with polythene coating (67.92%) was also higher than control but the difference was non-significant. The harvesting was significantly preponed in polythene bag, scurting bag, butter paper bag, muslin cloth bag whereas in newspaper bag, it was significantly delayed. The polythene bag (62.50 days) took minimum days for harvest after bagging. The treatments newspaper bag, butter paper bag, muslin cloth bag, brown paper bag and brown paper bag with polythene coating were at par with control (65.00 days) for days required for harvest after bagging. The abiotic factors *viz*. temperature and humidity play critical role in fruit growth and development. Bagging on fruits alters the microenvironment around fruits (Sharma et al., 2014). The early harvesting of fruits bagged with polythene bag and delay in harvesting of fruits bagged with news paper bag has been reported in Tomato, Litchi and Fuji Supreme Apple (Leite et al., 2014; Debnath & Mitra, 2007; Fallahi, Colt, Baird, & Chun, 2001).

Table 1. Effect of types of bag on fruit retention and days required for harvesting after bagging in mango fruit cv. Alphonso (2013-2014)

Treatments	Fruit retention (%)	Days required for harvesting after bagging
Newspaper bag	71.25	67.5
	(71.25±0)	(67.50±0)
Brown paper bag	71.67	66
	(71.67±0.72)	(66± 0)
Scurting bag	71.67	64.5
	(71.67± 1.90)	(64.50± 0)
Plastic bag with perforations	65	62.5
	(65± 0.72)	(62.50± 0)
Butter paper bag	68.75	64.5
	(68.75± 1.44)	(64.50±2)
Muslin cloth bag	68.58	64.5
	(68.58± 2.62)	(64.50± 0)
Brown paper bag with polythene coating	67.92	66
No Bagging	(67.92± 2.16)	(66± 0)
	66.25	65
Range	(66.25± 0.72)	(65±2.5)
Mean	65.00-71.67	62.50-67.50
S. Em ±	68.88	65.06
C. D. at 5%	0.86	0.61
P – Value	2.61	1.84
	0.000385	0.002389

Preharvest bagging with newspaper bag, brown paper bag and brown paper bag with polythene coating improved physical parameters *viz*: weight of fruit, length of fruit, diameter of fruit and pulp weight over unbagged control fruits, and the variation was statistically significant (Table 2). The fruits bagged in polythene bag produced the smallest fruit having (225.78 g) fruit weight and diameter of (7.43 cm). The polythene bag exhibited the fruits with best pulp to stone ratio (6.76). Preharvest bagging with newspaper bag, butter paper bag and muslin cloth bag also recorded superior pulp to stone ratio over unbagged control fruits. Covering fruit with a bag at a particular developmental stage may influence their growth and size. Reports on effects of fruit bagging on fruit size and weight opined that it may be due to differences in the type of bag used, fruit and cultivar responses. (Sharma et al., 2014). Bagging 'Nam Dok Mai 4' mango fruit with two-layer paper bags, newspaper, or golden paper bags increased fruit weight. (A. Watanawan, C. Watanawan, & Jarunate, 2008). Bagging increased fruit weight, size over unbagged control fruits. (Chonhenchob et al., 2011). Microenvironment created by news paper bag, brown paper bag and brown paper bag with polythene coating might have congenial effect on fruit growth of mango cv. Alphonso. All these three treatments recorded more period for harvesting than that of unbagged control fruits. The fruits bagged in polythene bag were harvested earlier than those of unbagged fruits. The preharvest bagging was found beneficial to increase to fruit weight of BC-2, Fuji Apple (Fallahi et al., 2001). Bagging promoted longan fruit development, resulting in larger-sized fruit (Yang et al., 2009).

Table 2. Effect of types of bag on physical parameters of mango cv. Alphonso (2013-2014)

Treatments	Weight of fruit (g)	Length of fruit (cm)	Diameter of fruit (cm)	Pulp weight (g)	Stone weight (g)	Pulp: stone ratio
Newspaper bag	264.07	8.87	7.79	206.55	32.89	6.37
	(264.07±10)	(8.87±0.14)	(7.79±0.10)	(206.55±9.09)	(32.89±1.81)	(6.37±0.03)
Brown paper bag	254.47	8.89	7.74	195.21	33.69	5.8
	(254.47± 8.48)	(8.89±0.13)	(7.74±0.12)	(195.21±8.91)	(33.69±0.87)	(5.80±0.07)
Scurting bag	243.53	8.53	7.56	185.79	31.15	5.98
	(243.53± 6.48)	(8.53±0.10)	(7.56± 0.02)	(185.79±3.77)	(31.15±1.04)	(5.98±0.02)
Plastic bag with perforations	225.78	8.37	7.43	182.57	28.78	6.76
	(225.78±15.38)	(8.37±0.25)	(7.43±0.26)	(182.57±11.07)	(28.78±0.74)	(6.76±0.05)
Butter paper bag	245.65	8.61	7.59	192.14	33.07	6.25
	(245.65±25.88)	(8.61±0.14)	(7.59± 0.12)	(192.14±5.81)	(33.07±0.57)	(6.25±0.10)
Muslin cloth bag	239.24	8.5	7.63	187.96	30.56	6.15
	(239.24± 4.14)	(8.50±0.03)	(7.63±0.05)	(187.96±3.95)	(30.56±0.20)	(6.15±0.02)
Brown paper bag with polythene coating	251.37	8.74	7.69	194.47	34.72	5.7
	(251.37±11.95)	(8.74±0.15)	(7.69±0.14)	(194.47±8.04)	(34.72±1.90)	(5.70±0.04)
No Bagging	232.46	8.3	7.45	180.62	31.06	5.92
	(232.46± 4.88)	(8.30±0.15)	(7.45± 0.10)	(180.62±4.89)	(31.06±0.77)	(5.92±0.07)
Range	225.78-264.07	8.30-8.89	7.43-7.79	180.62-206.55	28.78-34.72	5.70-6.76
Mean	244.57	8.6	7.6	190.66	31.98	6.11
S. Em ±	4.98	0.08	0.05	3.97	0.63	0.03
C. D. at 5%	15.09	0.26	0.17	12.04	1.91	0.09
P - Value	0.0020874	0.0014875	0.0083438	0.008764	0.000219	0.0000001

The pre-harvest bagging had non-significant effect on total sugars and ascorbic acid content of fruits at harvest (Table 3). The unbagged control fruits recorded the highest acidity (3.45%) and TSS (7.82 ^{o}Brix) which was significantly superior over all bagging treatments. The fruits of treatment polythene bag had significantly highest reducing sugars and βeta carotene. The variation observed in chemical composition of mango fruits can be attributed to the changed microenvironment around fruit during its growth and development. The bagged fruits recorded highest content of vitamin C, sucrose, glucose and fructose over control in Zill mango (Hongxia et al., 2009). The bagging of date palm fruits improved the total sugars (Harhash & Al-Obeed, 2010). Bagging enhanced carotenoid content in mango (Zhao, Wang, Zhang, Huan, & Gao, 2013).

Table 3. Effect of types of bag on chemical composition of mango cv. Alphonso fruit at harvest (2013-2014)

Treatments	Citric acid (%)	TSS (°Brix)	Reducing sugars (%)	Total sugars (%)	Ascorbic acid (mg/100 g)	β – carotene (µg /100 g)
Newspaper bag	2.97 (2.97±0.01)	7.46 (7.46±011)	1.13 (1.13±0.01)	2.13 (2.13±0.19)	76.81 (76.81±1.26)	312.1 (312.10±2.05)
Brown paper bag	3.01 (3.01±0.02)	7.44 (7.44±0.10)	1.21 (1.21±0.02)	1.97 (1.97±0.26)	77.36 (77.36±0.87)	306.75 (306.75± 3.12)
Scurting bag	3.39 (3.39±0.05)	7.61 (7.61±0.04)	1.16 (1.16±0.05)	2.3 (2.30±0.43)	75.78 (75.78±0.41)	316.58 (316.58± 1.23)
Plastic bag with perforations	2.89 (2.89±0.01)	7.58 (7.58±0.02)	1.35 (1.35±0.07)	1.92 (1.92±0.48)	74.77 (74.77±2.08)	317.56 (317.56± 0.71)
Butter paper bag	3.24 (3.24±0.02)	7.4 (7.40±0.02)	1.2 (1.20± 0.06)	1.75 (1.75±0.09)	77.97 (77.97±0.89)	311.51 (311.51± 1.86)
Muslin cloth bag	2.9 (2.90±0.04)	7.56 (7.56±0.12)	1.06 (1.06± 0.03)	1.7 (1.70±0.22)	76.84 (76.84±0.62)	312.76 (312.76± 1.82)
Brown paper bag with polythene coating	3.01 (3.01±0.03)	7.5 (7.50±0.02)	1.16 (1.16± 0.02)	1.89 (1.89±0.01)	74.05 (74.05±0.68)	309.45 (309.45± 2.52)
No Bagging	3.44 (3.44±0.04)	7.82 (7.82±0.05)	1.12 (1.12± 0.01)	2.03 (2.03±0.02)	76.74 (76.74±1.50)	310.63 (310.63± 4.24)
Range	2.89-3.44	7.40-7.82	1.06-1.35	1.70-2.30	74.05-77.97	306.75-317.56
Mean	3.1	7.54	1.17	1.96	76.28	312.16
S. Em ±	0.02	0.04	0.02	0.16	0.69	1.47
C. D. at 5%	0.06	0.13	0.07	NS	NS	4.46
P - Value	0.00000000001	0.000284	0.000052	0.263963	0.018502	0.002539

Fruits of newspaper bag exhibited the maximum TSS (16.10 ^{o}Brix) and reducing sugars (2.06%) at ripe stage (Table 4). It was followed by brown paper bag (15.99 ^{o}Brix, 2.05%). Fruits of polythene bag (0.44%) had maximum acidity followed by brown paper bag with polythene coating (0.43%), control (0.39%) and butter paper bag (0.37%) which were at par with each other. brown paper bag with polythene coating (7.48%) recorded the maximum total sugars which was significant whereas muslin cloth bag displayed the maximum ascorbic acid and βeta carotene at ripe stage. Sensory evaluation with respect to colour, flavour, texture was non-significant among various treatments under study. It indicated that the organoleptic qualities of fruit were not affected by pre-harvest bagging in mango cv. Alphonso. The bagging led to lower contents of chemical components such as sugar, phenols and organic acids in most of peach varieties (Lima, Angelo, Marcelo, Deyse, & Elisa, 2013). Fruit firmness was slightly increased by bagging treatments, whereas soluble solids content was decreased in apple (Feng, Mingjun, Fengwang, & Lailiang, 2014).

Table 4. Effect of types of bag on chemical composition and sensory evaluation of mango cv. Alphonso fruit at ripe stage (2013-2014)

Treatments	Citric acid (%)	TSS (°Brix)	Reducing sugar (%)	Total sugars (%)	Ascorbic acid (mg/100g)	β – carotene (µg /100 g)	Sensory Evaluation		
							Colour	Flavour	Texture
Newspaper bag	0.36 (0.36±0.01)	16.1 (16.10±0.37)	2.06 (2.06±0.12)	6.49 (6.49±0.01)	52.95 (52.95±0.78)	11143.69 (11143.69±260.59)	8 (8±0.25)	7.67 (7.67±0.14)	7.67 (7.67±0.38)
Brown paper bag	0.3 (0.30±0.02)	15.99 (15.99±0.49)	2.05 (2.05±0.03)	6.78 (6.78±0.16)	53.44 (53.44±0.09)	11067.61 (11067.61± 131.89)	8 (8±0.25)	7.83 (7.83±0.38)	7.83 (7.83±0.14)
Scurting bag	0.33 (0.33±0.01)	15.61 (15.61±0.34)	1.87 (1.87±0.04)	6.48 (6.48±0.09)	53.19 (53.19±0.19)	11533.38 (11533.38± 186.57)	7.75 (7.75±0.25)	7.75 (7.75±0.25)	7.83 (7.83±0.14)
Plastic bag with perforations	0.44 (0.44±0.03)	15.24 (15.24±0.15)	1.88 (1.88±0.03)	6.98 (6.98±0.02)	54.54 (54.54±0.63)	11572.08 (11572.08± 83.75)	7.5 (7.50±0.5)	7.58 (7.58±0.72)	8 (8±00)
Butter paper bag	0.37 (0.37±0.01)	15.78 (15.78±0.22)	2 (2.06±0.06)	6.43 (6.43±0.04)	52.66 (52.66±0.26)	11335.26 (11335.26± 95.76)	8 (8±0.25)	8.08 (8.08±0.14)	7.92 (7.92±0.29)
Muslin cloth bag	0.35 (0.35±0.02)	15.82 (15.82±0.24)	1.87 (1.87±0.04)	6.99 (6.99±0.04)	55.54 (55.54±0.32)	11754.34 (11754.34± 157.59)	8 (8±00)	7.92 (7.92±0.38)	8 (8±0.25)
Brown paper bag with polythene coating	0.43 (0.43±0.01)	15.85 (15.85±0.23)	1.97 (1.97±0.11)	7.48 (7.48±0.06)	53.94 (53.94±0.21)	11236.37 (11236.37± 58.42)	8 (8±0.5)	8 (8±0.5)	8.08 (8.08±0.29
No Bagging	0.39 (0.39±0.02)	15.64 (15.64±0.61)	1.86 (1.86±0.04)	6.81 (6.81±0.10)	52.73 (52.73±0.47)	11099.79 (11099.79± 271.45)	8 (8±0.43)	7.75 (7.75±0.5)	7.75 (7.75±0.43)
Range	0.30-0.44	15.24-16.10	1.86-2.06	6.43-7.48	52.66-55.54	11067.61-11754.34	7.50-8	7.58-8.08	7.67-8.08
Mean	0.37	15.75	1.95	6.8	53.62	11342.82	7.91	7.82	7.89
S. Em ±	0.01	0.22	0.04	0.01	0.26	104.83	0.19	0.21	0.17
C. D. at 5%	0.03	0.65	0.12	0.03	0.79	317.96	NS	NS	NS
P - Value	3.1157E-06	0.254194	0.003788	0.000000008	0.0000193	0.002677	0.51941	0.703268	0.674265

The unbagged control fruits of 'Alphonso' had shelf life of 15 days (Table 5). The fruits of newspaper bag (17.50 days), brown paper bag (16.50 days), brown paper bag with polythene coating (16.00 days) and muslin cloth bag (15.00 days) had greater shelf life than control (15.00 days). The fruit of scurting bag (13.50 days) had shortest shelf life. All bagging treatments showed fewer incidence of mealy bags and spongy tissue as compared to control. The fruits bagged in newspaper bag were totally free from mealy bags as well as spongy tissue. The fruits of polythene bag and butter paper bag were free from mealy bugs, whereas fruits of brown paper bag were free from spongy tissue. The maximum incidence of mealy bugs (9.63%) and spongy tissue content (9.00%) was recorded in control. Bagging modified the microenvironment near fruit especially in respect to temperature and humidity. The humidity as well as temperature in plastic bag was greater than that in news paper bag. The longer shelf life of bagged fruits indicated that the effect of bagging persisted after ripening. Bagging provided physical barrier between fruit and pests. The spongy tissue disorder is associated with convective heat (Katrodia, 1989) and exposure of fruit to sunlight (Om & Prakash, 2004). Bagging provides protection against both which helped in reducing occurrence of spongy tissue in fruits. In mango cv. Keitt white paper bags at approximately 100 days before harvest reduced anthracnose and stem end rot (Hofman, Smith, Joyce, Johnson, & Meiburg, 1997).

Table 5. Effect of types of bag on chemical composition and sensory evaluation of mango cv. Alphonso fruit at ripe stage (2013-2014)

Treatments	Shelf life (days)	Mealy bugs (%)	Spongy tissue (%)
Newspaper bag	17.5 (17.50±2)	0	0
Brown paper bag	16.5 (16.50±0)	4.17 (4.17±0)	0
Scurting bag	15 (15±0)	1.67 (1.67±0)	1.72 (1.72±0.48)
Plastic bag with perforations	13.5 (13.50±0)	0	6.17 (6.17± 1)
Butter paper bag	14.5 (14.50±0)	0	0.67 (0.67± 0)
Muslin cloth bag	15.5 (15.50±0)	2.84 (2.84±0)	0.84 (0.84±0)
Brown paper bag with polythene coating	16 (16±0)	3.33 (3.33±0)	2.39 (2.39±0.96)
No Bagging	15 (15±1)	9.63 (9.63±2.24)	9 (9± 0)
Range	13.50-17.50	00-9.63	00-9.00
Mean	15.43	2.7	2.59
S. Em ±	0.43	0.46	0.27
C. D. at 5%	1.3	1.39	0.82
P - Value	0.0004431	0.00000001	0.0000000000049

5. Conclusion

Thus, investigation revealed that preharvest bagging at 30 days after fruit set with various types of bag modified fruit retention, period required for harvesting, physico-chemical composition, shelf life, occurrence of spongy tissue and pest incidence in mango cv. Alphonso. Bagging with newspaper bag and brown paper bag improved fruit retention, weight of fruit, diameter of fruit, pulp weight, total soluble solids and reducing sugars at ripe stage and produced spongy tissue free fruits. The brown paper bag with polythene coating improved fruit retention, weight of fruit, pulp weight and decreased occurrence of spongy tissue and incidence of mealy bag. The butter paper bag, muslin cloth bag and scurting bag improved fruit retention, reduced occurrence of spongy tissue and incidence of mealy bag. Preharvest bagging with different types of bag did not change the sensory qualities of ripe fruits of mango cv.Alphonso.

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Presence of Adhesive Vesicles in the Mycoherbicide *Alternaria helianthi*

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Abstract

Alternaria helianthi conidia have been shown to cause disease on common cocklebur. *Alternaria helianthi* conidia grown at 18 °C are more virulent than those grown at 28 °C, and adhere to the leaves of the treated plants and causes necrotic lesions, stunting and mortality in common cocklebur at the 6- to 12-leaf stage. Using confocal laser scanning microscopy (CLSM) the virulent conidia produced multiple branched germ tubes. The distribution of the adhesive material on the conidial surface was varied, being evenly distributed on some conidia while appearing as globules on others. Examination by transmission electron microscopy (TEM) showed that virulent conidia had dense ribosomes and abundant endoplasmic reticulua indicating actively synthesizing cytoplasm. Adhesive vesicles, which appear to be the means of export of the adhesive from the cytoplasm, were often arranged along external cell walls. An osmophilic material, possibly the adhesive substance itself, was seen between the cell membrane and the cell wall. This substance may play an important role in the virulence of *A. helianthi* to common cocklebur and survival of this pathogen.

Keywords: ultrastructure, adhesive material, electron microscopy, fungi, conidia, biological control, temperature

1. Introduction

Common cocklebur (*Xanthium strumarium* L.) is an important weed in many areas and on many crops (Abbas et al., 1996; Abbas et al., 1999; Bloomberg et al., 1982; Buchanan & Bruns, 1971; Holm et al., 1977; Soltani et al., 2010; Weaver & Lechowicz, 1983). *Alternaria helianthi* (Hansf.) Tubaki and Nishihara] is a pathogen of cocklebur and other plants (Abbas et al., 1995; Morris et al., 1983; Quimby, 1989). *Alternaria helianthi* is an effective mycoherbicide for biological control of cocklebur (Abbas and Barrentine, 1995; Abbas and Egley, 1996; Abbas et al., 1996; Abbas et al., 2004; Sanyal et al., 2008; Quimby, 1989). We have previously reported that temperature is an important factor in the development of disease on common cocklebur caused by *A. helianthi*. Conidia of *A. helianthi* produced at 18 °C were more virulent than those grown at 28 °C.

There are many reports that many fungi such as *Alternaria* species, *Uncinuliella australiana* [(Ces.) Wils.], *Colletotrichum graminicola* [(McAlpine) R. Y. Zheng & G. Q. Chen] produce adhesive vesicles involved in their survival and pathogenicity (Mims et al., 1995a; Mims et al., 1995b; Mims et al., 1997; Silva et al., 2014).

Electron microscopy has been an excellent tool in determining the mechanism of infection of hosts by fungal pathogens (Cleary et al., 2013; Garcia et al., 2012; Silva et al., 2014). *Ascochyta anemones* causes leaf spot of windflower in China. It has been reported that windflower is infected by one tube from the fungus penetrating the leaf cuticle (Dan et al., 2012). In infection of barley by *Erysiphe gramimis*, the fungus dissolves the cuticle for a point of entry (Kunoh et al., 1988; Nicholson & Epstein, 1991). The fungus Zygophiala *jamaicensis* causes flyspec on fruits to secrete large amounts of mucilage creating a path for the fungal hyphae to penetrate the cuticle (Nasu & Kunoh, 1986). Deising et al., 1992 described what is termed an adhesion pad in *Uromyces* providing information that serine esterase is essential to the adherence of the pad to the leaf surface. It has been reported that the fungus *Colletotrichum graminicola* releases adhesive materials which are responsible for adherence to hydrophobic surfaces (Mercure et al., 1994; 1995). More recently Silva et al., 2014 described adhesive materials that play a very important role in the virulence of the fungus *Alternaria infectoria*.

In this research, we have attempted to determine by electron microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), how the adhesive apparatus of *A. helianthi* conidia attaches to the host, common cocklebur and establishes infection, causing disease and death of the plant.

2. Method

2.1 General

2.1.1 Conidia of *Alternaria helianthi*

Culture and growth of *A. helianthi* at various temperatures as well as histochemical and ultrastructural studies were described in detail by Abbas et al., 1995. In order for the conidia of *A. helianthi* to be used for the adhesive materials research, we ran quality control evaluation of conidia for their virulence using the assays described in detail in (Abbas & Barrentine, 1995; Abbas et al., 1995; Abbas et al., 2004). All the evaluations were measured visually by looking for damage symptoms on 3 replicates of 6 plants each in the greenhouse. Evaluations occurred after 10 – 14 days post inoculation. Symptoms included necrotic lesions, growth inhibition, and mortality (Figure 1). Conidia were evaluated for germination using light and electron microscopy for number, length of germ tube and also using visual assessment (Figure 2). Figures 1 and 2 were generated from the same conidia used to study the adhesive materials (Figures 3 and 4). This was done to make sure the research for the adhesive materials would be conducted on virulent conidia.

2.2 Adhesive Material Studies

All sample protocols including preparation of samples, materials, steps for cutting and bedding of samples, and conditions of electron microscopy in this study were as described before in detail by Abbas et al., 1992; and Abbas et al., 1995. Briefly, all specimens were fixed in 4% glutaraldehyde + 0.5% caffeine for 3 hrs at room temperature. After washing (1hr in buffer) some tissues were stained in block before embedding with mercuric bromphenol blue (MBB), a general protein stain (Abbas et al., 1995). Another set of tissues was set aside after initial fixation for sectioning and histochemistry. The rest of the samples were post-fixed in 1% OsO_4 for 2 hrs. All tissues were then washed in distilled H_2O, dehydrated in acetone and embedded in Spurr's resin. A set of samples from each of the following lists were semi-thin sectioned, attached to glass slides and stained in toluidine blue for general light microscope observation.

2.3 Confocal Laser Scanning Microscopy

Conidia cells were adhered to slides dipped in dimethyldichlorosilane solution (5% solution [v/v] in methylene chloride). This makes the surface hydrophobic, emulating leaf wax (Mercure et al., 1994). Aqueous suspensions of conidia were placed on the dipped surface and allowed to sit for 0.5 – 1 hr. The slides containing the conidia were rinsed twice in 1 ml 0.05 M glycine in phosophate buffered saline (PBS) and then stained 0.5 hr with FITC conjugated lectin Con A (Sigma-Aldrich) at a concentration of 200 µg per ml of PBS solution (Mercure et al., 1995). A drop of 50% glycerin water was placed over the adhered, stained conidia and the samples were observed using a Zeiss LSM 410 confocal microscope.

2.4 Transmission Electron Microscopy

Specimens were fixed in 4% glutaraldehyde + 0.5% caffeine for 3 hrs at room temperature. After washing 1 hr in cacodylate buffer, the tissue was post fixed in 1% OsO_4 for 2 hrs. The material was washed 1 hr in distilled water, dehydrated in a graded acetone series and embedded in Spurr's resin. Thin sections were stained with 1% uranyl acetate, post-stained in lead citrate, then observed and photographed using a Zeiss EM 10 transmission electron microscope.

2.5 Scanning Electron Microscopy

Conidia adhered to dimethyldichlorosilane coated (dipped) cover slips were fixed in osmium vapor overnight, allowed to air dry, then coated with 20 nm of Au/Pd. The specimens were observed and photographed in a JEOL JSM 840 scanning microscope.

3. Results

3.1 General Description of Samples

Conidia of *A. helianthi* produced at 18°C were virulent on common cocklebur plants. The treated plants exhibited damage symptoms of necrotic lesions on plant tissues (leaves and stems), growth inhibition, and mortality (Figure 1) to 6- to 12- leaf multiple seeded common cocklebur (MSC) and to 6- to 8- leaf normal common cocklebur (NCC) in greenhouse when plants were treated with 50,000 conidia /ml, after 10 to 14 days

after treatment.

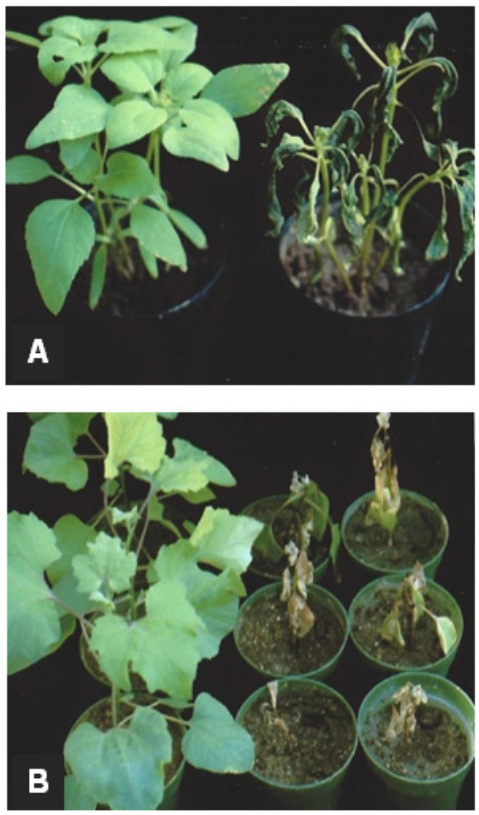

Figure 1. A. Multiple seeded common cocklebur (Abbas et al., 2004; Sanyal et al., 2008); and B. normal common cocklebur (Abbas and Barrentine, 1995; Abbas et al., 1996) plants treated with conidia produced at 18 °C, 14 days after treatment with control on the left , respectively

The conidia of *A. helianthi* are multicellular. There appears to be some division of labor within the individual conidium in that the individual segments often stain very differently. Some segments, even in the 18 °C control tissue appear empty. The number of empty segments increases with exposure to 28 °C temperature with the greatest change(s) occurring at 48 - 72 hrs. At 72 hrs, there are almost no unaffected conidia. Most conidia at this temperature consist of predominantly empty shells with only a few filled. At 28 °C, conidial segments filled with cytoplasm are rare, but make up the most visible aspect of the samples since the predominant structures are the empty shells of atrophied conidia. Also, under both light and electron microscopy, conidia grown at 18 °C produced aggressive, branched, and long and multiple germ tubes (Figure 2).

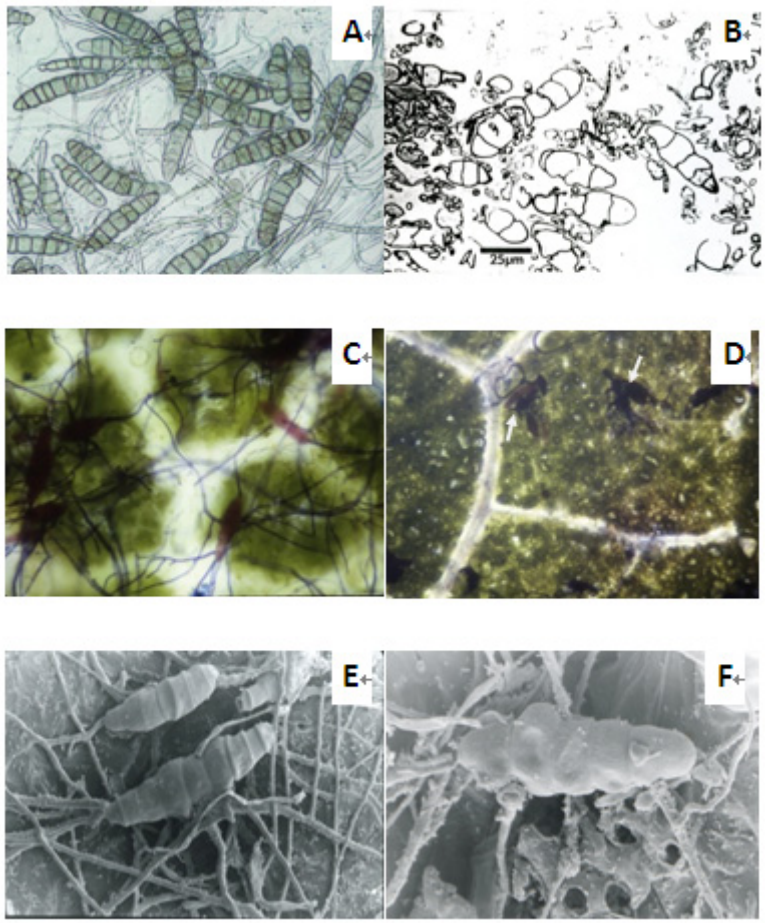

Figure 2. A. Conidia of *A. helianthi* grown at 18 °C. B. Conidia of *A. helianthi* grown at 28 °C. C. Germinating conidia on leaf surface of common cocklebur (18 °C). Note multiple germ tubes after 24 hrs after application. D. Germinating conidia on leaf surface of common cocklebur (28 °C). Note a very few, weak, short multiple germ tubes after 24 hrs after application in comparison with germinating conidia (18 °C) described previously in C. E & F. SEM of germinating conidia of *A. helianthi* grown at 18 °C. Note multiple germ tubes with clear point of the emerging of the germ tube from the cell of each conidia in close up as shown in F. (A, B, C, & D bars = 25 µm; and E & F bars = 10 µm)

Also, conidia produced at 18 °C under electron microscopy demonstrated the presence of adhesive materials (Figures 3 and 4). Conidia produced at 28 °C were less virulent, produced weak, short, non-branched germ tubes, and lacked adhesive materials in comparison to the conidia produced at 18 °C.

3.2 Adhesive Material Studies

3.2.1 Ultrastructural Effects

The individual segments of control (18 °C) conidia apparently vary in physiological state. Adjacent segments often have very different appearances related to inclusions present, cytoplasmic density and organelles visible. At higher magnification, one sees mitochondria (Mt), nuclei (N), microbodies (Mb) and certain inclusions (I). Very dense osmiophilic structures are present randomly spaced in the cytoplasm or lined up along the plasmalemma are apparently related to conidial adhesion, and possibly are carrying adhesive material to the plasmalemma to be exported to the conidial surface.

The conidia produced at 18 °C adhered to the dipped slides in larger numbers in comparison to the control conidia. The conidia grown at 28 °C did adhere, except rarely – most were rinsed off. This indicates a lack of ability of the heat-grown conidia to produce the adhesive material, stick to the surface, and be present for staining. This would affect only the confocal microscopy as described above, since it requires adhesion of the conidia for subsequent image processing. These results are summarized in detail in Figures 3 and 4.

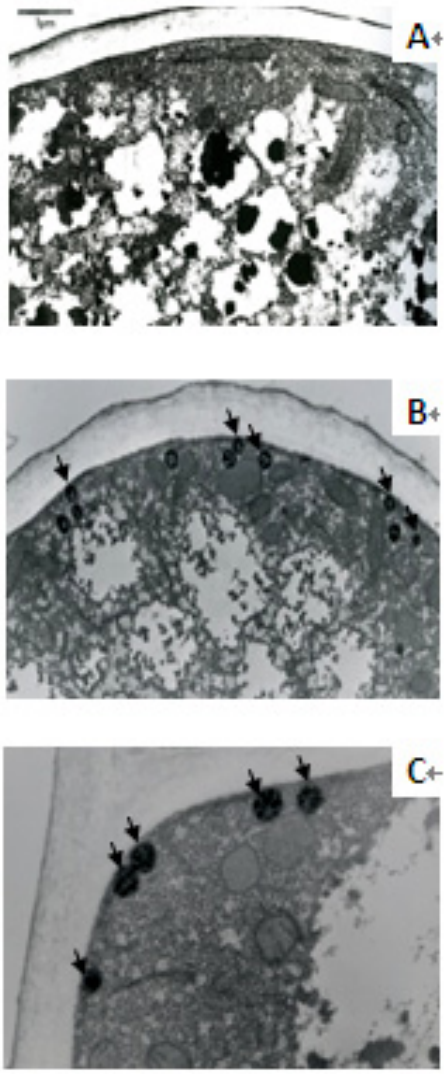

Figure 3. A. TEM of edge of conidium grown at 28 °C. Note the absence of adhesive vesicles when compared to 18 °C grown conidia. B. TEM of edge of conidium grown at 18 °C. Arrows denote adhesive vesicles which are lined close to the cell wall. And C. Close-up shot of B (All bars = 1 µm)

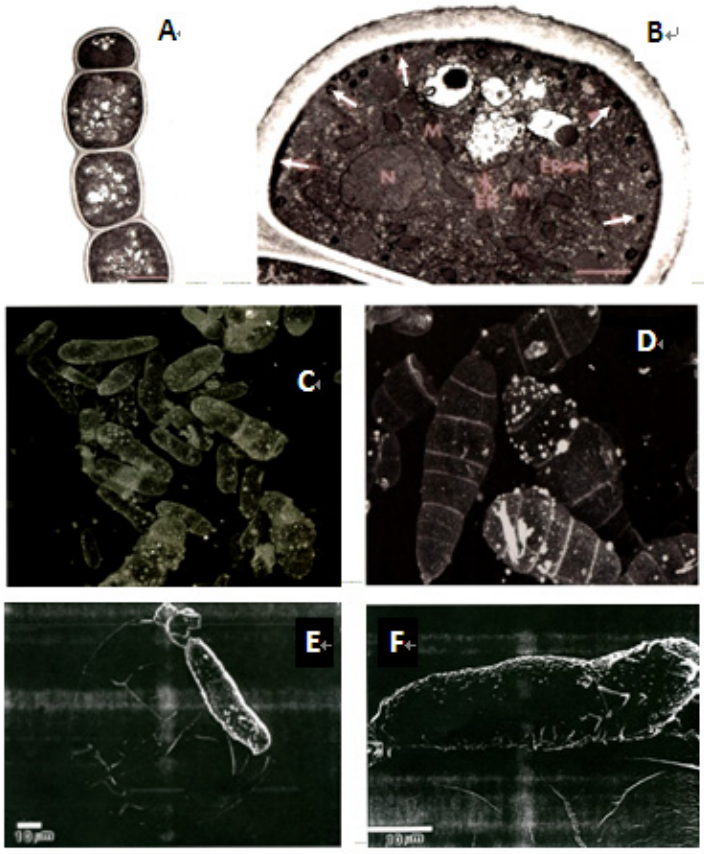

Figure 4. Transmission electron and confocal laser scanning micrographs of *A. helianthi* conidium grown at 18 °C. A. TEM of conidium shows 4 adjacent cells. B. Higher magnification micrograph of apical cell of conidium shown in Figure 4A. Small cytoplasmic inclusions (white arrows) are probably adhesive vesicles arrayed around the cell periphery. These may be involved in transporting adhesive or adhesive precursor material to the space between the cell wall and the cell membrane. A similar material seems to have accumulated in the region. C. Confocal laser scanning microscopy (CLSM) of conidia showing regions of staining by Con A. Some of the conidial cells show blobs of stained material on their surface. D. Close up of some conidia from C. E & F. Scanning electron micrographs of conidia attached to dipped coverslip. E. Overview of one conidium and its adhesive material. F. Tilted, higher magnification of conidium seen in Figure 4 E. Notice the globular protrusions on the conidium surface, and the heavy layer of adhesive material on its lower surface. All photographs made under Nomarski differential interference contrast optics. All bars = 10 µm

4. Discussion

The research of the effect of temperature on conidial formation at 18 °C and 28 °C of the fungus *A. helianthi* showed there are huge differences in gross appearance of these conidia as well as ultrastructural properties. Conidia grown at 18 °C were more aggressive, vigorous and virulent as shown by mortality of cocklebur plants. The conidia produced at 28 °C did not cause mortality or other symptoms on cocklebur and other hosts. These physical findings correlated with ultrastructural characteristics under both light and electron microscopy. These results are in agreement with our previous results studying the influence of temperature on the virulence of *A. helianthi* on several plant hosts including cocklebur (Abbas & Egley, 1996; Abbas et al., 1995; Abbas et al., 1996).

Using the CLSM, the adhesive material was distributed in different ways in various conidia. Some conidia had globules of material and other had a more even distribution. When the TEM was used, the 18 °C conidia had many ribosomes and much endoplasmic reticulum that indicated active synthesis cytoplasm was occurring. The adhesive vesicles were concentrated on or near cell walls. There was an osmophilic material between the cell wall and the cell membrane that presumably is the adhesive substance itself. It is possible when the correct stimulus is received, the material will pass through the cell wall and glue the conidium to the host plant. These

sticky materials have been referred to as adhesive vesicles (Mims et al., 1995a; Nicholson & Epstein, 1991). It has been reported that these adhesive materials appear to disappear with exposure to heat in that they are greatlyreduced in number after 24 hrs and are extremely rare after that (Hyde et al., 1991; Oliveira et al., 2013). When Deising et al., 1992 discovered the adhesion in *Uromyces*, they provided evidence that serine esterases are essential to the adherence of the pad to the leaf surface. In addition to two nonspecific serine esterases, a cutinase was found to be localized on the spore surface. These enzymes were released rapidly from the spore surface upon contact with an aqueous environment. Restoration of the enzymes to the adhesive pads of autoclaved spores made the dead spores adhesive again (Deising et al., 1992). Also, when Silva et al., 2014 addressed the role of the adhesive materials in the pathogenicity of *A. infectoria*, they found about 20 identified proteins, polysaccharides, enzymes involved in the synthesis of pigment, adhesion of material to the host cell and transport of vesicles and other cellular substances inside the cell. They concluded that extracellular vesicles might have a major role in the virulence of *A. infectoria* (Silva et al., 2014). It has been reported extensively in (Nicholson & Epstein, 1991; Oliveira et al., 2013) that multiple fungi have been determined to produce adhesive material that is important in the pathogenicity of the fungi, allowing fungal organisms to attach themselves to the host in a way that they are not easily dislodged. This study showed that these adhesive vesicles are important in the pathogenicity of *A. helianthi* to common cocklebur and other hosts. We have shown that conidia of *A. helianthi* produced at 18 °C are more vigorous than those produced at 28 °C. The electron microscopy demonstrated that adhesive vesicles are more numerous and produce more mucilage at 18 °C that 28 °C. This is consistent with the adhesive vesicles being the delivery system of *A. helianthi* to infect common cocklebur and other hosts. More recently, Oliveira et al., 2013 described extracellular vesicles and their contents. These extracellular vesicles were originally intracellular vesicles produced by endoplasmic reticulum. Vesicles were found to contain virulence factors for fungi. This correlates with the adhesive vesicles produced by *A. helianthi* that seem to enable the conidia to attach to the host plant.

5. Conclusion

Overall, conidia produced at 18 °C are the most virulent, as confirmed by microscopic studies. Production temperature is an important factor to consider for biocontrol of common cocklebur by *A. helianthi.* Adhesive vesicles are present in conidia produced at 18 °C and not in conidia produced at 28 °C. This observation might indicate an important role for adhesive vesicles in the virulence of *A. helianthi* conidia. This substance may play an important role in the virulence of *A. helianthi* to common cocklebur and survival of this pathogen. Further research would be helpful to understand the identity of the adhesive substance and its synthesis in *A. helianthi.*

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Differential Regulation of Superoxide Dismutase Activity in Selected Strawberry Lines Exposed to *Mycosphaerella fragariae*

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Abstract

The effect of leaf infection by the fungus *Mycosphaerella fragariae,* on total superoxide dismutase activity and induction of SOD isozymes was studied under controlled conditions using four selected strawberry cultivars: Kent (HS, highly susceptible), Joliette (HR, highly resistant) and two advanced strawberry lines, SJ8976-1 and APF029-4 (MR, moderately resistant). Observations were made of conidia morphology of *M. fragariae* grown in strawberry leaf agar (SLA) at different stages of conidia development and of infective symptoms of the leaves after inoculation. Inoculation of strawberry leaves with *M. fragariae* increased protein content and SOD activities in all four cultivars. In all cases, total SOD increased 1 day (d) after inoculation, reaching a peak 2 d after inoculation, and slowly declining thereafter. Total SOD activity in Joliette, SJ8976-1 and APF029-4 two days after inoculation was 4376, 4433 and 4283 U g^{-1} FW, respectively, and significantly was lower for Kent (3656 U g^{-1} FW). From the electrophoresis profile of the strawberry cultivars, 2 newly synthesized isozymes were found in infected Joliette and SJ8976-1, (R_f = 0.31 and 0.34), which are believed to be associated with leaf spot resistance.

Keywords: superoxide dismutase, SOD, Kent, Joliette, *M. fragariae*, synthesized isozymes, leaf spot

1. Introduction

During normal cellular activities in plants, various processes, including enzymatic reactions, electron transportation and self-oxidation of small molecules inside the cells, produce reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals ($OH^\bullet$) (Hernández et al., 1995; Foyer & Noctor, 2000; Anderson, 2002). Plant cells can produce ROS when exposed to an unhealthy environment, such as air or metal pollution, UV or water stress, extreme low or high temperatures, pathogen invasion, or herbicide action. In general, ROS are produced in both stressed and non-stressed cells, with the primary sites being the cytosol, chloroplasts, mitochondria, and/or the apoplastic space (Mittler, 2002). There are several mechanisms plants employ to protect them from the toxicity of ROS. Environmental stresses enhance ROS production, resulting in an imbalance between oxidative stress and the detoxification defense systems (Smirnoff, 1998; Kuźniak & Sklodowska, 2004). To limit the damage initiated by ROS, plants have developed a complex and perfect antioxidant system, which includes a number of enzymatic and non-enzymatic ROS detoxifying agents that are distributed in most cellular compartments and have been well characterized (Bowler et al., 1992; Casano et al., 1994). Superoxide dismutases (SOD, EC 1.15.1.1) are the first scavengers of ROS in enzymatic systems and are crucial in defending cells against the associated effects. SODs are metal chelated enzymes that are present in virtually all organisms, including animals, plants, and microorganisms, except for strict anaerobes, and in subcellular compartments where oxidative stress is likely to arise. These enzymes catalyze the dismutation of superoxide to H_2O_2 and O_2 (McCord & Fridovich, 1969); this reaction occurs at a 10 000-fold faster rate than spontaneous dismutation (Bowler et al., 1992). Subsequently, H_2O_2 is catalyzed into H_2O and O_2 by catalase (CAT) and various kinds of peroxidase (POD), such as PAX and the

ascorbate-glutathione cycle.

Leaf spot, caused by *Mycosphaerella fragariae*, is a common foliar disease of strawberry in North America (Dale & Fulton, 1957; Fall, 1951; Fulton, 1958; Plakidas, 1965). In earlier years it was considered the most devastating strawberry disease due to its detrimental economic impact. With increased emphasis on the development and use of resistant cultivars, leaf spot, although still an important foliar disease, is now of less concern (Maas, 1984). Strawberry cultivars differ in their sensitivity to leaf spot; host and environmental conditions during infection are factors that affect the lesions produced by *M. fragariae* (Delhomez et al., 1995; Carisse et al., 2000). Joliette is HR (highly resistant) and Kent HS (highly susceptible) (Delhomez et al., 1995; Khanizadeh et al., 1996). Based on examination and observations of infected leaves in a production context, the two selected strawberry lines, SJ8976-1 and APF029-4, are considered to be MR (moderately resistant).

The objective of this y research was to analyze the activity of SOD during infection of the four strawberry cultivars by leaf spot disease.

2. Materials and Method

2.1 Plant Material and Inoculation

Forty plants of four strawberry cultivars (*Fragaria×ananassa* Duchesne)—Kent (HS), Joliette (HR) and two selected lines, SJ8976-1 and APF029-4 (MR)—were propagated by stolons into 160 pots containing sand, peat moss, and mineral soil in a ratio of 1:1:1 by volume. Plantlets were grown in a greenhouse for 2 months at 25 ºC with a 16-h photoperiod, until inoculation.

The method used to produce *M. fragariae* (Tul.) Lindau (*Ramularia tulasnei* Sacc.) inoculum was formerly described by Delhomez et al. (1995). Stock cultures were temporarily maintained on potato agar slants at 3 ºC. Because pathogenicity may decline during storage, strawberry leaves from Kent were inoculated with the pathogen, and the fungus was re-isolated using a method described by Carisse and Kushalappa (1989), before production of the inoculum. The fungus was then transferred to strawberry leaf agar (SLA) and incubated at room temperature (20 to 25 ºC) for 2 weeks. Spores were produced by inoculating the mycelial suspension onto SLA plates (2 mL/plate), and the spore production plates were incubated at room temperature (20 to 25 ºC) for 1 week. On the day of plant inoculation, a conidial suspension was prepared from the cultures by pouring a solution containing 10 mL of sterile distilled water and 0.01% Tween 80 into each plate. Observations of spore morphology were made using an Infinity 2 digital camera mounted on a Leica DM LB microscope. The final spore suspension was adjusted to 1.14×10^5 spores/mL using a hemocytometer.

Twenty plants of each cultivar were inoculated by spraying the spore suspension onto both faces of their leaves. The plants were then transferred to a growth chamber and maintained at 25 ºC with a 16-h photoperiod and high humidity (about 100%). Another twenty non-inoculated plants, were held in an independent growth chamber under identical conditions and served as controls. After 48 h, the humidity was reduced to 85% in both chambers until the end of the study.

2.2 Enzyme Extraction

Triplicate samples of fully expanded young leaves from the inoculated and control plants were collected 1, 2, 3, 4, 10, and 20 d after inoculation. The samples were immediately frozen in liquid nitrogen and stored at −80 ºC until utilization. All methods of enzyme extraction were performed at 0–4 ºC. For total protein extracts, frozen leaves (0.5 g) were homogenized with a mortar and pestle in 3 mL of ice-cold 0.05 M sodium phosphate buffer (pH 7.8) containing 1 mM EDTA-Na_2, 0.1% ASA (w/v), 15% glycerol (v/v), 5% PVP, 0.1% Triton X-100, 0.01 mM phenylmethanesulphonyl fluoride and 0.05% β-mercaptoethanol (v/v). The homogenate was centrifuged at 12 000 g for 20 min and the supernatant was used for the SOD assay and electrophoresis.

2.3 SOD Activity

An adapted version of the method introduced by Beauchamp and Fridovich (1971), which is based on the photo-reduction of nitroblue tetrazolium (NBT), was used to obtain the total SOD activity in the extracts. The reaction mixture consisted of 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) containing 13 mM methionine, 75 µM NBT, 0.01 mM EDTA-Na_2, 0.002 mM riboflavin, and 0.1 mL of 25%, 50%, 75% and 100% concentrations of sample. Lastly, Riboflavin was added as a source of superoxide. Cuvettes containing the reaction mixtures were illuminated by two 15-W fluorescent lamps until mixtures without SOD achieved a moderated blue color. The absorbance of the reaction mixtures was measured at 560 nm using a spectrophotometer (DU 640, Beckman). The non-irradiated reaction mixtures served as controls and were deducted from the A_{560} of the samples. The volume of the extracts corresponding to 50% inhibition of the reaction was considered to be one enzyme unit. Total SOD activity was expressed in units per gram fresh weight

(U g^{-1} FW), and specific SOD activity in U mg^{-1} protein. Total protein content (TPC) of the extracts was quantified by Bradford's method (Bradford, 1976) using BSA as a standard.

2.4 Native Polyacrylamide Gel Electrophoresis and SOD Visualization

Thirty µg of concentrated proteins were loaded onto polyacrylamide slab gels (18×14×0.15 cm) prepared according to Davis (1964), with slight modifications. The concentrations of the stacking and resolving gels were 4% and 10–12%, respectively. Riboflavin was used for photopolymerization of the gel in place of ammonium persulfate. Electrophoresis was performed at 15–20 mA for 10 to 12 h, until the bromophenol blue dye marker migrated to the bottom of the gel. The gel was stained by the riboflavin/NBT method (Beauchamp and Fridovich, 1971). For the comparison of different isozymes, the relative distance (R_f value) of the bands was determined as described by Manganaris and Alston (1992), from the origin of each band (R_f = 0) and from the migration distance of the dye marker (R_f = 1).

2.5 Statistical Analysis

Data were analyzed using the ANOVA and GLM procedures of SAS (1989), and the means were separated using the least significant difference (LSD) test at the 0.05 level.

3. Results

3.1 Conidia Observation and Symptoms of Infected Strawberry Leaves

M. fragariae conidia are elliptical to cylindrical, hyaline, and 0-4 septate. They measure 20–40×3–5 µm and are often formed in short chains (Figure 1). Conidiophores are short, hyaline, unbranched, and frequently curved or bent; they have prominent conidial scars, as described by Maas (1984).

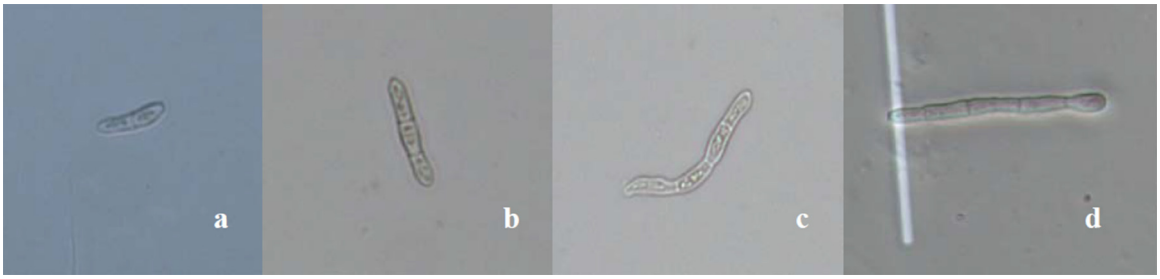


Figure 1. Conidia of *M. fragariae* (250×). a, b, c, and d are 1, 2, 3, and 4 septate conidia, respectively

Symptoms of leaf spot first appear as circular, deep purple spots on the upper leaf surface. These spots enlarge and the centers turn brown to grayish and then white on older leaves. A definite reddish purple to rusty brown border surrounds the spots. Colonization of strawberry leaf tissues by *M. fragariae* 30 d after inoculation caused macroscopic red spots of varying severity depending on the cultivar (Figure 2). From Figure 2, we can see clearly that susceptibility to leaf spot varies by the cultivar.

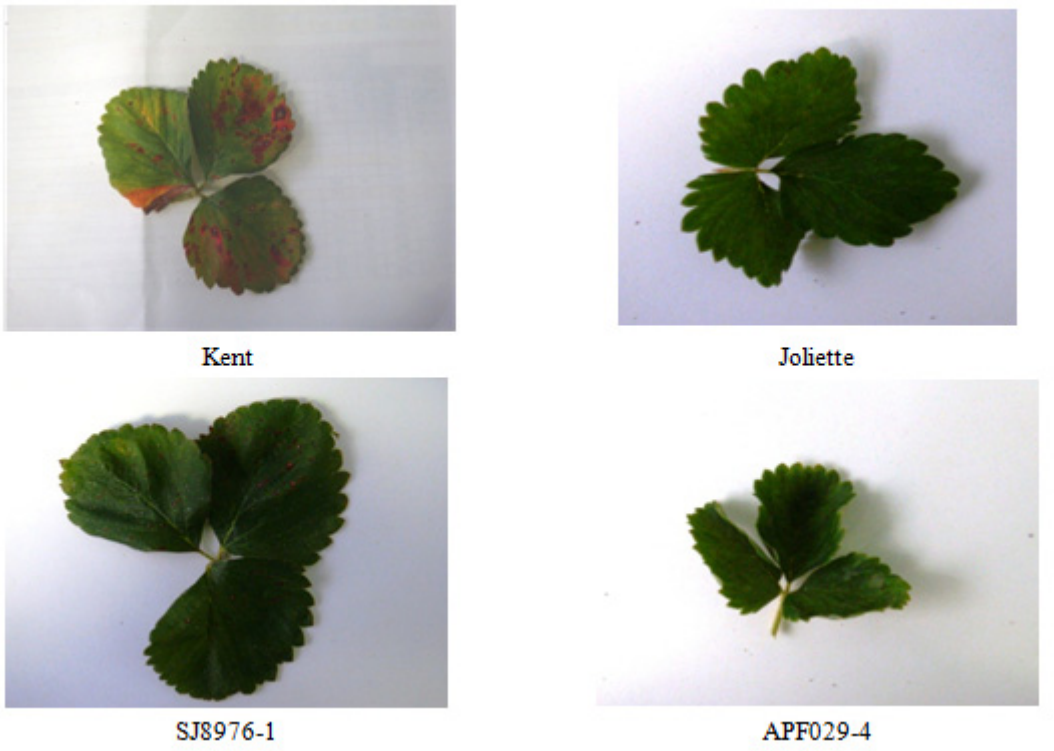


Figure 2. Leaf spot lesions caused by *M. fragariae* on Kent (HS), Joliette (HR) and SJ8976-1 and APF029-4 (MR) 1 month after inoculation

3.2 Analysis of SOD Activity

TPC and total and specific SOD activities for Kent, Joliette, SJ8976-1, and APF029 up to 20 d after inoculation with *M. fragariae,* are presented in Table 1.

Table 1. TPC and total and specific SOD activities of the four strawberry cultivars inoculated by *M. fragariae*

Cultivar	Day after inoculation	Total protein content (mg g-1 FW)	Total SOD activity (U g-1 FW)	Specific SOD activity (U mg-1 protein)
Kent	0	5.50b	2245b	408 d
	1	5.79b	2540b	438cd
	2	7.22a	3656a	506cd
	3	7.07a	3420a	483cd
	4	5.00c	2561b	513c
	10	3.58d	2273b	637b
	20	3.05e	2310b	757a
$LSD_{0.05}$	-	0.39	346.38	60.23
[a]OC	-	2.54ns	19.72**	47.76***
[b]OPC	-	L***	ns	L***
Joliette	0	5.09bc	2197d	432d
	1	5.34b	3729b	699b
	2	5.86a	4376a	746ab
	3	5.39b	3624b	673bc
	4	5.21bc	3137c	602c
	10	4.96c	2961c	596c
	20	3.01d	2397d	798a
$LSD_{0.05}$	-	0.38	319.63	58.37
[a]OC	-	0.83ns	106.36***	149.44***
[b]OPC	-	L***	L*	L*
SJ8976-1	0	5.40d	2274e	422e
	1	5.90c	3689c	625c
	2	7.11a	4433a	623c
	3	6.92ab	4208b	609c
	4	6.52b	3535c	543d
	10	4.61e	3048d	663b
	20	3.07f	3188d	1038a
$LSD_{0.05}$	-	0.41	200.61	21.77
[a]OC	-	4.00ns	389.60***	1140.85***
[b]OPC	-	L***	ns	L***
APF029-4	0	4.88d	2808d	577cd
	1	5.91c	3661bc	619c
	2	7.22a	4283a	593cd
	3	7.16ab	3940b	550de
	4	6.51bc	3366c	516e
	10	4.20e	2884d	687b
	20	3.29f	2958d	901a
$LSD_{0.05}$	-	0.46	301.9	48.26
[a]OC	-	26.65**	43.09***	15.25*
[b]OPC	-	L**	L*	L***

Triplicate samples of fully expanded young leaves from both control and inoculated plants were collected at 1, 2, 3, 4, 10, and 20 d after inoculation. Each value represents the mean of three independent plants. $LSD_{0.05}$: Least

Significant Different at 0.05 level. *=(P＜0.05), **=(P＜0.01), ***=(P＜0.001), ns=non-significant (P＞0.05). [a]OC: Orthogonal Contrast day 0 vs all days; [b]OPC: Orthogonal polynomial contrast; L=liner, ns=not significant.

Kent: total SOD activity increased after inoculation, reached a peak after 2 d (3656 U g^{-1} FW), and declined slowly thereafter. The orthogonal contrast (OC) value was 19.72^{**}, which indicates that, after inoculation, total SOD activity was significantly higher than in the control (2245 U g^{-1} FW), although there were no significant linear effects. TPC reached its highest peak after 2 d (7.22 mg g^{-1} FW), showing an increase of 112% relative to the control (5.50 mg g^{-1} FW). Although there was no significant difference between the control and all other days for TPC, inoculation showed a significant linear effect. Specific SOD activity (enzyme concentration based on total protein, U mg^{-1} protein) showed a significant linear effect.

Joliette: total SOD activity increased after inoculation, reached its highest level after 2 d (4376 U g^{-1} FW) and slowly declined thereafter. Inoculation had a significant linear effect on total SOD activity, which was very pronounced in the control vs. all other days (OC = 106.36^{***}). As with Kent, the highest TPC value was recorded after 2 d (5.86 mg g^{-1} FW). Although there was no significant difference between the control and all other days for TPC, inoculation showed significant linear effects. Specific SOD activity showed a significant positive linear effect.

SJ8976-1: as in the case of Kent and Joliette, total SOD activity increased after inoculation, reached its highest level after 2 d (4433 U g^{-1} FW) and slowly declined thereafter. Total SOD activity was significantly higher than for the control (2274 U g^{-1} FW); however, no significant linear effect was found after inoculation by *M. fragariae*. The TPC value was recorded after 2 d (7.11 mg g^{-1} FW), showing an increase of 133% relative to the control value (5.40 mg g^{-1} FW). There was no significant difference between the control and all other days for TPC; however, inoculation showed significant linear effects. Specific SOD activity showed significant positive linear effects after inoculation.

APF029-4: as in the case of the other three cultivars, total SOD activity increased after inoculation, reaching its highest level after 2 d (4283 U g^{-1} FW), then slowly declined thereafter. Inoculation had a significant linear effect on total SOD activity; the effect was very pronounced for the control compared with all other days (OC = 43.09^{***}). The variation in TPC was similar to that for total and specific SOD activities, with inoculation showing a significant linear effect.

For Kent, Joliette, SJ8976-1 and APF029-4, the lowest level of TPC was recorded after 20 d: 3.05, 3.01, 3.07 and 3.29 mg g^{-1} FW, respectively.

Figure 3 illustrates the variation of total SOD activity in the four cultivars on different days after inoculation with *M. fragariae*. Total SOD activity was significantly higher in APF029-4 than in the other three cultivars on the control day, but increased in all four cultivars after inoculation. There was no significant difference between Joliette, SJ8976-1 and APF029-4, with all three cultivars showing significantly higher effects than Kent 1, 2, 4 and 10 d after inoculation. Total SOD activity was significantly higher in SJ8976-1 and APF029-4 than in Kent and Joliette 20 d after inoculation.

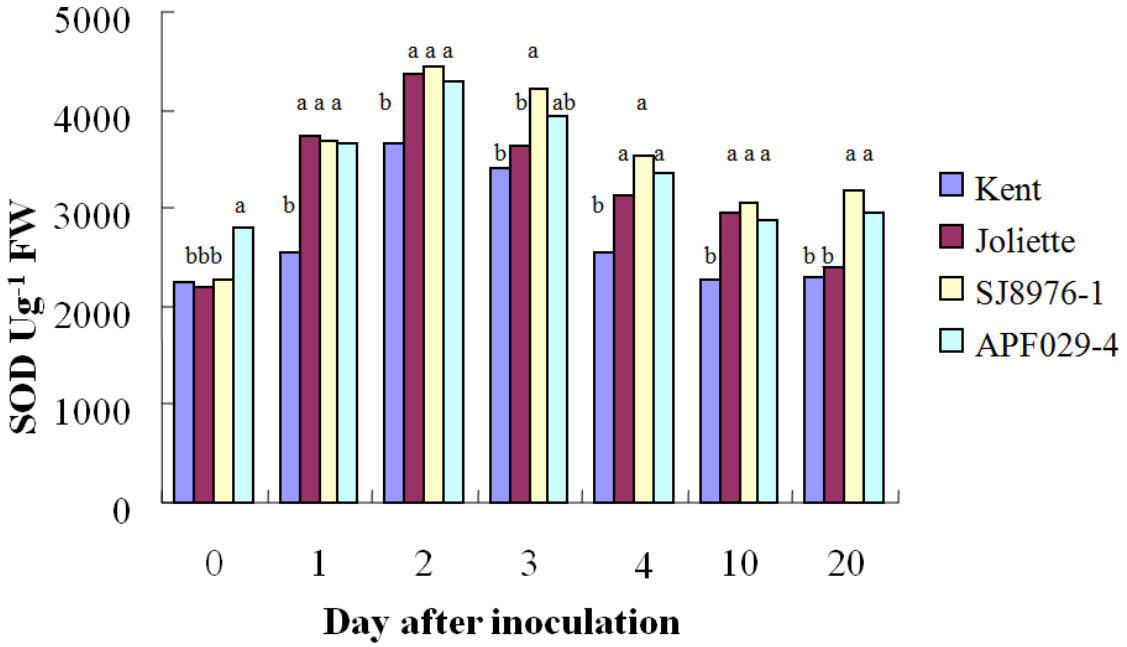


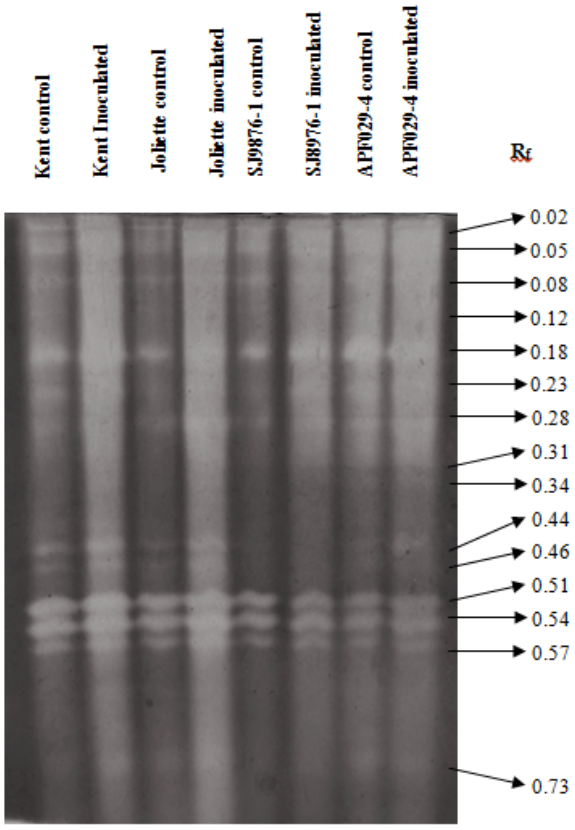
Figure 3. The effect of *M. fragariae* infection on total SOD activity in the four strawberry cultivars 20 d after inoculation. Triplicate samples of fully expanded young leaves from both control (0 d) and inoculated plants were collected at 1, 2, 3, 4, 10, and 20 d after inoculation. Each bar is the average of three independent plants. Bar with different letter whithin the same cultivars are significantly different ($P \leq 0.05$)

3.3 Native PAGE

Native polyacrylamide gel electrophoresis (PAGE) was performed on the samples several times, giving similar results. For this reason, the data from only two experiments are discussed. The isozyme pattern of SOD changed between the four cultivars in response to inoculation with *M. fragariae* (Figure 4 and Figure 5).

Initially, a total of 14, 13, 13 and 15 SOD isozymes were detected in the control plants of Kent, Joliette, SJ8976-1 and APF029-4, respectively. After inoculation, Kent and APF029-1 showed no difference; however, the number of SOD isozymes in Joliette and SJ8976-1 increased to 15 with the identification of two newly synthesized SOD isozymes (i.e., $R_f = 0.31$ and 0.34) without reduction of bands. It is considered that the two synthesized SOD isozymes are associated with leaf spot resistance.

In the present study, inoculation also affected the intensity of the isozymes bands, which became more intense and thicker in the 4 inoculated strawberry cultivars as compared to the controls.



Rf	Kent		Joliette		SJ8976-1		APF029-4	
	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
0.02	1	1	1	1	1	1	1	1
0.05	1	1	1	1	1	1	1	1
0.08	1	1	1	1	1	1	1	1
0.12	1	1	1	1	1	1	1	1
0.18	1	1	1	1	1	1	1	1
0.23	1	1	1	1	1	1	1	1
0.28	1	1	1	1	1	1	1	1
0.31	0	0	0	1	0	1	1	1
0.34	1	1	0	1	0	1	1	1
0.44	1	1	1	1	1	1	1	1
0.46	1	1	1	1	1	1	1	1
0.51	1	1	1	1	1	1	1	1
0.54	1	1	1	1	1	1	1	1
0.57	1	1	1	1	1	1	1	1
0.73	1	1	1	1	1	1	1	1

Figure 4. Isozyme pattern of SOD activity of four strawberry cultivars at 0 d and 2 d after inoculation with *M. fragariae*. Arrows correspond to SOD bands along with R_f value in table

R_f: = Relative distance of the band as described in the Materials and Methods section (0 and 1 indicate the presence and absence of SOD bands, respectively).

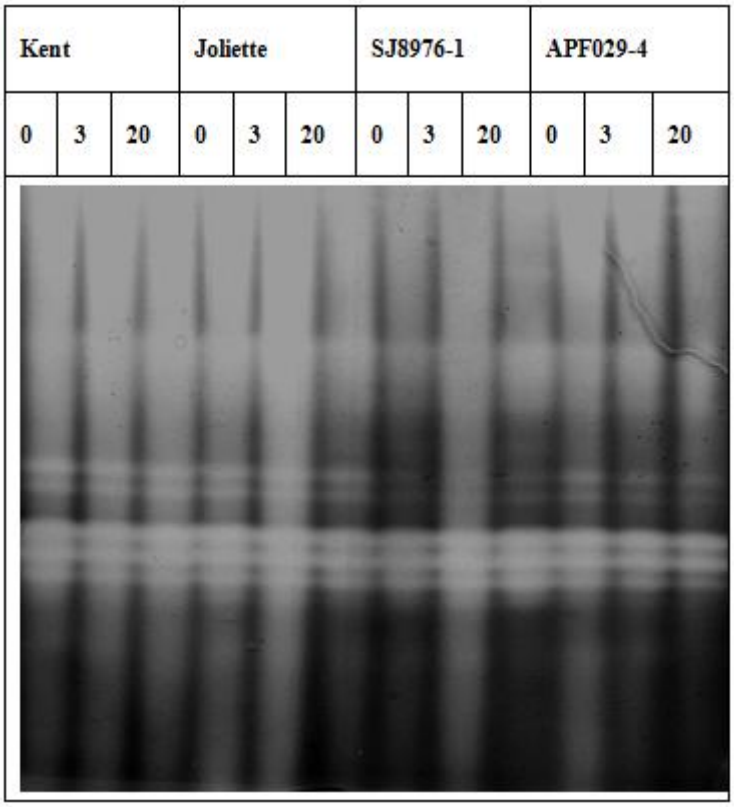


Figure 5. Isozyme pattern of SOD in the four strawberry cultivars at 0 d and 3 and 20 d after inoculation with *M. fragariae* (Kent 0, 3, 20 d; Joliette 0, 3, 20 d; SJ8976-1 0, 3, 20 d and APF029-4 0, 3, 20 d from left to right, respectively)

4. Discussion

Environmental stresses lead to increased ROS production in plant cells, which can damage proteins, membrane lipids, DNA and other cellular components. Tolerance to various environmental stresses, such as pathogen attack in plants (Reuveni et al., 1992; Miyazawa et al., 1998; Ros Barceló et al., 2003; Kuźniak & Sklodowska, 2004; Moghaddam et al., 2006)., has been known to be associated with both antioxidant concentrations in plants as well as the activity of ROS scavenging enzymes (Chaitanya et al., 2002).

SOD enzymes protect against ROS and play a key role in the biology of cells and tissues. These enzymes have therefore become a focus of research in the fields of botany, biochemistry and biology as well as in medical, food, animal and veterinary sciences. SOD activity has been reported to increase under stress conditions such as high irradiance, low temperature, air pollutants, etc. (Tsang et al., 1991; Scandalios, 1993). Changes in SOD activity are used as an indicator of changes in superoxide production, since the level of SOD production depends on superoxide production (Wang et al., 2004).

Various environmental stresses can induce SOD gene expression. Total SOD activity has been found to increase in maize, wheat, tobacco and other plants following exposure to herbicides, illumination, heat shock, mechanical damage and ethylene (Perl-Treves & Galun, 1991; Matters & Scandalios, 1986; Tsang et al., 1991; Wu et al., 1999). Kuźniak and Sklodowska (2004) reported that infection of tomato leaves with the necrotrophic fungus *Botrytis cinerea* resulted in substantial changes in enzymatic and non-enzymatic components of the ascorbate-glutathione cycle as well as in changes in SOD, glutathione peroxidase (GSH-Px), glutathione transferase (GST), and L-galactono-γ-lactone dehydrogenase (GLDH) activities. Total SOD activity began to increase 1 d after inoculation and remained, on average, 66% higher in the mitochondria of the control plants (Kuźniak & Sklodowska, 2004). In the present study, we used extracts of non-infected leaves as controls and leaves infected with *M. fragariae* to study SOD activity in response to *M. fragariae* infection. The level of concentration in inoculated strawberry cultivars may be indirect evidence because of the enhancement of superoxide radical production in the plants. In the present study, a significant increase in SOD activity was observed in both resistant and susceptible strawberry plants 2 d after inoculation, which is similar to what was

found in the study by Ehsani-Moghaddam et al. in 2006. Joliette, SJ8976-1 and APF029-4 showed significantly higher levels of total SOD activity 2 d after infection compared to Kent, indicating that these three cultivars have a higher tolerance to the oxidative stress induced by the pathogen.

Changes in SOD activities were examined in leaves of bean (*Phaseolus vulgaris* L. cv. Tendergreen) undergoing compatible and incompatible interactions to race 6 and race 3 strains, respectively, of the halo-blight bacterium *Pseudomonas syringae* pv. *phaseolicola*. Resistance of cv. Tendergreen to race 3 is determined by the *R3* gene and was expressed by a hypersensitive reaction, which was associated with a rapid increase in lipid peroxidation between 8 and 12 h after inoculation. Five main isoforms of SOD were resolved by native PAGE. Three further minor isoforms of SOD showed a strong increase in activity during the hypersensitive reaction (Ádám et al., 1995). In previous studies, the most severe infection of strawberry leaves by *M. fragariae* was found to occur within 12 to 96 h of leaf wetness and inoculation (Elliott, 1988; Carisse et al., 2000). With the growth of conidia, total SOD activity in the four strawberry cultivars increased and then peaked 2 d after inoculation, a pattern that presumably reflects the oxidative effects of *M. fragariae*. With the maturity and senescence of strawberry leaves, TPC and total SOD activity began to slowly decrease 4 d after inoculation; however, specific SOD activity showed a significant positive linear effect after inoculation. This provides evidence that SODs play an important role in protecting cells and tissues against biological stress.

SODs are metal-containing enzymes that are present in virtually all living organisms, including animals, plants and microorganisms, and are closely related to the metabolism of trace elements, e.g. copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn). Mn SOD is located in mitochondria, Fe SOD in chloroplasts and Cu and Zn SODs in both the cytosol and chloroplasts (Bowler et al., 1994). In a previous study, six isozymes were detected in control plants of Kent (HS), Honeoye (MR) and Joliette (HR), and some synthesized isozymes appeared or disappeared after inoculation (Ehsani-Moghaddam et al., 2006). In our study, a larger number of SOD isozymes were detected in control plants of Kent (HS), Joliette (HR), SJ8976-1 and APF029-4 (MR), that is, 14, 13, 13 and 15, respectively. After inoculation, there were no differences for Kent and APF029-1, but for Joliette and SJ8976-1, the number of SOD isozymes increased to 15 as a result of two newly synthesized SOD isozymes (i.e., R_f = 0.31 and 0.34) without reduction of bands. The two synthesized SOD isozymes are believed to be associated with leaf spot resistance. This finding also indicates that the kinds of SODs vary by strawberry cultivar. Inoculation induced changes in SOD isozymes, in both quantity and quality, which could be linked to the differing susceptibility of strawberries to *M. fragariae* and other environmental stresses.

In conclusion, based on the analysis of SOD activity and electrophoretic analysis of isozymes, the four strawberry cultivars showed different susceptibilities to *M. fragariae*. This demonstrates that HR and MR cultivars possess an advanced antioxidant defense system, which is partly attributable to the increase in SOD activities and changes in concentration of the SOD isozymes. Future research should focus on separating the different SOD isoforms and controlling experimental conditions for different strawberry cultivars, in order to select highly disease-resistant cultivars through hybridization or transgene technology.

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Registration of 'AMBERICHO' a Newly Released Field Pea (*Pisum sativum* L) Variety for the Southern Highlands of Ethiopia

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Abstract

Ambericho (IG-51664) with a large and white seeded field pea variety was selected and developed by Areka Agricultural Research Center, southern Ethiopia. This variety was selected from the regional variety trial tested together 15 other test genotypes including local and standard checks at 8 environments. Finally the variety was officially released for wider production in the southern highlands of Ethiopia.

Keyword: Ambericho, variety, field pea

1. Introduction

Field pea (*Pisum sativum* L.) is an annual climbing, herbaceous plant, showing very considerable variation in form and habit. It is probably originated in south-western Asia and has spread to the temperate zones throughout the world and is grown as a cool season crop in sub-tropics, and the higher altitude in the tropics. It requires a cool; not excessively cold climate, evenly distributed a rainfall of about 800-1000 mm/annual and cultivated in wide range of soil type with PH range 5.5-6.5 provided that the derange is good (Key, 1979). In Ethiopia field pea with other pulse covers a wide range of about 11-15% of the total 6-7 million hectares of the area and is the third most important staple food legume among the highland pulses (CSA, 2009). The estimated annual consumption of field pea per person in rural Ethiopia is 6kg in form of spilt, milled and unmilled (Asfaw et al., 1994). Field pea is nutritious food staff when fully matures and they are valuable food legume, often being ground in to flour and used extensively in the manufacture of soups. Fresh green peas are almost universally accepted as nutritious vegetable. Green peas are in fact the leading processed vegetables; a large quantities being grown for canning, freezing or dehydrating (Kay, 1979). Asfaw et al (1994) indicated that field pea is a 'break' crop with the cereal rotation, especially with barley and wheat, which serves to restore soil nitrates and minimize weeds, insects pest and disease of cereals.

2. Methodology

Around 100 lines were acquired from Holetta Agricultural Research Center in 2002 and tested as nursery at Location Angacha for one year. Out of the tested lines of field pea 16 materials were screened for yield and agronomic traits in randomized complete block design in 2005 and 2006 in multi-location yield test within the southern region at Angacha, Hossana, Waka and Bule major pea growing areas. All the materials were evaluated together with local check and 'Tegegnech' standard check in a total of eight environments. The elite line with pedigree IG-51664 was named **AMBERICHO** after official release for commercial production by the national seed releasing committee.

3. AMBERICHO

AMBERICHO is common name for the field pea variety with the pedigree name of IG-51664. It is developed and released by Areka Agricultural Research Center for major field pea growing areas of Southern Ethiopia. Ambericho was evaluated for two years (2004-2005) at Angacha, Hossana, Waka and Bulle stations and had better mean grain yield than the standard check. Ambericho was moderately tolerant to *ascochyta* blight and powdery mildew. It has creamy white seed coat colour with no black spot and with smooth round seed character. The result of multi-location trials showed that Ambericho had above-average grain yield performance across tested locations and years.

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Antinociceptive and Anti-Inflammatory Activities of the Aqueous Leaf Extract of *Tamarindus indica* L. in Albino Rats

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Abstract

The research was conducted to investigate the phytochemical constituents, acute toxicity, the anti-inflammatory and antinociceptive activities of the aqueous extract of *Tamarindus indica* L. (AQETI) leaves. Phytochemical constituents present in AQETI were found to be flavonoids, cardiac glycosides, tannins and terpenoids which might be responsible for the established anti-inflammatory and antinociceptive activities in the plant extract. The leaf extract of the plant was found to be moderately toxic with an intraperitoneal acute toxicity (i.p. LD_{50}) of 566 mg/kg. AQETI produced significant ($p < 0.05$) and dose-dependent anti-inflammatory and antinociceptive activities. AQETI (400 mg/kg) exhibited a maximum percentage inhibition (56.97%) in acetic acid-induced writhing response and at 30 min increased the pain threshold by 100 % in the tail immersion test. In the hot plate method, AQETI (400 mg/kg) showed a similar percentage inhibition (84.62%) at 30 and 60 min. The results demonstrate that the aqueous extract of *Tamarindus indica* L. leaves contains some pharmacologically active substances, was moderately toxic and possessed significant anti-inflammatory and antinociceptive acivities.

Keywords: *Tamarindus indica,* anti-inflammatory, antinociceptive, acetic acid, thermal nociception, tail immersion

1. Introduction

Plants are very important in many parts of the world since creation of mankind and have been used by man as source of food, medicine, shelter, clothing, cosmetics, flavours and spices (Gamaniel, 2000; Cordell, 2006; Tor-Anyiin et al., 2006). The use of medicinal plants in West Africa is probably as old as the duration of human settlement in the region (Abdulrahman et al., 2010).

Tamarind or *Tamarindus indica* L. of the Fabaceae, is an important food in the tropics. It is a multipurpose tree of which almost every part finds at least some use (Kumar & Bhattacharya, 2008), either nutritional or medicinal. Tamarind is indigenous to tropical Africa but it has been introduced and naturalized worldwide in over 50 countries. The major production areas are in the Asian countries (India and Thailand, Bangladesh, Sri Lanka and Indonesia). In America, Mexico and Costa Rica are the biggest producers. Africa on the whole does not produce tamarind on a commercial scale, though it is widely used by the local people. Minor producing countries in Africa are Senegal, Gambia, Kenya, Tanzania and Zambia (El-Siddig et al., 2006). The plant is well known in traditional medicine (Morton, 1987; El-Siddig et al., 2006; Sidhuraju, 2007) and has been utilized as remedy for a number of ailments (Rimbau et al., 1999; Kristensen and Balslev, 2003; El-Siddig et al., 2006) for its antioxidant (Perez et al., 1995; Ramos et al., 2003; Tsuda et al., 2004; Sudjaroen et al., 2005; Al-Fatimi et al., 2007; Siddhuraju et al., 2007), antihelminthic (Das et al., 2011), Cytotoxic (Al-Fatimi et al., 2007), laxative (Bhat et al., 1990), analgesic (Dighe et al., 2009; Khalid et al., 2010), antiasthmatic (Tayade et al., 2009), hepatoprotective (Pimple et al., 2007), hypolipidaemic and Weight-Reducing (Jindal et al., 2011), antimicrobial (Meléndez and Capriles, 2006; Al-Fatimi et al., 2007; Warda et al., 2007) anti-diabetic, antihelminthic (El-Siddig

et al., 2006), hypolipidaemic (Martinello et al., 2006), anti-inflammatory (Useh et al., 2004; Fook et al., 2005) activities.

Inflammation is the local response of living mammalian tissues to injury due to any agent. It is the body's defense reaction in order to eliminate or limit the spread of injurious agents as well as to remove the consequent necrosed cells and tissue (Anupama et al., 2012). It is a manifestation of the body's response to tissue damage and infection. The result of each inflammatory reaction may be beneficial (defends the body against agents deranging its homeostasis) or harmful (damage surrounding tissues) (Reynold, 1993). It is part of a multifaceted biological phenomena of vascular tissues to injurious stimuli due to pathogens, injured cells or irritants. It is a defensive attempt by the organism to remove such injurious stimuli and commence the healing process (Maldini et al., 2008), but the symptoms like swelling, tightness, joint pain and irritation associated with inflammation cause patients discomfort. Combating the inflammation can improve circulation and aid healing as well as lessen pain.

Pain is an unpleasant feeling often associated with tissue damage. Tissue injury is the immediate cause of pain as it releases different chemical mediators like prostaglandins, bradykinins and substance P which act on the nociceptors causing this sensation. The nociceptive stimulus is transmitted to the CNS by small myelinated Aδ-fibres or by unmyelinated thin C-fibres (Otsuka & Yanagisawa, 1990). It is often classified as chronic and acute. Acute pain may be characterized by its quick onset and short duration, lasting for hours. On the other hand, chronic pain is often associated with persistent pain over a large period of time (Merskey & Bogduk, 1994; Mark, 1999). Pain and fever are the most common complaints associated with inflammation. The NSAIDs used in the inflammatory conditions do not cure and remove the underlying cause of the disease but they only modify the inflammatory response to the disease. Large numbers of NSAIDs are available in the market with their advantages and disadvantages. Though there are standard drugs like Aspirin, Indomethacin, Phenylbutazone, etc., these drugs are not entirely free of side effects and have their own limitation (Reynold, 1993). Thus, there is still a need to develop newer and safer anti-inflammatory drugs. NSAIDs use is frequently limited by gastrointestinal side effects, ranging from dyspepsia to life threatening bleeding from ulceration. It is believed that NSAIDs by inhibiting cyclooxygenase (COX) pathway causes inhibition of prostaglandins synthesis, which are responsible for maintaining gastric mucosal integrity (Kalra et al., 2010). Herbal medicines used in Ayurveda remain the major source of health care for the world's population. The World Health Organisation (WHO) has recognized herbal medicine as an essential building block for primary health care of many countries like India, China and African countries.

Conventional medicines such as steroids and non-steroidal anti-inflammatory drugs (NSAIDs) have shown only limited achievement against all forms of inflammatory circumstances. Furthermore, the unpleasant (adverse) side effects associated with NSAIDs such as bleeding and mucosal damage and other gastrointestinal disturbances as well as tolerance and dependence induced by opiates, the use of these drugs as analgesic agents have not been successful in all the cases making the treatment difficult (Goldsby et al., 2003; Ukwuani & Hassan, 2014).

Considering the probable adverse effects of these drugs, as well as their limited ability to provide long-term remission, there is a need for a new, safe and cost-effective anti-inflammatory agent which can reduce pain and other associated symptoms (Fleischman et al., 2004). To overcome all these problems, preparations from plant origin have become important in modern medicine and are widely prescribed in traditional systems. Anti-inflammatory and antinociceptive effects in rats may be induced by acetic acid, hot plate (thermal nociception), tail immersion, tail flick or carrageenan hind-paw oedema. In view of the reported uses of this plant and its economic value, the present study investigated the safety and the anti-inflammatory and antinociceptive effects of the aqueous leaf extract of *Tamaridus indica* L. in rats (Wistar strain albino rats).

2. Materials and Methods

2.1 Plant Collection and Identification

Fresh sample of the leaves of *Tamarindus indica* L. were collected from the tamarind population in the University of Maiduguri Campus, Maiduguri, Borno State, in March, 2014. The fresh plant material was taken to the Department of Biological Sciences, University of Maiduguri, where it was identified and authenticated by a plant Taxonomist, Prof. S.S. Sanusi of the same Department.

2.2 Extraction

The identified leaves of *Tamarindus indica* L. were carefully detached from the stalks, washed with distilled water (to remove sand particles) and air-dried (away from sun, dust and intense heat) under the shade in the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, University of Maiduguri, for one week. The

leaves were then pulverized using a wooden mortar and pestle to powder, weighed and stored in a specimen bottle. Two hundred and fifty gram of the pulverized leaves of was refluxed (hot extraction) exhaustively with freshly distilled water at 80 °C. The aqueous extract was filtered, concentrated *in vacuo* and stored in a specimen bottle for use throughout the study. The yield was 62.17 g (24.87%) with respect to the starting material with a light brown colour, fine texture and characteristic taste and odour.

2.3 Animals

Fifty three healthy adult Wistar strain albino rats of both sexes weighing 96-193 g and 20 healthy adult mice of both sexes weighing 20-36 g purchased from the Faculty of Pharmacy Animal House were housed in standard wire meshed plastic cages in the Department of Physiology, Pharmacology and Biochemistry Laboratory, Faculty of Veterinary Medicine, University of Maiduguri. The animals were allowed to acclimatize to this environment for a period of two weeks where they were allowed access to food (Vital® Feed) and water *ad libitum.* All the animals were handled according to the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985) as certified by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Maiduguri.

2.4 Preliminary Phytochemical Screening of AQETI

Qualitative phytochemical tests were carried out to determine the presence or absence of some pharmacologically active secondary metabolites in the leaves of *Tamarindus indica* L. using standard methods (Brain & Turner, 1975; Vishnoi, 1979; Markham, 1982; Silva et al., 1998; Sofowora, 2008; Evans, 2009).

2.5 Acute Toxicity Studies (LD_{50} Determination)

The acute toxicity AQETI was determined as described by Lorke (1983). The experiment was divided in two phases both using the intraperitoneal route of adminisration (i.p) as follows:

Phase I

Nine (9) healthy Wistar strain albino rats of both sexes weighing 103-168 g were randomly selected and divided into three groups (labelled A, B and C) of three animals each. The animals in each group were weighed and labelled with picric acid on either the head, back or tail (as required), as a mark of identification. The groups were then treated respectively with the extract at incremental doses of 10 mg/kg, 100 mg/kg and 1000 mg/kg intraperitoneally. The animals were then observed for 24 hours for signs of toxicity and mortality.

Phase II

Four (4) randomly selected healthy Wistar strain albino rats weighing 109-193 g were grouped into four groups A, B, C and D of one animal each, weighed and given identification marks. The animals were then exposed to graded doses of AQETI intraperitoneally (200 mg/kg, 400 mg/kg, 800 mg/kg and 800 mg/kg) respectively based on the result of phase I. The rats were allowed access to food and water *ad libitum* and were observed for 24 hours for signs of toxicity and death after which the LD_{50} was calculated using the formula below:

$$\text{LD } 50 = \sqrt{a \times b}$$

Where a = lowest dose that kills an animal, 1/1

b = highest dose that does not kill any animal, 0/1 (Lorke, 1983).

2.5 Acetic Acid-induced Writhing Test in Mice

This was carried out according to the method described by Koster et al. (1959); Singh and Majundar (1995). Twenty adult mice of both sexes (weighing between 20 and 36 g) were randomly separated into four groups (A, B, C and D) of five mice each. They were deprived of food for 24 hr before the commencement of the experiment. Those in group A received distilled water (3 ml/kg) to serve as negative control group while those in groups B and C received 200 and 400 mg/kg respectively of the AQETI; while those in group D received pentazocine (30 mg/kg) to serve as the positive control group. All drug and extract administration were carried out intraperitoneally (i.p). Twenty minutes later, 10 ml/kg of 0.6% acetic acid solution (in 0.9% w /v normal saline) was administered i.p. to all groups to induce writhing. Writhing response was observed as described by Turner (1965). The number of writhes was counted from five minutes after acetic acid administration for thirty minutes. A reduction in the number of writhing as compared with the negative control group was considered as evidence of analgesia. The percentage protection was obtained using the formula described by Hernandez-Perez et al. (1995) as shown below;

$$\% \text{ inhibition} = \frac{\text{Mean no. of writhes in negative control group} - \text{Mean no. of writhes in test group}}{\text{Mean no. of writhes in negative control group}} \times 100$$

2.6 Tail Immersion Test

The tail immersion method was used to evaluate the central mechanism of analgesic activity (Ramabadran et al., 1989).This was based on the method described by Singh and Majundar (1995). Twenty rats of both sexes (weighing between 96-159 g) were randomly divided into four groups (A, B, C and D) of five rats each. They were deprived of food for 24 hr before the commencement of the experiment. Those in group A (negative control) received distilled water (10 ml/kg) while those in groups B and C received 200 and 400 mg/kg respectively of AQETI whilst those in group D (positive control) received pentazocine (30 mg/kg). All treatments were by i.p route. Thirty minutes later, the tail (upto 10 cm) was dipped into a water bath maintained at 55 ± 0.5 °C. The time (in seconds) to withdraw the tail clearly out of the water was taken as the reaction time. The latent period of the tail response was determined at 30, 60, 90 and 120 min after the administration of drugs and extract. The percentage (%) increase in pain threshold (latency) was calculated by the formula below;

$$\% \text{ increase pain threshold} = \frac{\text{Mean reaction time in rats in test group} - \text{Mean reaction time in rats in negative control group}}{\text{Mean Reaction time in rats in negative control group}} \times 100$$

2.7 Hot Plate (Thermal Nociception) Test

This was carried out according to the method described by Badilla et al. (2003). Twenty rats of both sexes (weighing between 121-161 g) were randomly divided into four groups (A, B, C and D) of five rats each. They were deprived of food for 24 hr before the commencement of the experiment. Those in group A (negative control) received distilled water (10 ml/kg) while those in groups B and C received 200 and 400 mg/kg respectively of AQETI whilst those in group D (positive control) received pentazocine (30 mg/kg). All treatments were by i.p route. Thirty minutes later, they were placed on Eddy's hot plate maintained at 55 ± 0.5 °C. This was repeated every thirty minutes for 2 hrs. The reaction time was taken as the time for the rats to jump out of the stainless steel plate on the hot plate. The percentage (%) inhibition was calculated by the formula below;

$$\% \text{ inhibition} = \frac{\text{Mean reaction time in rats in test group} - \text{Mean reaction time in rats in negative control group}}{\text{Mean reaction time in rats in negative control group}} \times 100$$

2.8 Statistical Analysis

Data generated during the study were expressed as mean ± standard deviation (S.D.) and analyzed by one way analysis of variance (ANOVA) using statistical package for Social Sciences windows 16.0 version (SPSS, 2007) and $P < 0.05$ was considered significant.

3. Results

3.1 Phytochemistry

Phytochemical screening revealed that AQETI contained carbohydrates, cardiac glycosides, flavonoids, steroidal nucleus, tannins, terpenoids, but alkaloids, anthraquinones, saponins and starch were absent (Table 1).

Table 1. Preliminary phytochemical analysis of the AQETI

S/No	Constituent	Test	Result	Observation
1	Alkaloid	General Test:		
		(i) Dragendorff's Test	-	No ppt formed
		(ii) Mayer's Test	-	No ppt formed
2	Anthraquinones	(i) Free anthraquinones Test:		
		Borntrager's Test	-	No colour formed
		(ii)Combined AnthraquinonesTest	-	No violet colour formed
3	Carbohydrates	(i)General (Molisch's) Test	+	Purple colouration
		(ii) Free Reducing Sugar (Fehling's) Test	-	No colour change
		(iii)CombinedReducing Sugar	-	No colour change
		(iv) Ketosis	-	No colour change
		(v) Pentoses	-	Red colouration
4	Cardiac Glycosides	(i)Liebermann-Buchard's Test	+	Bluish-green colouration
		(ii) Salkowski's Test	+	Reddish-brown colouration
5	Flavonnoids	(i) Ferric Chloride Test	+	Bluish-green Colouration
		(ii) Lead Ethanoate Test	+	Buff Coloured ppt
		(iii) Shinoda's Test	+	Light-Pink colouration
		(iv) Sodium Chloride Test	-	Yellow colouration
6	Saponins	Froth test	-	No Foam Formed
7	Starch	Soluble Starch Test	-	No Dark-blue Colouration
8	Steroidal Nucleus	Liebermann-Buchard's Test	+	Bluish-green colouration
9	Tannins	(i) Ferric Chloride Test	+	Deep Blue-black colouration
		(ii) Lead Ethanoate Test	+	White ppt. Formed
10	Terpenoids	General Test	+	Violet ppt. formed

Key: + = Present; - = Absent.

3.2 Acute Toxicity (LD_{50}) Studies of the Aqueous Leaf Extract of Tamarindus indica L.

The i.p. LD_{50} was 566 mg/kg (Table 2).

Table 2. Intraperitoneal Acute Toxicity (LD50) Test of the crude aqueous leaf extract of *Tamarindus indica* L

Phase	No. of Rats	Dose (mg/kg)	Clinical Sign	Mortality
1	3	10	None	0 / 3
1	3	100	None	0 / 3
1	3	1000	None	2 / 3
2	1	200	None	0 / 1
2	1	**400**	None	0 / 1
2	1	**800**	None	1 / 1
2	1	1600	None	1 / 1

$$\text{i. p. LD50} = \sqrt{a \times b} = \sqrt{800 \times 400} = 566 \text{ mg/kg}$$

Where a = least dose that kills the animal = 1/1; b = highest dose that does not kill the animal = 0/1

3.3 Acetic Acid-Induced Writhing

AQETI (200- and 400 mg/kg) dose-dependently reduced the number of writhes induced by 0.6 % Acetic Acid Solution. The highest dose (400 mg/kg) produced a significant ($P < 0.05$) percentage inhibition of 56.97% while pentazocine (30 mg/kg) gave 86.63%, a value higher than that of the extract (Table 3).

Table 3. Effect of aqueous leaf extract of *Tamarindus indica* L. on acetic acid induced writhing in mice

Group	Dose (mg / kg)	No. of Writhes (secs) (Mean ± S.D)	% Inhibition
A (Distilled Water)	3 ml	80.40 ± 1.14^{a}	-
B (AQETI-200)	200	39.60 ± 0.55^{b}	50.70
C (AQETI-400)	400	34.60 ± 0.55^{b}	56.97
D (Pentazocine)	30	10.75 ± 0.55^{b}	86.63

n = 5 = Number of rats in each group, A (Distilled water) = Negative control, D (Pentazocine) = Positive control. Means with different superscripts (b) are statistically significant ($p < 0.05$) among the groups when compared with group A (negative control).

3.4 Tail Immersion Test

The aqueous leaf extract of *Tamarindus indica* L. at the different post-treatment times induced a dose-dependent increase in pain threshold (latency) for removal of tail. The maximum latency, (highest significance, $p < 0.05$), irrespective of post-treatment time for the extract at 200 and 400 mg/kg were 5.60 ± 0.55 and 6.40 ± 0.55 sec. respectively compared with a value of 9.40 ± 0.53 for pentazocine. Pentazocine produced a greater inhibition compared with the exract (400 mg/kg) at all post-treatment times. Peak antinociceptive effect for the extract occurred at 30 min. post-treatment (Table 4).

Table 4. Effect of aqueous extract of *Tamarindus indica* L. on tail immersion test in wister albino rats

Treatment		Reaction Time in secs (Mean ± SD)							
Group	Dose (mg/kg)	30 min (sec)	% Inhibition	60 min (sec)	% Inhibition	90 min (sec)	% Inhibition	120 min (sec)	% Inhibition
A (Distilled water)	10 ml	3.40 ± 0.89^{a}	0.00	3.60 ± 1.14^{a}	0.00	4.20 ± 0.84^{a}	0.00	3.60 ± 1.14^{a}	0.00
B (AQETI-200)	200	5.20 ± 0.45^{b}	52.94	5.60 ± 0.55^{b}	55.56	5.40 ± 0.55^{b}	28.57	4.40 ± 0.55^{b}	22.22
C (AQETI-400)	400	6.80 ± 0.45^{b}	100.00	6.40 ± 0.55^{b}	77.78	5.40 ± 0.55^{b}	28.57	4.60 ± 0.55^{b}	27.78
D (Pentazocine)	30	9.40 ± 0.55^{b}	176.47	9.40 ± 0.45^{b}	170.50	9.40 ± 0.55^{b}	123.81	8.80 ± 0.55^{b}	144.44

n = 5 = Number of rats in each group, A (Distilled water) = Negative control, D (Pentazocine) = Positive control. Means with different superscripts (b) are statistically significant ($p < 0.05$) among the groups when compared with group A (negative control).

3.5 Hot Plate (Thermal Nociception) Test

Aqueous leaf extract of *Tamarindus indica* L. (200-400 mg/kg) dose-dependently significantly ($p < 0.05$) increased the pain threshold (latency) 30 min after intraperitoneal administration with a percentage inhibition of 53.85 and 84.62% respectively administration reaching peak in 60 min. The inhibition produced by the extract was however lower when compared with that of the standard drug, pentazocine which was 138.46%, but it was significant ($P < 0.05$) (Table 5). It was also observed that the percentage inhibition for both the extract and the drug decreased with time.

Table 5. Effect of aqueous extract of *Tamarindus indica* L. on hot plate test in wister albino rats

Treatment		Reaction Time in seconds (Mean±SD)							
Goup	Dose (mg/kg)	30 min (sec)	% increase in pain threshold	60 min (sec)	% increase in pain threshold	90 min (sec)	% increase in pain threshold	120 min (sec)	% increase in pain threshold
A (Distilled water)	10 ml	2.60±0.55 [a]	0.00	2.60±0.55 [a]	0.00	2.80±0.45 [a]	0.00	2.80±0.55 [a]	0.00
B (AQETI-200)	200	4.00±0.71 [b]	53.85	4.00±0.71 [b]	53.85	4.00±0.71 [b]	42.86	4.00±0.71 [b]	42.86
C (AQETI-400)	400	4.80±0.45 [b]	84.62	4.80±0.84 [b]	84.62	4.40±0.55 [b]	57.14	3.80±0.45 [b]	35.71
D (Pentazocine)	30.00	6.20±0.45 [b]	138.46	6.60±0.5 5 [b]	153.85	5.60±0.55 [b]	100.00	4.60±0.55 [b]	64.29

n = 5 = Number of rats in each group, A (Distilled water) = Negative control, D (Pentazocine) = Positive control. Means with different superscripts (b) are statistically significant ($p < 0.05$) among the groups when compared with group A (negative control).

4. Discussion

The results of the phytochemical screening revealed that AQETI contained carbohydrates, cardiac glycosides, flavonoids, steroidal nucleus, tannins and terpenoids, while alkaloids, anthraquinones, saponins, and starch were absent. These phytochemicals have been reported to influence physiological activities of the body. The presence of carbohydrates in this plant forms the basis for its wide use as a food source by man and animals.

Cardiac glycosides have strong activity on the heart and some have been used in the treatment of congestive heart failure (CHF). Cardiac glycosides may also have pesticidal properties (Okwute, 1992; Harbone, 1998).

Bioactive compounds such as tannins and flavonoids as found in the extract possess analgesic and anti-inflammatory activities (Ahmad et al., 2005).The profile of polyphenols and flavonoids in *Tamarindus indica* include proanthocyanidine in various forms likeapigenin, anthocyanin, procyanidine, catechin, epicatechin, along with taxifolin, eriodictyol andnaringenin (Samina et al., 2008). Out of these phytoconstituents, polyphenols and flavonoids have been well known to exhibit anti-inflammatory and antinociceptive action (Loggia et al., 1986; Gonzalez et al., 2007; Rao et al., 2008). Flavonoids have been reported to play a role in analgesic activity primarily by targeting prostaglandins (Rao et al., 1998; Rajnarayan et al., 2001). These flavonoids may interact directly with the prostaglandin system and inhibit the substitute cofactor for the prostaglandin generation and also inhibit arachidonic acid lipoxygenation as well as enzymes involved with inactivation or biotransformation of prostaglandins (Panthong et al., 1989; Recio et al., 1995). Flavonoids are also implicated in having antipyretic, anti-inflammatory and antioxidant properties (Evans, 2009). It has been reported that the seeds of *Tamarindus indica* have antiulcer, anti-asthmatic, anti-diabetic and antioxidant activity (Pankaj et al., 2011; Nurhanani et al., 2012). Also seeds of *Tamarindus indica* are rich in phenolic compounds, polymeric tannins and fatty acids, flavonoids, saponins, alkaloids and glycosides (Valko et al., 2006; Boots et al., 2008). Flavonoids, tannins, saponins and alkaloids are responsible for the anti-inflammatory and analgesic activity (Doughari, 2006).

The presence of the steroidal nucleus, the backbone of steroidal agents such as cortisone and prednisolone among others is purported to be responsible for the established anti-inflammatory effect of *Tamarindus indica* L. (Gonzalez et al., 2007).

Tannins have astringent properties which are important in wound healing (Pondrimoli & Grazi, 1996). There are also reports on the role of tannins in anti-nociceptive activity (Vanu et al., 2006).

Terpenes are known to have anti-inflammatory properties. The antinociceptive activity observed may also be through the effect of the extract on the inflammatory process.

One or a combination of these compounds detected might be responsible for the established antinociceptive and anti-inflammatory effects.

These phytochemicals together with amino acids, proteins, fatty acids and minerals (macro- and micro-elements) have been reported elsewhere (El-Sidigg et al., 2006; Martinello et al., 2006;) in the whole plant and the leaves in particular. Amino acids are building blocks for proteins and are necessary for the synthesis of endogenous peptides such as *enkephalins, endorphins and dynorphins* among others, which are endogenous pain modulators released by the body especially during acute inflammation and pain.

Saponins, though absent in this plant extract, cause haemolysis if given intravenously (Patrick-Iwuanyanwu and Sodipo, 2007). The absence of alkaloids and other constituents and or the differences in constituents found in the literature are likely to be due to differences in genetic strains, stages of maturity at which the plant parts were collected, growing conditions, harvesting and handling techniques as well as to differences in analytical methodologies.

The result of the acute toxicity, i.p. LD_{50} of 566 mg/kg shows that the substance is moderately toxic. According to Clarke and Clarke (1977) and Sodipo et al. (2007) any substance whose i.p. LD_{50} in rats falls between 50 and 500 mg/kg is regarded as toxic, between 500 mg/kg but less than 1,000 mg/kg is moderately toxic and greater than 1,000 mg/kg is non-toxic.

The antinocieptive activity of the crude aqueous extract of *Tamarindus indica* leaf produced significant graded dose effects in all the three models employed viz; acetic acid-induced writhing, hot plate (thermal nociception) and tail immersion. Writhing induced by chemical substances (e.g. acetic acid, phenylbenzoquinone) injected i.p. are due to sensitization of nociceptors by prostaglandins (Nunez et al., 1997; Yongna et al., 2005) and this test is useful for the evaluation of mild analgesic non-steroidal anti-inflammatory compounds (Eekankopf et al., 1988; Ferreira & Vane, 1974).

The inhibition of writhing in mice by the aqueous extract suggests a peripheral mechanism of action possibly via inhibition of prostaglandins among several possibilities. Pain sensation in acetic acid-induced writhing method is elicited by triggering localized inflammatory response resulting from the release of free arachidonic acid from tissue phospholipid (Ahmed et al., 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte et al., 1988). In other words, the acetic acid induced writhing has been associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products (Deraedt et al., 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria et al., 2008). The acetic acid induced writhing method has been found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte et al., 1988; Ferdous et al., 2008). The significant pain reduction of the plant extract might be due to the presence of analgesic principles acting within the prostaglandin pathways (Ukwuani & Hassan, 2014). The abdominal writhing induced by acetic acid has been reported to be less selective (Collier et al., 1968) and proposed to act indirectly by releasing endogenous mediators stimulating neurons that are sensitive to other drugs such as narcotics and centrally acting agents (Toma et al., 2003). The effect of the extract was however; lower than that of the standard drug, pentazocine, in all the three tests and at the doses used. Peak antinociceptive effect was observed at a dose of 400 mg/kg in all the tests. In order to further confirm the antinociceptive effect of the extract, the tail immersion and the hot plate tests were carried out. Thermal nociceptive tests are more sensitive to opioid μ receptors and non-thermal tests are to opioid κ receptors (Abbott & Young, 1988; Furst et al., 1988). The tail immersion test is considered to be selective for the drugs acting central. It measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity (Sabina et al., 2009). The effects of the extract in the tail immersion and hot plate methods confirmed its analgesic action. This goes further to suggest also a central mechanism of action for the extract. It is known that centrally acting analgesic drugs elevate the pain threshold of albino rats towards heat and pressure (Singh & Majumdar, 1995). In the hot plate and tail immersion experiments, pentazocine's effect as an analgesic has a higher effect than that of the extract. The analgesic superiority is expected, since pentazocine is a narcotic analgesic used to alleviate deep-seated pain (Turner, 1965; Besra et al., 1996). Since there were significant activities recorded in both methods (tail immersion and hot plate), the extract could be said to act both peripherally and centrally in producing analgesia. Noxious stimuli cause release of chemicals such as prostaglandins, decarboxylated amines (histamine and serotonin), there by inducing pain locally (Nunez et al., 1997; Yongna et al., 2005). Peripherally acting analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting release of prostaglandins (Wagner et al., 2004; Ngulde, 2010). The centrally acting analgesics such as pentazocine act through their receptors in the central nervous system (CNS) by increasing the threshold response to pain stimuli (Singh and Majumdar, 1995). Opiod analgesics inhibit both peripheral and central mechanisms of pain, while NSAIDs inhibit only peripheral pain (Elisabetsky et al., 1995; Pal et al., 1999).

Goyal et al. (2013) evaluated the analgesic effect of the ethanol extract of *Tamarindus indica* leaves in experimental rats (Adult Swiss Albino Mice) using the tail immersion method in which they reported significant ($p < 0.05$) analgesic activity in 30 min after oral doses of 200 and 400 mg/kg. Similar results have been obtained for the seeds (Anupama et al., 2012) and whole stem (Ukwuani & Hassan, 2014).

The crude aqueous leaf extract of *Tamarindus indica* contained many pharmacologically active compounds such

as flavonoids, terpenoids tannins and cardiac glycosides and some of these may be responsible for the local or central antinociceptive effect.

5. Conclusion

In conclusion, the aqueous extract of *Tamarindus indica* L. is moderately toxic, contained some pharmacologically active constituents (cardiac glycosides, flavonoids, steroidal nucleus, tannins and terpenoids) and possessed analgesic and anti-inflammatory activities purported to be mediated via peripheral (probably through inhibition of prostaglandin synthesis) and central mechanisms. This supports the use of the plant in ethnomedical and folkloric practices in alleviating pain.

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Assessment of Nutritional Status of Different Genotypes of Common Bean (*Phaseolus vulgaris* L.)

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Abstract

The objective of this study was to evaluate the nutritional state of 20 different genotypes of common bean, which were six cultivars and 14 breeding lines. The experiment was conducted in the field, between August and November of 2002, in an Oxysoil, Cristalina – GO, Brazil. Yield and leaf diagnosis were evaluated, 45 days after the emergence of the culture (total foliar rates of N, P, K, Ca, Mg, S, B, Mn, Cu and Zn), by the DRIS method, using the nutrient concentrations, taken two by two. Nutrient absorption was different for the genotypes. In most of them, Zn was the less absorbed nutrient, while S was the nutrient in excess. DRIS correlation was different for nutrient interactions, as for a positive correlation for P x Zn (0,928), as for a negative correlation, for N × S (-0,947). DRIS shows nutrient deficiency and nutrient excess, for manure recommendations, and it can be used as a routine for bean culture, based on a leaf diagnosis.

Keywords: nutrient uptake, leaf diagnosis, DRIS, yield

1. Introduction

The common bean (*Phaseolus vulgaris* L.) represents an important protein source in Brazil. Due to its easy adaptability to many climatic conditions in this country, it can grown in different regions, and can be cultivated over the year (up to three harvests) using several systems ranging from subsistence farming in small farms to high-tech agriculture on large scale. This crop reached a year production of 3.5 million ton in 2010 (MAPA, 2012), however actually this area decreased to about 4.1 million hectares (Ruas, 2010).

The utilization of high-tech agriculture, especially with irrigation, the production becomes economically viable, including the use of lime, proper fertilization, high yield cultivars, severe control of diseases, pests and weeds (Thung & Oliveira, 1998).

The bean productivity in Brazil is considered low as compared to the potential of the varieties recommended by researchers. The explanation for this low productivity could be several factors, such as, inadequate farming management, poor soil and lack of using of improved breed varieties, and finally the inappropriate fertilization. In high-tech agriculture, fertilizer application usually occurs with large amounts, increasing production costs, which leads the necessity to optimization of this practice, in order to maximize the yield at low cost (Malavolta, 1992).

However, in cases of insufficiency, excess of imbalance of one or more nutrients the plant growth become restricted, because the nutrients coexist in complex interactions involving energy metabolism, and plant physiology. Thus, deficiency symptoms may be due to low soil nutrient supply or low plant genetic ability to uptake and transport ions, but also by their interaction with other ions (Creste et al, 1999; Schulte & Kelling, 2002).

Schulte and Kelling (2002) reported the role of plant nutrition analysis to detect the deficiency, toxicity or non-balance of nutrients, identifying especially “hidden hunger”, to assess fertilization programs. This

information determines the availability of elements not detected by other methods, the interaction between nutrients and completes the soil nutritional analysis.

To provide more data besides the chemical analysis of soil, the leaf analysis quantitatively can determine the nutrients concentration in plant tissues, which allows an assessment of plant nutritional status *in situ* (Bonilla & Bolaños, 2010; Creste et al., 1999). The plant nutrient analysis of a specific organ at a phonological stage have been used to evaluate the nutritional status and in the fertilizer recommendation (Creste & Echer, 2010; Malavolta et al., 1998).

The DRIS (Diagnosis and Recommendation Integrated System) is a method to understand leaf analysis, which takes into account all interactions between nutrients and minimize the main limiting factors for the critical level method. The DRIS method uses the ratios between the concentrations of nutrients to understand the leaves and soil (Malavolta et al, 1998; Schulte & Kelling, 2002). The utilization of DRIS can alleviate the effects of nutrients concentration or dilution on dry matter. When the DRIS value become negative, suggests that the plant is deficient in those nutrients, otherwise the positive values indicate the excess of nutrients, and near the zero means that the plants are nutritionally balanced (Lana et al., 2010).

Costa (2002) used the DRIS system in commercial bean farms in Jussara city (Goiás state, Brazil), whose preliminary results showed that the method has been effective to diagnosis the crop nutritional status. This indicates non-balance degree and the range between the excess and deficiency, which are fundamental for fertilizer recommendation.

According to Schulte and Kelling (2002), the advantage of DRIS system would be that the growth stage, plant parts and cultivars are not essential to detect the critical level of a particular nutrient. However, a research conducted by Wadt et al. (1999) and Silveira (2000) with eucalyptus and Ferreira (2003) in *Heliconia latispatha* using the DRIS demonstrated a distinct behavior according to the evaluated genotype.

The current research aimed to assess the nutritional status through the DRIS method in different bean genotypes, observing the differences between cultivars to seek a understanding of the evaluated traits. (foi isso que vc quis dizer)

2. Materials and Methods

The study was conducted at Pantanal Farm, owned by Prezzoto Sementes Company Ltd., Crystalline City, Goias State, Brazil. on an clay texture Red Oxisol. The weather was defined as Aw (Köppen), with two distinct seasons (wet and dry). The experimental area was primarily used for pasture, and for the implementation the bean was sown in 2002.

The experimental design was composed by a randomized block design with three replicates. The plot size was 5 × 3 m, with a space of 0.50 m between rows having 12 plants per m, where 0.5 m of the boarder lines was deducted, totaling 6 m^2 area per plot. The three central rows within each plot were chosen for planta analysis ahd yield.

Soil analysis was performed before the experiment: pH in water, 5,2; Organic Matter, 55 g dm^{-3}; Al^{3+}, 0.03 $cmol_c$ dm^{-3}; Ca^{2+} + Mg^{2+}, 4.29 $cmol_c$ dm^{-3}; H^+ + Al^{3+}, 8.26 $cmol_c$ dm^{-3}; P, 18,3 mg dm^{-3}; K^{2+} 0,10 $cmol_c$ dm^{-3}. The soil amendment and fertilization (600 kg / ha of NPK 7-21-16 + FTE BR-12 and 200 kg / ha of ammonium sulfate at 22 DAE) were applied based in the fifth matching and according to technical recommendations for bean from EMBRAPA-Brazil (Stone & Sartorato, 1994).

In August 17, 2002 the bean was sown and the weeds and pests control were done as usual in the farm where the experiment was located. Considering the topography and the existence of sub-soil water a sub-surface irrigation (by capillarity) was used too.

Six cultivars of beans and 14 lines were used, composing 20 genotypes as follows: FT-84-105, FT-Nobre, FT-97-512, FT-97-708, FT-91-625, FT-Soberano, FT-97-837, FT-91-3168, FT-206, FT-9768, FT-96-1117, FT-Magnifico, FT-97-176, FT-97-119, FT-84-113, FT-90-1535, FT-97-255, Carioca, Bonito and Bionobre, provided by FT - Sementes Ltda. The agronomic characteristics of these genotypes, obtained in different assays are described in Table 1.

Five plants were randomly collected from each plot for leaf analysis 45 days after crop seeding. From these plants the first young leaves from the apical part were removed (Oliveira, 2002). Then the samples were drought in oven at 70 °C for 72 hours, ground and 20 g was taken for macro and micronutrients analysis, performed at the Laboratory of Soil Fertility and Plant Nutrition of CAMPO Co., Paracatu City - Minas Gerais-Brazil.

The nutrients N, P, K, Ca, Mg, S, B, Zn, Fe, Mn and Cu were determined, whose concentrations were expressed

in g kg^{-1} and for macronutrients mg kg^{-1} for the micronutrients. The leaf tissue analysis was performed according to the methods described by Malavolta et al. (1998).

Table 1. Agronomical characteristics of the bean genotypes used in the experiment, as informed by FT- Sementes Ltda, Brazil

Genotype	Grain type	Prod(1)	Standard	kg/ha(2)	Number(3)	Stability(4)	Antrac.(5)	FS(6)	Curtobac.(7)	Type(8)
FT 91-3168	Carioca	99	Pérola	2341	26	15 in 26	R	4,4,0,0	1,2,2	SP
FT 91-625	Carioca	96	Pérola	2341	26	14 in 26	R	4,4,2,0	2,3,2	SE/E/SP
FT 97-68	Carioca	93	Pérola	2341	26	11 in26	R	4,3,2,1	2,1,1	SE -SP/SE
FT Magnífico	Carioca	116	Pérola	2341	26	25 in 26	MR	0,0,0,0	2,3,2	P/SP
FT 97-175	Carioca	116	Pérola	2341	26	25 in 26	MR	0,0,0,0	2,3,2	P/SP
FT 97-119	Carioca	99	Pérola	2341	26	16 in 26	AE	1,1,0,0	2,1,2	SE/SP
FT 90-1535	Carioca	104	Pérola	2341	17	13 in 17	MS	0,5,1,3	SI	SP
FT 97-255	Carioca	99	Pérola	2341	17	14 in 17	AS	3,1,0,1	2,3,2	E/SE
Carioca	Carioca	102	Pérola	2341	17	18 in 26	S	4,5,0,1	SI	P
Bonito	Carioca	101	Pérola	2341	10	6 in 10	MS	SI	SI	SP
FT206	(9)	(9)	(9)	(9)	(9)	(9)	(9)	(9)	(9)	(9)
FT 84-105	Preto	96	Nobre	2340	19	13 in 19	AR	3,5,2,0	SI	SE - P
FT 84-105	Preto	96	Nobre	2340	19	13 in 19	AR	3,5,2,0	SI	SE - P
FT 97-512	Preto	95	Nobre	2340	19	9 in 19	MR	3,5,0,0	SI	E/SE - P
FT 97-708	Preto	97	Nobre	2340	19	10 in 19	MS	5,5,4,5	SI	E-SP
Soberano	Preto	109	Nobre	2340	19	13 in 19	AR	0,0,0,1	SI	SP/P - SE
FT 97-837	Preto	94	Nobre	2340	19	9 in 19	MR	0,2,0,0,	SI	E/SE
FT 96-1117	Preto	110	Nobre	2340	19	12 in 19	AR	2,3,2,5	SI	SP/SE
FT 84-113	Preto	97	Nobre	2340	19	11 in 19	MR	4,5,2,5	SI	SE/SP
Bionobre	Preto	101	Nobre	2341	19	12 in 19	AS	2,0,0,0	SI	SE/SP P

1) Relative productivity compared to the standard (%).

2) Average productivity (kg ha^{-1}) of the standard.

3) Total number of assays.

4) Number of assays equals of greater than the standard.

5) R(resistant), AR (strongly resistant), MS (moderate susceptible), S (susceptible), AS (strongly susceptible).

6) Soil fungus (*Fusarium* and *Rhizoctonia*) - 0 to 5.

7) Curtobactérium (0 a 5) - SI= no information.

8) Type: E (Straight), P (supported), SE-SP (semi-straight or semi-supported).

9) Data not informed.

The grain yield was determined at 90 days after seeding, when the plants reached the physiological maturity, with the grain mass moisture corrected to 13%. The concentration of the nutrients N, P, K, Ca, Mg, S , B, Zn, Fe, Mn and Cu in leaves and yield (kg ha^{-1}) were used as a database for DRIS application (Beaufils, 1973; Creste et al, 1999; Costa, 2002).

The DRIS index estimation was done by merging the genotypes based on average yield in two groups: group A (productivity less than 3000 kg ha^{-1}), and group B, with yield equal or more than 3000 kg ha^{-1}. The Group B was considered high yield ones denominated "control group" as described in Table 2.

The DRIS rates obtained from different bean genotypes were calculated using computer software developed by

Prof Sebastiao Oliveira, (University of Brasilia – Brazil) (Lana et al., 2010; Oliveira et al., 2009).

Table 2. Leaf concentration of macro e micronutrients and productivity of 20 bean genotypes (average of three replicates)

Genotypes	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	Yield
	g/kg						mg/kg					kg/ha
FT-84-105	36.30	3.40	23.10	27.77	5.23	3.00	61.7	13.0	348.7	266.3	32.7	1846.66
FT-Nobre	34.77	4.07	28.17	24.55	5.25	3.10	72.4	13.0	386.0	283.0	38.3	3714.07
FT-512	36.04	3.70	28.67	22.4	4.70	2.94	78.3	13.1	350.3	204.3	35.0	2962.00
FT-97-708	35.77	3.70	28.67	22.4	4.70	2.94	56.3	13.3	350.3	204.3	35.0	3892.65
FT-91-625	41.90	3.74	26.40	21.47	4.64	2.97	66.0	13.0	330.7	281.0	34.7	2487.77
FT-Soberano	40.34	3.85	24.30	25.57	4.87	2.74	70.7	13.0	309.0	255.3	34.3	2942.22
FT-97-837	35.04	3.54	25.20	23.06	4.03	2.74	74.7	12.0	328.0	234.7	36.0	2591.11
FT-91-3168	39.07	3.37	27.13	20.43	5.03	2.77	80.7	10.3	355.7	210.3	34.0	3436.74
FT-206	41.53	3.77	28.27	23.3	13.80	3.34	68.0	12.3	300.0	243.3	35.7	3513.41
FT-9768	37.36	3.87	28.36	21.80	5.06	2.80	63.0	14.3	303.0	214.7	34.7	4109.63
FT-1117	40.00	3.53	27.57	23.24	4.77	2.64	68.0	13.3	331.3	247.3	35.3	3024.37
FT-Magnífico	39.30	4.03	24.40	23.83	6.77	2.96	82.0	14.0	418.7	246.7	36.7	3810.81
FT-97-176	32.53	3.43	29.73	21.03	5.47	3.23	67.7	12.7	296.0	248.3	29.7	4208.15
FT-97-119	36	3.20	20.80	20.63	5.5	2.63	62.0	11.0	344.3	189.7	32.7	3757.85
FT-84-113	40.3	3.30	25.67	21.33	4.13	2.70	56.3	12.0	265.3	200.3	30.0	3690.37
FT-90-1535	34.5	3.50	25.40	21.2	5.17	2.97	65.7	12.3	310.0	192.7	29.3	2636.82
FT-97-255	34.26	3.63	27.17	20.5	4.87	3.17	70.7	12.3	519.3	235.7	33.0	1328.37
Carioca	34.8	3.53	23.36	22.8	5.40	2.83	64.0	12.7	360.7	213.3	33.0	1897.11
Bonito	36.03	3.83	24.80	23.66	5.00	2.90	73.7	14.0	247.7	261.0	33.7	3467.78
Bionobre	33.53	3.43	26.93	21.8	4.40	2.96	69.0	11.7	298.0	221.3	33.0	1767.93

3. Results and Discussion

The nutrients concentration in leaves and productivity of the genotypes are shown in Table 2. Leaf analysis genotypes corroborates with the data of macro and micronutrients which are appropriate for the beans, described by Malavolta et al. (1998). The yield ranged from 1328 to 4208 kg ha $^{-1}$ (Table 2).

The calculation of DRIS index for each nutrient in all genotypes and the series values were obtained. Then these values were ranked within each series, in order of limitation importance, the criteria in descending order (Table 3) was used. When the index was more negative, the nutrient limitation was higher, and more positive indicates less limiting and depending on the situation by limiting excess.

The results in Table 3 showed that Zn was the most limiting nutrient in 40% of the genotypes, whose yields were below than those of the Group B, in 55.5% of cases. B and K were the most limiting in 15% of cases, and 66.6% of these belonged to control group. The Fe and S had the same level of limitation, 10%, and the yields obtained were lower than the standards in 50% of the genotypes. And finally, the most limiting nutrients were N and Cu with 5% of occurrences in the control group. P, Ca, Mg and Mn had not any results in terms of limitation in any situation. In summary, the limitation sequence of nutrient scarcity in the genotypes in percentages was as follows: Zn (40%) > K = B (15%) > S = Fe (10%) > N = Cu (5%) > = P = Ca = Mg = Mn (0%).

Among 20 analyzed genotypes, 40% (eight) showed the S as the nutrient most limiting by excess. Within this group, 75% (six) had Zn as the most limiting nutrient for scarcity. And in both genotypes and FT-97-837 and FT-1117, where the S was considered the limiting by scarcity, Zn was the limiting nutrient by excess, suggesting a trend of antagonism between these two elements i.e. the S and Zn are contradictory in terms of absorption in plants. Other nutrients have been limiting for excess: Mg (genotypes), Ca (two), K (two), Mn (one), B (one) and

Cu (one) (Table 3).

Table 3. Primary DRIS index and limiting sequence of nutrients in different bean genotypes

Genotype	DRIS index											Limiting sequence
	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	
FT-84-105	-62	-55	-55	111	33	95	-81	23	-36	59	-115	Zn>B>N>P=K>Fe>Cu>Mg>Mn>S>Ca
FT-Nobre	-32	31	7	31	12	31	32	6	32	48	42	N>Cu>K>Mg>P=S=Ca>B=Fe>Zn>Mn
FT-512	-35	-13	41	-27	-20	37	-45	-3	-17	-34	-31	B>N>Mn>Zn>Ca>Mg>Fe>P>Cu>S>K
FT97-708	-34	-11	41	-10	-24	39	-80	18	-16	-34	-32	B>N=Mn>Zn>Mg>Fe>P>Ca>Cu>S>K
FT-91-625	4	-5	36	-19	-26	67	-5	-6	-52	40	-37	Fe>Zn>Mg>Ca>Cu>B=P>N>K>Mn>S
FT-Soberano	36	17	-54	75	-5	-35	12	23	0	47	26	K>S>Mg>Fe>B>P>Cu>Zn>N>Mn>Ca
FT-97-837	29	30	-13	36	-43	-50	62	-16	32	37	96	S>Mg>Cu>K>N>P>Fe>Ca>Mn>B>Zn
FT-91-3168	1	-30	7	-87	20	-3	102	-104	29	-33	8	Cu>Ca>Mn>P>S>N>K>Zn>Mg>Fe>B
FT-206	-60	-61	78	-66	-50	157	-46	-49	-128	-3	-143	Zn>Fe>Ca>P>N>Mg>Cu>B>Mn>K>S
FT-9768	-6	19	28	-19	-10	1	-29	35	-23	-19	-7	B>Fe>Ca=Mn>Mg>Zn>N>S>P>K>Cu
FT-1117	58	28	-30	48	-21	-85	18	21	38	24	81	S>K>Mg>B>Cu>Mn>P>Fe>Ca>N>Zn
FT-Magnífico	-15	11	-81	6	119	-31	72	-9	87	-4	17	K>S>N>Cu>Mn>Ca>P>Zn>B>Fe>Mg
FT-97-176	-210	-111	152	-139	31	350	-69	-48	-210	-2	-333	Zn>Fe=N>Ca>P>B>Cu>Mn>Mg>K>S
FT97-119	0	-18	-81	8	83	-40	24	-28	80	-32	9	K>S>Mg>Cu>P>N>Ca>Zn>B>Fe>Mg
FT-84-113	-11	-57	45	-31	-54	81	-86	-11	-111	-21	-124	Zn>Fe>B>P>Mg>Ca>Mn>Cu=N>K>S
FT-90-1535	-138	-91	71	-103	42	210	-61	-44	-123	-50	-241	Zn>N>Fe>Ca>P.B>Mn>Cu>Mg>K>S
FT-97-255	-114	-47	104	-109	-2	204	-5	-45	-129	0	-164	Zn>Fe>N>Ca>P>Cu>B>Mg>Mn>K>S
Carioca	-46	-21	-34	14	61	34	-14	-2	22	-10	-47	Zn>N>K>P>B>Mn>Cu>Ca>Fe>S>Mg
Bonito	-34	12	15	13	-5	75	13	32	-96	57	-63	Fe>Zn>N>Mg>P>Ca=B>K>Cu>Mn>S
Bionobre	-72	-37	64	-51	-24	119	-2	-41	-75	3	-88	Zn>Fe>N>ca>Cu>P>Mg>B>Mn>K>S

The excess S and the Zn deficiency might be related to the soil richness in organic matter (OM), because it can induce to this situation (Fageria, 2002; Oliveira et al., 1996). The fact that in most of the genotypes the S was the limiting nutrient by excess may be due to the application of ammonium sulfate as cover fertilizer and / or because of the high amount of OM in the soil experiment. According to Oliveira et al. (1996), soils rich in OM that received high doses of P and areas that were planned or soil moved from surface may exhibit Zn deficiency. In the present experiment the P dosage used was the one recommended for the crop, but the experiment place has the same characteristics as described above. This may explain the limitation of Zn by scarcity. Thung & Oliveira (1998) reported that the S concentration in bean leaves increases until the end of crop cultivation.

The productivity of the genotypes that presented the Zn as limiting nutrient by scarcity was below to those of "control group" in five genotypes, however in three of them it was higher, suggesting that despite of DRIS analysis indicate Zn limitation by scarcity, the productivity was not affected (Table 3). According to Oliveira et al. (1996) there are varieties of bean less susceptible to Zn deficiency, which enables the beans cropping even in soil poor with Zn deficiency.

In the genotype FT-Nobre, where N was the strongest limiting factor, Mn showed the highest index, suggesting that despite of Mn to be a toxic element, when in excess, the general conditions of the plot did not show their deleterious effect (Table 2 and 3).

For the genotypes FT-Magnifico and FT97-119, it was observed that K and Mg were the most and least limiting nutrients respectively, indicating that the excess of Mg affected the uptake of K in these genotypes (Table 3). This result corroborates with Malavolta et al. (1998), who reported that excess of Mg leads to lack of Ca and K.

The Table 3 presents a scarcity of B in three genotypes (FT-512, FT-97-708, FT-9768) which can be explained by

its low mobility in plants (Malavolta et al., 1998), probably these genotypes have more difficulty to mobilize B than others. The B has a low range between sufficient concentration in substrate and toxic level, and the relative tolerance of plants to toxicity is linked to transportation rate from roots to shoots (Malavolta et al., 1998). In the case of FT-91-3168, the B excess could be attributed to the phenomenon mentioned above.

The availability of Fe is reduced in flooded soils (Oliveira et al., 1996). The genotypes FT-91-625 and Bonito, where there was scarcity of Fe, may be due to the sub-surface irrigation during the experiment and/or to big sensitivity of these materials to flood (Table 3).

Oliveira et al. (1996) reported that bean does not respond to potassium fertilization, similar situation observed in this experiment, because 66.6% of the genotypes that showed K as the most limiting by scarcity belong to the "control group".

Phosphorus has most increased the beans production compared to other elements (Malavolta, 1987; Oliveira et al., 1996; Westermann, 2011). In the currently research, the P was not limiting by scarcity nor excess, because due to fertilization applied with and appropriate P concentration, made difficult the detection of genotypes tolerant to P deficiency.

The potassium, boron, sulfur and copper showed an interesting role in this experiment, limiting by deficiency and excess, which indicates the sensitivity of these nutrients to the fertilizer management. Costa (2002), when using DRIS in irrigated commercial beans, found the following results: N, P, K and Cu as limiting by scarcity and Mn, Cu and Zn limiting by excess. Some genotypes of this study behaved similarly: N, K and Cu limiting by scarcity and Mn, Cu and Zn by excess (Table 3).

The correlation matrix between DRIS rates for different bean genotypes (Table 4) led to identify interactions between nutrients estimating the necessity for fertilizations in the future. In the genotypes could be observed that the highest positive correlations between the nutrients levels were: P × Zn (0.928), N × Zn (0.920), Fe × Zn (0.885), K × S (0.829), N × P (0.823), N × Fe (0.754) and Ca × Cu (0.735). Negative correlations were: S × Zn (-0.963), N × S (-0.947), S × Fe (-0.908), K × Fe (-0869), P × S (-0.855) and K × Ca (-0.815). However, to construct the correlation matrix between DRIS rates the data of all genotypes were used together, and would be interesting to make this correlation matrix for each genotype. Epstein (1975) pointed out that chemical analyzes of different varieties of same species grown in similar environments, can vary widely in the plant tissue nutrient concentration.

According to Malavolta et al. (1998), the excess of P can cause deficiency of heavy metals such as Fe, Cu, Zn and Mn, thus the higher level of this nutrient increase the necessity of the other, i.e. they correlate negatively. In the currently research, the P content was in the proper range thus there was a negative correlation of P with the heavy metals mentioned above. N and P are the most limiting factors in bean production, confirming the positive correlation found (Thung & Oliveira, 1998).

The values of Nutritional Balance Index (NBI) of the genotypes ranged from 192 to 1655. In DRIS applied to bean in low productivity crops (lower than 300 kg ha^{-1}), when the NBI was below 30, the limiting factor in the productivity is not nutrition, however above 60 the productivity was limited by a nutritional factors (Costa, 2002). This author had NBI values ranging between 26 and 220, lower than those in this research. The Figure 1 shows the effect of NBI on bean productivity, but there was no correlation between them. Costa (2002) demonstrated that although the correlation value has not been shown, the graph indicates a correlation between NBI and productivity. However, as the conditions of each experiment are different and the study of DRIS on bean is recent, all of comparisons of different studies should be carefully done.

Table 4. Correlation matrix between the DRIS rate in different bean genotypes

	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
N	1,000	0.823	-0,713	0,673	-0,195	-0,947	0,496	0,393	0,754	0,255	0,920
P		1,000	-0,613	0,657	-0,081	-0,855	0,586	0,568	0,745	0,416	0,928
K			1,000	-0,815	-0,455	0,829	-0,473	-0,400	-0,869	-0,267	-0,734
Ca				1,000	0,049	-0,704	0,149	0,735	0,630	0,612	0,672
Mg					1,000	-0,048	0,284	-0,104	0,378	-0,178	-0,043
S						1,000	-0,565	-0,400	-0,908	-0,153	-0,963
B							1,000	-0,242	0,634	0,199	0,649
Cu								1,000	0,246	0,475	0,372
Fe									1,000	0,059	0,885
Mn										1,000	0,269
Zn											1,000

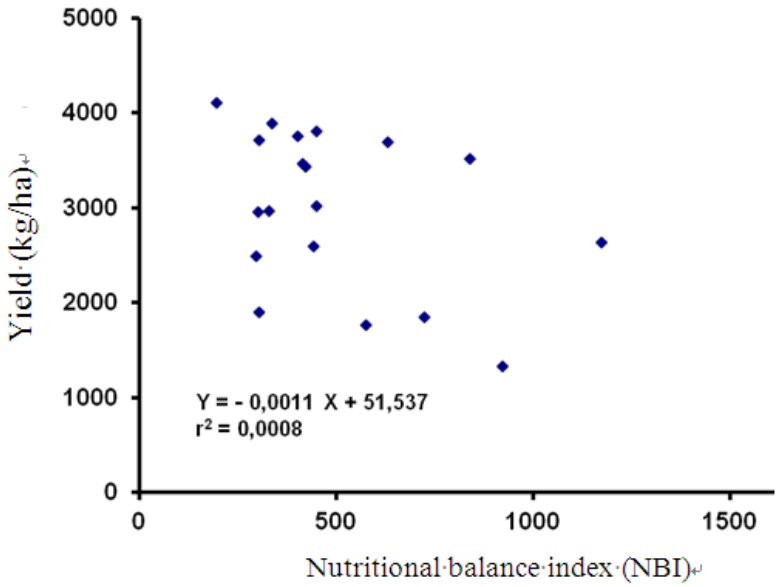

Figure 1. Correlation between yield (kg/ha) of 20 bean genotypes and the nutritional balance index (NBI)

The results of this study indicate that the DRIS is a relatively efficient methodology to determine the trustable sufficiency levels in soil, plant and in commercial crops. The DRIS performance could be explained by the fact that the forms are quite general, composed of a database with many variables, such as different genetic materials with large differences in yield potential and more (Silva et al., 2013; Wadt & Novais, 1999).

Thus, this study showed that the DRIS analysis must take into account the cultivars, because in general each genotype showed different responses to the same fertilization.

4. Conclusions

1). The nutritional status of the genotypes obtained from leaf analysis are suitable for cropping.

2). The elements with high probability of response to fertilization in descending order of percentage genotypes are Zn (40%) > K = B (15%) > S = Fe (10%) > N = Cu (5%) > P = Ca = Mn = Mg = (0%).

3). The NBI shows no correlation with productivity for the genotypes studied.

4). The DRIS can be used to select genotypes for the efficient use of nutrients in bean production.

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Phytochemical Screening and *in-Vitro* Antimicrobial Activities of the Leaf Extract of *Acanthospermum hispidum* DC (Asteraceae)

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Abstract

The study into the chemical contents and *in-vitro* antimicrobial activities of the methanolic leaf extract of *Acanthospermum hispidum* were carried out. The extract was evaluated for its antibacterial activity against four Gram positive (*Staphylococcus aureus, Streptococcus pyogenes, Corynebacteria specie* and *Bacillus subtilis*) and four Gram negative bacteria (*Salmonella typhi, Klebsiella phumoniae, Pseudomonas aeruginosa* and *Escherichia coli*). The fungal strains used were *Aspergilus niger, Penicillium sp.* and *Candida albicans*. The sensitive microorganisms (*Conynebacteria* sp., *Pseudomonas aeragunosa, Klebsiella pneumoniae, Bacillus subtilis* and *Staphylococcus aureus*) had zones of inhibition ranging from 12.20 ± 1.06 mm to 24.00 ± 1.00 mm at 100 mg/ml, while the standard drug (tetracycline 250 mg) had zones of inhibition in the range 20.27 ± 0.64 mm to 27.23 ± 0.68 mm against all the microorganisms tested in this study. The MIC/MBC against the tested organisms ranged from 25 mg/ml to 50 mg/ml and 50 mg/ml to 100 mg/ ml respectively. The results obtained from this study revealed that the leaf extract of *Acanthospermum hispidum* possesses antibacterial activity against some pathogenic microorganisms tested. The study confirmed the use of the leaf of *Acanthospermum hispidum* in some parts of Northern Nigeria as a remedy against diarrhoea, dysentery and other related diseases. In view of the reported uses of this plant, the present study investigated the antimicrobial effect of the methanol extract of *A. hispidum* on some Gram positive, Gram negative and some fungal species.

Therefore, this study validates the medicinal use of *Acanthospermum hispidum* tradicinally in some parts of Northern Nigeria.

Key words: *Acanthospermum hispidum,* phytochemical screening, antimicrobial, methanolic extract

1. Introduction

*Acanthospermum hispidum (*DC) (Bristly starbur) from Asteraceae family is found in the tropical and sub-tropical temperate regions of the world. In Nigeria, the plant is common as a weed along the roads and in moist habitats and is reported to have various medicinal values (Smith, 2002). *A. hispidum* can be potential sources of useful drugs (Faleye et al., 2012). *Acanthospermum hispidum* is adapted to a wide range of soil and climatic conditions. It is particularly adapted to light textured soil but also grows well in heavy textured one. It is commonly found in cultivated upland crops, roadsides, pastures, waste area, around corrals, along railroads and cattle trails. Both seed and leaves contain phenolic acids that are allelophatic to other plants (Holm et al., 1997). It is found in a wide range of habitats, commonly on roadsides, disturbed areas and around settlements. It is known to invade pastures and out-compete more desirable native species. It is also a weed of crops and a serious contaminant of wool (Smith, 2002). Sub-inhibitory concentrations of *A. hispidum* (5 mg/ml) enhanced the activity of amoxicillin against *Staph. aureus* and *B. subtilis* but reduced slightly the activity against Kl. pneumoniae. Combining ciprofloxacin with the sub-inhibitory concentrations (5 mg/ml) of A. hispidum extract modulated the resistance of all the organisms to ciprofloxacin. The resistance modulatory activity of the extracts

on amoxicillin and ciprofloxacin is more pronounced with Gram positive organisms than Gram negative organisms (Adu et al., 2011). Bioactivity-directed purification of the leaf of *A. hispidum* using anti-trichomonal assay yielded subfractions C_6 and C_7 which had activity comparable to metronidazole, the positive control and had better activity than the mother ethyl acetate extract. The study further showed the potential usefulness of *A. hispidum* in treating protozal infections (Deepa et al., 2004).

The leaves and stems of *Acanthospermum hispidium* were extracted with distilled ethanol using cold extraction and concentrated using a rotary evaporator at 37 °C. The crude extract was partitioned successively using hexane, benzene and methanol. Fractions 19, 20 and 21 purified on Sephadex LH-20 gave a compound elucidated to be 1, 3, 6, 8-tetrahydroxyl-9-anthracene carbonaldehyde, using the state-ofart tools of spectrometry. The results of the antimicrobial test on the isolated compound show activity against *P. mirabilis, B. subtilis, P. aeruginosa, C. albican, S. typhi and B. cereus* at minimum inhibitory concentration (MIC) value of 100 ppm Olajide et al., (2014). Early workers have reported the use of the stem bark for medicinal purposes (Chakraborty et al., 2012). *Acanthospermum hispidum* plant is important for its medicinal properties. In Nigeria, from information available from the indigenous traditional healers, the crushed herb is used in the form of a paste to treat skin ailments and the leaf juice is taken orally to relieve fevers (Mshana et al., 2000).

2. Materials and Methods

2.1 Sample Collection and Identification

Fresh samples of the leaves of *Acanthospermum hispidum* were collected from Uvaha village, Gwoza Local Government Area, of Borno State in November, 2012. The plant was identified and authenticated by a plant Taxonomist Prof. S.S. Sanusi, in the Department of Biological Sciences, University of Maiduguri. A voucher specimen No. Chem/09/01 was deposited in the Research Laboratory, Department of Chemistry. The leaves were cleaned, air-dried under shade for seven (7) days, then pulverised to powder and coded ‘‘plant material’’.

2.2 Extraction of Plant Material

The air-dried powdered plant material (1000 g) was extracted exhaustively with 85% methanol using a Soxhlet apparatus as described by Evans (2002). The methanolic extractobtained was concentrated to dryness at 45 °C on a water bath and coded ‘‘CMLE’’ Crude methanolic leaf extract. About 200 g of the CMLE concentrate were subjected to preliminary phytochemical screening and *in-vitro* antimicrobial susceptibility test while the MIC and MBC determined accordingly.

2.3 Qualitative Phytochemical Screening

The crude methanolic leaf extract ‘‘CMLE’’ of *A. hispidum* was subjected to qualitative phytochemical screening for identification of the various classes of active chemical constituents such as flavonoids, alkaloids, sterols, terpenes, saponins, tannins as methods described by the Harbone (1973), Awe and Sodipo (2001), Evans (2002); Sofowora (2008).

3. Atimicrobial Studies

3.1 Test Microorganisms

A number of microorganisms consisting of both Gram positiveand Gram negative (-ve) bacteria were used. The Gram positive (+ve) organisms used were *Streptococcus faecalis, Staphylococcus aureus, Corynbacterium spp*, *and Bacillus cereus, w*hile the Gram negative ones were: *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and some fungi species such as: *Candida albicans Aspergillus niger* and *Penicillium* species were also used. The microorganisms were obtained from the University of Maiduguri Teaching Hospital, Maiduguri, (UMTH) Nigeria.

3.2 In vitro Disc Antibacterial Activity of Methanolic Extract of Leaf

The *in vitro* disc diffusion method was used to test for antibacterial activity of the extract as described by Cheesbrough (2004).

3.3 Preparation of Culture Media

The culture media used in this study were nutrient agar (Biotec Medical Market, UK) for bacteria and C*andida albicans* and sabouraud-2% glucose agar (Merk, Darstadt, Germany) for *Penicilium spp. and Aspergillus niger* The nutrient agar was prepared according to the manufucturer’s specifications (by dissolving 18.5 g powder in 500 ml distilled water) and strerilized at 121 °C for 15 minutes. After autoclaving, the pH was 7.2-7.4 (Bello, 2002). This was poured in 90 mm diameter sterile, disposable plastic petri-dishes to a depth of 4 mm (about 25 ml per plate). Care was taken to pour the plates on a level surface so that depth of the medium would be uniform. The

plates were dried upside down in an incubator at 37 °C with their lids opened and inverted so that water would not condensed back in to the agar. The sabouraud-2%-glucose agar was prepared according to the manufacturer's specification (by dissolving 18.5 g in 400 ml of distilled water) and sterilized at 121 °C for 15 minutes. 1 ml each of the different concentrations of the CMLE (200 mg/ml, 400 mg/ml, 800 mg/ml and 1600 mg/ml) was pipette into eight (8) sterile, disposable petri-dishes i.e. 2 plates for each CMLE concentration 25 ml of the sabouraud-2%-dextrose agar was poured in to the plate, swirled round to mix very well with the CMLE, then allowed to set at low temperature. The other plates were also prepared, but without the CMLE, to act as the control. All the eleven plates were then incubated upside down, with their lides opened at 37 °C in an incubator to dry (Sodipo et al., 2012)

3.4 Preparation of Test Organisms

The microorganisms were propagated and stored on nutrient agar at 4 °C for 24 hours prior to antimicrobial testing.

3.5 Preparation of Agar Medium

Nutrient agar (25 g) was dissolved in one litre (1L) of distilled water in a sterilized conical flask and heated to dissolve by stirring. The prepared medium was tightly corked with aluminium foil and sterilized in an autoclave at 121 °C at 15 mm Hg for 15 minutes. The conical flask was put on a water bath at 55 °C to cool for 20 minutes. 10 ml of peptone water was put into a sterile inoculating bottle and sterilized in an autoclave at 121°C at 15 mm Hg for 15 minutes. Normal saline was prepared by the same process for 30 minutes. The nutrient agar was poured into sterilized petri dishes and allowed to set and dry in an incubator at room temperature (Sodipo et al., 2010*).*

3.6 Preparation of Discs Containing Graded Concentrations of the CMLE of the Leaves of A. hispidum and Tetracycline Discs

Whatman filter paper No.1 was punched into circular discs (each 6 mm indiameter), with the aid of an office punch. The discs were then put in a glass petri-dish and sterilized in a hot air oven at 60 °C for 30 minutes. 1 ml of each of the different concentrations of the extract were put in sterile glass plates and thirteen (13) sterile discs were put in their using sterile forceps to soak the extract, then they were allowed to dry. The discs were checked to be sure that they were not sticking together (Lamikanra, 1999). These CMLE discs were used for the antibacterial tests and that of *Candida albicans*. One capsule tetracycline 250 gm powder was dissolved in 1 ml distilled water. Sterile discs were then put inside it so as to be soaked with the tetracycline and then left to dry. This gave tetracycline discs of 250 mg/ml which is equivalent to 2.5×10^5 µg/ml. This concentration of tetracycline disc was prepared because the pilot study revealed that the commercially available tetracycline disc, 50 mg/ml is too low to be effective on both the bacterial and fungal species under test (Sodipo et al., 2012).

3.7 Disc Diffusion Antibacterial Selectivity Test

A stock solution was prepared with sterile distilled water at a concentration of 1 mg/ml. Tetracycline was used as a standard drug. Solutions of different concentrations (200, 400, 800, 1600, 3200, 6400 mg /ml) of the methanolic leaf extract and tetracycline (250 mg / ml) were prepared. Filter papers of 7 mm in diameter were cut and sterilized for one hour at 160 °C. The paper discs were placed on the prepared solutions to allow for absorption of the solution. The standard discs were allowed to dry in the oven and then placed on the dried surface of the culture plates already inoculated with bacteria (Agar Plates) and left at 37^oC for incubation. The zones of inhibition were measured after 24 hours (Soodipo, *et al.,* 2010) and recorded if it was greater than 10mm (Vlietinek et al., 1995).

3.8 Disc Diffusion Antifungal Selectivity Test

The antibiotic disc tetracycline (2.5×10^5µg/disc) were placed on the already prepared sabouraud-2%-dextrose agar containing graded concentrations of the CMLE (8 in all) and the control (2 pates). The *Penicillium spp.* and the *Aspergillus niger* were then removed from their pure culture with a pair of sterile forceps and placed on the plates so that the organisms could spread on the antibiotic disc and the extract in the plates. The plates were incubated at 25-30 °C and examined every 2-3 days and kept for four weeks before being considered negative for the fungi (Bello, 2002).

3.9 Minimum Inhibitory Concentration (MIC)

MIC can be defined as the lowest concentration where no visible turbidity is observed in the test tube. MIC is a technique employed to know at what concentration the extract can inhibit the microbial activity. MIC was determined using the broth dilution technique Vollekova et al. (2001). The minimum inhibitory concentration was determined from micro-organisms that where sensitive to the extract under study (leaf extract). Equal volume of nutrient broth was dispensed in to tubes (bijou bottles) were known concentrations of the extract diluted at concentration ranging from lowest to highest i.e 12.5 mg/ml to 200 mg/ml were prepared. Also 0.2 ml suspension

of microbial isolates (*Pseudomonas aeruginosa* and *Corynebacteria* species) were inoculated to various concentrations.

3.10 Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed (Reuben et al., 2009). MBC was determined by using the broth dilution technique described by Usman et al. (2007) by assaying the test tubes resulting from MIC determinations. A loop of the content of each test tube was inoculated by streaking on a solidified nutrients agar plate incubating at 37 °C for 18 hours and observed for bacterial growth. The lowest concentration of the subculture with no growth was considered the minimum bactericidal concentration.

4. Results

The result of the qualitative phytochemical analysis of methanolic leaf extract is shown in Table 1. The result showed the presence of flavonoids, cardiac glycosides, alkaloids, steroids, terpenes, saponins, carbohydrates and tannins, while anthraquinones and Phlobatanins were absent.

Table 1. Phytochemical analysis of the leaf extract of *Acanthospermum hispidum*

S/No	Plant Constituents	Test	Result	Observation
1.	Tannins	Ferric Chloride Test	+	Deep blue black
2.	Alkaloids	General test: Dragendorff's Test	+	Orange-red
		Mayer's Test	-	No ppt formed
3.	Saponins	Froth Test	+	Foam formed
4.	Cardiac Glycosides	(i) Keller-Killiani's Test	+	Greenish colour
		(ii) Liebermann-Burchard Test	-	Bluish green
		(iii) Salkowski Test	+	Reddish brown
5.	Steroids	Liebermann-Burchard Test	+	Bluish green
6.	Flavonoids	(i) Ferric Chloride Test	+	Bluish green
		(ii) Lead Ethanoate Test	+	Buff color ppt
		(iii) (Shinada's Test)	+	Light pink colour
		(iv) (Sodium Hydroxide Test)	-	Yellow colour
7.	Phlobatanins	Hydrochloric Acid Test	-	No color change
8.	Anthroquinones	(i) Free Anthroquinone Test	-	No colour formed
		(ii) Combined Anthroquinone Test	-	No colour formed
9.	Carbohydrates	(i) General test (Molish's Test)	+	Purple colour
		(ii) Monosaccharides (Barfoed's Test)	+	Brick red ppt
		(iii) Free Reducing Sugar	+	Deep brick red
		(iv) Combined Reducing Sugar	+	Deep brick red
		(v) Ketoses (Resorcinol or Selivanoff's Test)	+	Deep rose colour
10.	Terpenenoids	General Test	+	Pink colour

Key: + = present; - = absent (not detected).

In vitro disc diffusion method was used to study the antimicrobial activity of methanol leaf extract. The result of the antimicrobial activity on some bacterial pathogens shows that the extract at various concentrations inhibited the growth of *Staphylococcus aureus Conynebacteria sp. Bacillus subtilis, Klebsiella pnumoniae* and *Pseudomonas aeruginosa* were presented in Table 2, but there was no activity against *Streptococcus pyogene, E. coli, Salmonella typhii, Candida albican, Aspergillus niger* and *Pencillium* species. Among the sensitive microorganisms, *Conynebacteria* specie recorded the largest zone of inhibition (24.00 ± 1.00 mm) and *Staphylococcus aureus* recorded the least zone of inhibition (12.20 ± 1.06 mm). While other microorganisms such

as *Bacillus* cereus, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* had 13.00 ± 1.00 mm, 13.33 ± 1.15 mm and 18.23 ± 1.08 mm zone of inhibition respectively at 1000 mg/ml concentration. The standard drug (tetracycline 250 mg) inhibited the growth of the microorganisms tested in this study. The result of the minimum inhibitory concentration and minimum bactericidal concentration (/MIC/MBC) assay are as presented in Table 3 and 4 respectively. They revealed the concentration of the extract which could inhibit the growth of the bacterial species under test (bacteriostatic / bactericidal concentrations) with the values ranges from 25 to 50 mg/ml and 50 mg/ml to 100 mg/ml respectively.

Table 2. In vitro disc antimicrobial susceptibility test of the methanolic leaf extract of *A. hispidum*

Exract/drug conc. mg/ml	**Micro organisms/Diameter of zones of inhibition (mm)**										
	Staph. aureus	***Strep. Pyogene***	***Con. bacterium specie***	***Bacillus Subtilis***	***Salmo nella typhi***	***Klebsiella Pnumoniae***	***Pseudo monas aeruginosa***	***E. coli***	***Asper gillus niger***	***Penici llium specie***	***Con. Albican***
200	7.00±1.00	R	14.50±0.50	7.07±1.07	R	7.93±0.12	9.67±1.53	R	R	R	R
400	8.66±0.58	R	18.33±0.58	9.00±0.00	R	8.53±0.50	14.33±0.58	R	R	R	R
600	10.00±1.00	R	21.33±0.58	9.67±1.53	R	10.67±0.58	14.00±1.00	R	R	R	R
800	11.00±0.00	R	23.67±1.53	11.67±0.58	R	12.00±0.00	16.00±1.00	R	R	R	R
1000	12.20±1.06	R	24.00±1.00	13.00±1.00	R	13.33±1.15	18.23±1.08	R	R	R	R
*250 mg (TTC)	23.33±0.58	23.00±1.00	27.23±0.68	22.17±0.77	22.00±1.00	20.27±0.64	23.00±1.00	27.23±0.68	20.70±1.54	21.00±1.00	20.07±1.05

Key: R = Resistant (i.e not sensitive), TTC = Tetracycline, * = standard drug (2.5 x 105µg/disc), Data are presented as means±S.D and n = 3.

Table 3. The minimum inhibitory concentration (MIC) values of some organisms to the methanolic extract of *A. hispidum*

Bacteria	**Extract Concentrations (mg/ml)**				
	12.5	25	50	100	200
Conynebacterium species	+	β	-	-	-
Pseudomonas aeruginosa	+	+	β	-	-

Key: - = No growth (inhibition of bacterial growth); + = There was growth (Resistant); β = Least concentration showing no turbidity (MIC).

Table 4. The Minimum Bactericidal Concentration (MBC) of some organism to the methanolic extract of *A. hispidum*

Bacteria	**Extract Concentration (mg/ml)**				
	12.5	25	50	100	200
Conynebacterium species	+	+	+	-	-
Pseudomonas aeruginosa	+	+	+	β	+

Key: - = No growth; + = There was growth; β = Minimum bactericidal concentration (MBC).

5. Discussion

Previous research in to the phytochemistry of the leaves of *Acanthospermum hispidum* revealed the presence of flavonoids, cardiac glycosides, alkaloids, terpenes, saponins, carbohydrates and tannins, while steroids, amino acid and Phlobatanins were absent (Harekrishna et al., 2010). These secondary metabolites are responsible for most physiological and chemotherapeutic effects exhibited by plant extractives both in *vitro* and *vivo* (Usman, 2012). The extract under study had shown the presence of tannins and flavonoids; these phytochemicals had been known to inhibit the growth of microorganisms (Havagiray et al., 2004; Ogundaini, 2005; Usman et al.,

2007; Usman, 2012).

Alkaloids in the extract under study produce analgesic, anti-inflamatory and adaptogenic effects which help to develop resistance against diseases and endurance against stress (Gani, 1990; Gupta, 1994; Aska, 2008).

Carbohydrates in this extract occupy an important position in metabolism so the method of their detection is useful in phytochemistry. Carbohydrates have no therapeutic actions but they possibly increase the effectiveness of the biological active principles in the plant, thus most therapeutic principles isolated from plants occur in combination with sugar as glycosides (Iwu, 1984; Vollekowa et al., 2001).

In vitro disc diffusion method was used to study the antimicrobial activity of methanol leaf extract. The microorganisms tested in the study are widely distributed in air, water, faeces and can contaminate our food, thus producing enterotoxin which leads to some infections of the gastrointestinal tract including diarrhoea (Lucas and Gilles, 2003). The result of the antimicrobial activity on some bacterial pathogens shows that the extract at various concentrations inhibited the growth of *Staphylococcus aureus Conynebacteria sp. Bacillus subtilis, Klebsiella pnumoniae* and *Pseudomonas aeruginosa* were presented in Table 2, but there was no activity against *Streptococcus pyogenes, E. coli, Salmonella typhii, Candida albicans, Aspergillus niger and Pencillium* species. The result obtained showed that methanolic leaf extract of *Acanthospermum hispidum* exhibited inhibitory activities against some microorganisms tested at varying degrees of concentration as demonstrated by the diameters of the zones of inhibitions. These result where in conformity with those earlier reported by Harekrishna et al. (2010) and Chakraborty et al. (2012). The ability of the extract to inhibit the growth of these organisms *in vitro* may be due to the presence of some active chemical constituents found in the extract (Ogundaini, 2005).

The MIC/MBC data obtained from the evaluation of CMLE against the tested organisms ranges from 25 mg/ml to 50 mg/ml and 50 mg/ml to 100 mg/ml respectively, shown in Table 3 and 4. There is a need to consider the use of this leaf extract that have shown some measures of antimicrobial activities, judging by the antimicrobial activity, low Minimum Inhibitory Concentration (MIC) and low Minimum Bactericidal Concentration (MBC) on tested microorganisms.

6. Conclusion

In conclusion, the extract was found to be effective against the tested pathogenic microorganisms at varying concentrations. Therefore, this study validates the use of the leaf of *Acanthospermum hispidum* traditionally in some parts of Northern Nigeria.

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A Short Season Canadian Soybean Cultivar Double Cropped After Winter Wheat in Uzbekistan With and Without Inoculation with *Bradyrhizobium*

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Abstract

Agricultural systems in Uzbekistan are dominated by the production of cotton and winter wheat as these crops are subject to state-prescribed quotas. An experiment was conducted in the Fergana valley, in Uzbekistan, Central Asia, to determine the feasibility of growing a short-season Canadian soybean (*Glycine max* [L.] Merr.) cultivar after the harvest of winter wheat in early July. An inoculated treatment was compared to a non-inoculated control in a randomized complete block design with four blocks. While the inoculation did not establish well in 2003, in 2004, the yield of inoculated soybean was twice that of the non-inoculated control (106% increase). Inoculation in 2004 increased seed weights by 30%, final pod number by 29%, biomass dry weight at the pod-filling stage (56%) and at harvest (56%), as well as the harvest index by 22%. Nodules were, in general, only present in the inoculated treatments, which indicated that appropriate indigenous rhizobial strains were not present in these soils, but ineffective rhizobial competitors to commercial inoculants were also absent. Soybean production could be possible in Uzbekistan without competing with state prescribed crops such as cotton and winter wheat. Based on prices from 2004, this represents an additional income of more than 300$ ha^{-1}. More research is needed to determine the optimal conditions for inoculation success in hot and dry climates.

Keywords: double cropping, farming systems, *Glycine max*, inoculation, Uzbekistan

1. Introduction

Agricultural policies in Uzbekistan emphasize the culture of cotton, an important component of the Uzbek economy, and to a lesser degree, winter wheat. Both are subject to state regulation through a system of quotas, and little agricultural land is left for other crops. A typical rotation starts with cotton being planted in April, and harvested from September to December. Winter wheat is planted in November to December, in between rows of cotton, and harvested late June to mid-July in the following year. The land is then kept fallow until April when cotton is planted once again. To improve land productivity and food security in the region, the introduction of food legumes as double crops after the harvest of winter wheat was previously suggested (Bourgault et al., 2013). In many systems, the intensification of agriculture with double cropping is a major tool to improve the efficiency of resource use, such as water and radiation use efficiency (Van Opstal et al., 2011; Fouli et al., 2012). In the Fergana valley, the period between July and mid-October represents over 40% of the total yearly radiation (data taken from Pereira et al., 2009; Table 2). In addition, legumes in rotation with other crops can break disease cycles, improve the fertility and structure of the soil and encourage the development of mycorrhizal associations (Subbarao et al., 1995).

Soybean (*Glycine max* [L.] Merr.) is now the world's most important legume crop (Giller, 2001). Its annual production totalled 276 million tonnes in 2013 on over 110 million hectares averaging producer prices of US

$525 tonne^{-1} in 2012 (FAOSTAT, 2015). Soybean is used both as a food crop and for its oil, and the meal resulting from oil extraction is an important protein source for livestock. International markets for soybean are well developed and easy to access, and its production has been growing including in several dry areas such as Australia, Brazil and the United States (FAOSTAT, 2015). While short-duration soybean cultivars have been developed in Canada to avoid cold temperatures, no such short-duration cultivars are available to Uzbek farmers. Local Uzbek soybean cultivars generally mature in at least 120 days, and are thus in direct competition with government-prescribed production of cotton and winter wheat. As such, we hypothesized that the soybean cultivar Costaud, which matures in 90 to 100 days under Canadian conditions, would represent a good candidate for production under the conditions of Uzbekistan.

While the benefits of inoculating soybean are well established, the ability of native rhizobial populations in the soils of Fergana valley to form functional symbioses with soybean was not known prior to our work, and the performance of both the Canadian cultivar and the inoculum had to be assessed before encouraging farmers to grow soybean. Thus, the objective of this experiment was to determine the feasibility of growing a short-season Canadian soybean cultivar after the harvest of winter wheat in early July, and to evaluate the benefits from inoculation with *Bradyrhizobium japonicum* under these circumstances.

2. Materials and Methods

2.1 Location and Field Preparation

The experiment was conducted in the Fergana valley, in Uzbekistan, Central Asia (40°23'N, 71°45'E) from mid-July to mid-October, in the growing seasons of 2003 and 2004. During this period, the climate was hot and dry, with typical daily high temperatures of 40°C and daily low temperatures of 20°C. Rain was infrequent, except in early October: from July 15th to September 30th, 2003 and 2004, we recorded a total of 8.8 and 7.6 mm of rainfall, respectively, at our field sites. Climatic data (Figure 1) were collected using an on-site Vantage Pro Meteorological station (Davis Instruments Corp., Hayward, CA, USA), located approximately 200 m from the field site.

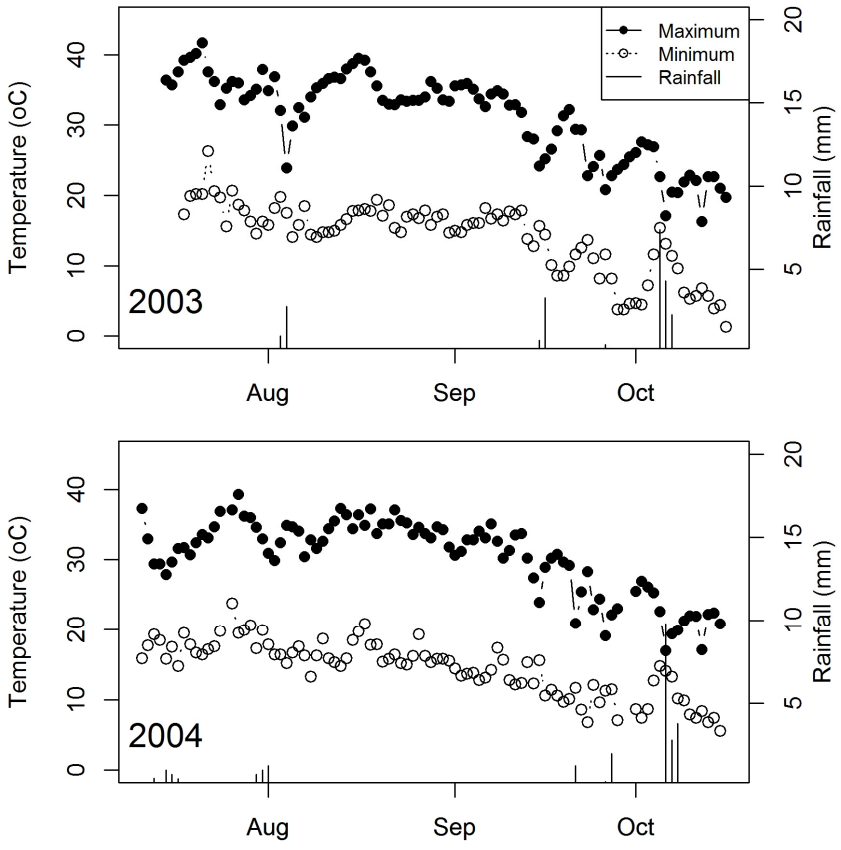

Figure 1. Climatic data for the growing seasons of 2003 and 2004 in the Fergana Valley, Uzbekistan (40°23'N, 71°45'E) from the beginning of July until the end of October

Based on textural analyses, soil at the experimental sites was silt loam. The available water content was 96 mm in 2003 and 75 mm in 2004, in the top 60 cm. The organic matter content was less than 2% and the soils had a well-developed plough pan at 30-40 cm depth.

Each field site produced winter wheat immediately prior to our experimentation. The wheat had been harvested, the straw and stubble burned, and the field ploughed and levelled, all following standard practices in the region. Neither experimental site had any history of soybean production. Sixty-centimeter-wide furrows were formed on the field site with a tractor-drawn lister.

2.2 Irrigation Scheduling

Irrigation scheduling was performed using a water balance and evapotranspiration estimates from climatic data as described in Allen et al. (1998) and in Webber et al. (2006). There were 5 irrigation events in both years (including an irrigation just prior to planting), which brought back soil moisture to field capacity, for a total irrigation applied of approximately 3650 and 4000 m^3 ha^{-1} in 2003 and 2004 respectively.

2.3 Experimental design

Plots consisted of 9 raised beds 5 m in length. Each plot was separated by a double row of mutant non-nodulating soybean plants. The plots were organized on the field site following a randomized complete block design with four blocks and two treatments. The treatments consisted of inoculated plots, and control non-inoculated plots.

2.4 Inoculation and Planting

The cultivar used was Costaud (Agrocentre Belcan, Ste-Marthe, QC, Canada), one of the shorter duration cultivar available in Canada at the time of the experiment. Seeds were covered with a slurry prepared from 10 g of commercial peat-based inoculant containing *Bradhyrhizobium japonicum* strain 532C (Nitragin, EMD Crop Bioscience, Milwaukee, WI, USA), and 15 mL of water, as directed on the package. Nitragin guarantees a minimum of 250 million viable bacterial cells per gram. Planting was done on July 22nd in 2003, and on July 13th in 2004. Seeds were sown by hand, with the non-inoculated control planted first to avoid contamination. Seeds were sown at 5 cm depth, on both sides of the raised bed to achieve a planting density of 50 plants m^{-2}. Weed control was done manually throughout the season.

2.5 Data Collected

Yield was measured by harvesting all pods in three randomly selected 2-m length sections of row in each plot, but at least 1 m away from the edge of the plot, and two outside rows were not utilized for data collection. Pods were threshed by hand, and seed yield was corrected for moisture content (to 0% moisture), and converted in kg ha^{-1} from plant population estimates before statistical analysis. The number of seeds per pod was evaluated on ten randomly selected pods from these areas, and seed weight was evaluated from one hundred seeds randomly selected from the harvested seeds from each plot. These were then oven-dried at 65-70 °C for 24 h, or until completely dry, to determine seed moisture level. Plant population estimates were determined by counting the number of plants in three 2-m length sections per plot.

To determine crop height and the number of flowers and pods, six plants were labelled at the beginning of the season and measurements were made on a weekly basis on these same plants until harvest. Above-ground biomass was also evaluated three times during the season at the flowering, pod-filling and harvest stages by sampling randomly 0.5 m of row. Plants were dried at 70°C for at least 24 h, until completely dry. The number of nodules and their dry weight were also determined in these destructive samplings. The nitrogen content and carbon to nitrogen ratio of the above-ground biomass was also determined with an elemental analyser (NC 2500 Elemental Analyzer, CE Instrument Inc., Milan, Italy). Non-nodulating soybean nitrogen contents were also looked at separately to ensure there was no spatial variability within the field.

2.6 Statistical Analyses

Statistical analyses were performed by analysis of variance (ANOVA) using the SAS/STAT software and GLM procedure (SAS, Cary, NC, USA). Because there were few nodules on the control plants, the analysis for the number of nodules was performed with a non-parametric approach using proc RANK prior to proc GLM.

3. Results

In both years, a soybean crop was grown to maturity using a short-season Canadian variety after the harvest of winter wheat in the Fergana valley, in Uzbekistan, Central Asia. Soybean yields averaged 1.0 and 1.4 t ha^{-1} in 2003 and 2004, respectively (Table 1), for inoculated soybean. Inoculation increased yields by 106% in 2004, but did not significantly increase yields in 2003. In fact, very few nodules were observed in 2003 (Table 1).

The higher yield observed due to inoculation in 2004 is mostly explained by higher seed weights (30% increase) and higher final pod numbers (29% increase), as compared to the control non-inoculated soybean plants (Table 1). The number of seeds per pod, however, was unaffected by inoculation. Nodules were not generally found on plants in the non-inoculated control treatment plots (Table 2).

Table 1. Yield and yield components of soybean grown in Uzbekistan

	2003		2004	
Parameters	Inoculated	Control	Inoculated	Control
Yield (kg/ha)	1047 a	968 a	1476 A	715 B
Seeds per Pod	NA*	NA	2.45 A	2.34 A
100- Seed Weight (g)	NA	NA	16.5 A	12.7 B
Final number of Pods	41.2 a	39.6 a	33.6 A	26.0 B
Above ground biomass (kg/ha)	2573 a	1887 a	3151 A	2020 B
Harvest index (%)	41.4 a	57.1 a	47.6 A	39.1 B

Values given are means of three (2003) or four blocks (2004) with the same treatment. Values associated with the same letter within the same year are not significantly different at $p<0.05$. Equipment malfunction in 2003 resulted in grains being lost before being counted.

*NA = "Not available". Due to oven malfunction, data on seeds per pod and 100-sed weight were not available in 2003.

Table 2. Average number (per 0.5 m row) and dry weight of nodules found in soybean grown in Uzbekistan in 2004

	Flowering		Pod-Filling		Harvest	
Treatment	Number	Dry weight (g)	Number	Dry weight (g)	Number	Dry weight (g)
2003						
Inoculated	2.3 a	NA*	8.7 a	NA*	16.0 a	NA*
Control	0 b	0	0 b	0	0 b	0
2004						
Inoculated	NA	0.185 a	122.5 a	2.338 a	13.5 a	0.285 a**
Control	0	0.000 b	0.75 b	0.005 b	0 b	0.000 a

Values given are the means of four blocks with the same treatment. Values associated with the same letter within the same sampling are not significantly different at $p < 0.05$.

*NA = "Not available". Dry weights for the 2003 season were not available.

**The probability of significance between the inoculated and control treatments was $p = 0.0505$.

In 2004, the above-ground biomass dry weight began to show increasingly significant differences by the pod-filling stage ($p=0.0533$; not shown), and showed very clear differences at the harvest stage ($p = 0.0098$; Table 1). The number of flowers was not affected by inoculation in either year on any of the days of observation, but the number of pods started to become greater in the inoculated treatment by mid-September (data not shown). Similarly, no differences were found in nitrogen content or nitrogen-carbon ratio at the flowering sampling. However, clear differences were detected for above-ground biomass at pod-fill and for grains at harvest (Table 3). The lack of differences in nitrogen content in leaves at the harvest stage suggests that most of the nitrogen from the symbiosis was translocated to grains. Our data suggest that the benefit of inoculation was greatest in the late stages of plant development.

Table 3. Nitrogen content and Nitrogen-Carbon Ratio of Soybean Grown in Uzbekistan in 2004

Treatment	Flowering		Pod-Filling		Harvest		Grains	
	N content (%)	N-C ratio	N content (%)	N-C ratio	N content (%)	N-C ratio	N content (%)	N-C ratio
Inoculated	3.98 a	0.0374 a	3.06 a	0.0245 a	2.65 a	0.0178 a	6.07 a	0.0491 a
Control	4.12 a	0.0352 a	2.41 b	0.0187 b	2.62 a	0.0148 b	5.27 b	0.0399 b

Values given are the means of four blocks with the same treatment. Values associated with the same letter within the same sampling are not significantly different at $p<0.05$.

4. Discussion

4.1 It is Feasible to Grow Soybean in Uzbekistan as a Double Crop

The cultivar Costaud, although originally developed for colder climate conditions prevalent in Canada, is able to grow and yield as a double crop in the hot and dry conditions of Fergana valley, Uzbekistan, provided some irrigation is available. It averaged 1.0 and 1.4 t ha^{-1} in 2003 and 2004, respectively after the harvest of winter wheat. This provides farmers with a possible alternative crop that does not compete with the production of cotton and winter wheat, which are subject to state quotas. However, yields were low compared to the world averages of 2.3 and 2.2 t ha^{-1} for 2003 and 2004 respectively (FAO, 2015), which suggest that genetic improvement for better adapted germplasm and inoculants could lead to greater yields. While the crop was irrigated, transient water stress during the day (with temperatures as high as 40 °C) were likely.

The production of an additional crop in the cotton-wheat rotation does require additional water. However, excessive water is often applied to furrow irrigated cotton and wheat in the region, and various strategies have been put forward to reduce irrigation amounts applied while maintaining yields (Horst et al., 2007; Pereira et al., 2009). For example, Horst et al. (2007) show that by irrigating cotton with surge-flow irrigation in alternate furrows, the irrigation water could be decreased by 44% (3891 m^3 ha^{-1}), which is about the amount of water necessary to irrigate soybean. Therefore water savings in cotton could compensate for the irrigation water necessary for soybean production. In 2004, the price of soybean was US \$211 t^{-1} (FAO, 2015), so this additional crop would increase income by approximately US \$300 ha^{-1}.

4.2 Importance of Environmental Conditions at Planting for Successful Nodulation

In 2003, the inoculation of seeds at planting was done in the afternoon, but due to technical difficulties in getting irrigation water, the plots remained dry until the next morning. In 2004, the inoculation and planting were done early in the morning and irrigation water was applied immediately afterwards, such that all operations were completed by midday. We suspect that in 2003 the soil was too hot and dry for the survival of rhizobial cells in the commercial inoculant, leading to poor nodulation. The success of 2004 however seems to indicate that with proper care, this inoculant can perform relatively well.

Environmental factors such as high temperature and drought affect nodulation and the ability of rhizobia to colonize plants (Hungria & Vargas, 2000). Our findings indicate that best management practices for soybean inoculation need to be developed for Uzbekistan as the benefits can be substantial. The manufacturer's instructions on the package (and quality assurance) are not taking into account environmental conditions that might be prevalent outside of Canada and the United States. In Australia, where the summer climate is similar, the current extension message to farmers is to inoculate and plant within 24 h (GRDC, 2013). Our experience suggests that even this might be too challenging for rhizobia survival. There is a real need to investigate genetic and agronomic solutions to this problem. Large variability in rhizobial strains has been documented in a number of semi-arid areas (Arun & Sridhar, 2005; Hungria et al., 2006; Giongo et al., 2008) and are potential genetic resources. Research with Brazilian inoculants and native rhizobia isolated from calcareous soils (prevalent in the region) is also being performed in the region (Egamberdiyeva et al., 2004).

To some extent, it is advantageous that no native bacteria were found in the soils of the Fergana Valley, as this indicates that there are no ineffective competitors to compete with introduced rhizobia.

4.3 Soybean Breeding Objectives for the Region Should Include Short Duration, Heat and Drought Tolerance

While Canadian, northern U.S. and northern Chinese cultivars could be genetic resources of short duration traits, Brazilian and Australian germplasm could be used as source of heat and drought tolerance. In addition, large genetic variability in nodulation sensitivity to water deficit stress among soybean cultivars has been

demonstrated (Serraj & Sinclair, 1998), and low petiole ureide content has been associated with the maintenance of nitrogen fixation under water stress (Sinclair et al., 2000). This could provide a relatively simple method for screening soybean cultivars and rhizobial strain combinations for higher nitrogen fixation in drought-prone areas.

5. Conclusion

Soybean production is possible in Uzbekistan without competing with state prescribed crops such as cotton and winter wheat. This could provide farmers with an additional income to their current production systems. More research is needed to determine the optimal conditions for inoculation success in hot and dry climates and heat and drought tolerance should be breeding objectives in the region.

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Biochemical Changes in Relation to Brown Leaf Spot (*Drechslera oryzae*) Resistance in Different Rice Genotypes

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Abstract

Brown leaf spot resistance in twelve rice genotypes *viz* IRRON M2 201, IRRON M2 202, IRRON M2 203, IRRON M2 204, IRRON M2 205, IRRON M2 302, IRRON M2 303, IRRON M2 304, IRRON M2 401, IRRON M2 402 and IRRON M2 501 were tested that the genotypes were showing 5.10 -20.16, 6.15-30.31 and 9.30-38.71 per cent disease severity at vegetative, panicle initiation and milk dough stages of plant growth, respectively which has also indicated that stages of plant growth also give variable response to disease. The mechanism of resistance revealed that the higher amount of total phenol and soluble protein in rice leaves resulted lower disease incidence. The rice genotype IRRON M2 205 is having maximum amount of soluble protein, representing 22.46, 21.94 and 21.27mg/gm and total phenol of 1.79, 1.68 and 1.61mg/gm of fresh leave at vegetative, panicle initiation and milk dough stage, respectively. Correlation between total phenol and soluble protein with disease severity at different growth stages shows that there was a negative correlation in all the rice genotypes. The rice genotype namely IRRON M2 205 was showing correlation value (r) -0.3310, -0.3415 and -0.3510 at vegetative stage, panicle initiation stage and milk dough stage, respectively. Banding pattern of protein resolving in SDS-PAGE showed that the maximum number of protein bands (13) was found in genotype IRRON M2 205. The protein banding patterns might be the responsible factors for resistance in rice against brown leaf spot pathogen.

Keywords: brown leaf spot, rice, resistance, soluble protein, total phenol

1. Introduction

Rice (*Oryza sativa* L.) is the food of millions of people since down of civilization, especially in Asia and the West Indies. It is cultivated in 114 countries and ranks third-highest in term of production in the world after maize (corn) and wheat FAO, 2010). In India, rice is grown under diverse agro ecological conditions ranging from puddle wet soils of the deltas to arid soil of Rajasthan in west and from the coastal areas at the mean sea level to the hilly tracts of about 2200 m high in north. The country produced 141.2 million tones of rice from 44.26 million hectares of land in 2010. The annual productivity of rice in India is 2240 kg/ha and in Uttar Pradesh 2119 kg/ha. It ranks second in term of production in the world being next to China (FAO, 2010). During 1950-51, the country produced 30 million tonnes of rice out of 50.8 million tonnes of total food grain and in 2010-11 the total food grain production reached 244.4 million tonnes from 125.3 m ha of land and rice alone produced 95.20 million tonnes from 44.24 m ha of land. It is grown on about one-fourth of the total crop area and provides food to about half of the country's population. In India, the slogan "Rice is Life" is most appropriate as this crop plays a vital role in our national food security. Because of wide adoptability of the crop growing in different parts of the country and different seasons of the year, several disease have been found to occur resulting causes extensive damage to the crop. Fungi alone account for nearly 30 diseases of rice in the country (Rangaswami & Mahadeven, 1999). Among these, a few occur in epiphytotic form in many parts of India and one of the important disease is brown leaf spot of paddy caused by *Drechslera oryzae* Subramanian and Jain (*Heliminthosporium oryzae* Breda de Hann) which caused havoc loss in Bengal during 1942-43.

The pathogen can infect paddy in all stages of crop growth *viz*, seedling, tillering, panicle initiation and grain filling. However, the occurrence of the disease is more when the crop approaches to maturity (Padmanabhan & Ganguly, 1954). The losses can be occurred up to 45% in case of severe infection and 12% in moderate infection

(Anonymous, 2003). Yield loss due to this disease has been estimated to be about 0.15 million tonnes annually in Eastern India (Variar, 1996). Bedi and Gill (1960) reported from Punjab that the disease caused 4.58 - 29.5% loss in weight of rice grain. The disease severity and yield loss varies from genotypes to genotypes. Therefore, characterization of genotypes and it response to disease is very important which can help us to do further research work in field of host pathogen interaction, defense mechanism, development of resistant variety etc. Keeping all these points in view, the studies have been undertaken in the present investigation.

2. Materials and Methods

2.1 Collection of Rice Genotypes

The seeds of rice genotypes namely IRRON M2 201, IRRON M2 202, IRRON M2 203, IRRON M2 204, IRRON M2 205, IRRON M2 302, IRRON M2 303, IRRON M2 304, IRRON M2 401, IRRON M2 402 and IRRON M2 501 were collected from Rice Breeders, Department of Genetics and Plant Breeding, C.S Azad University of Agriculture and Technology, Kanpur, India.

2.2 Isolation, Purification and Identification of Pathogen

The infected leaves of rice showing brown leaf symptom were collected from Nawabganj Farm, C.S Azad University of Agriculture and Technology, Kanpur, during crop season of 2010-11. The diseased leaves were collected and washed in sterilized water to remove dust and other surface contaminants. Small leaf bits from margin of newly emerged spot were cut with the help of a sterilized scalper. The bits surface were sterilized with 0.1% sodium hypochloride for 1-2 minute and washed 3-4 times with distilled water to remove the last traces of disinfectant. Excess moisture was removed by placing the bits in between the fold of sterilized filter paper.

The pieces were then transferred with the help of sterilized forceps into Petri plates, which are previously poured with sterilized 2% potato dextrose agar medium. The Petri plates used for isolation were also previously sterilized at 160 ± 1 °C for 2 hrs. in hot air oven. Two leaf bits were put in every Petri plate at equal distance and these were kept in B.O.D incubator at 25 ± 1 °C for incubations of pathogen. As soon as the mycelia growth was visible around the pieces, the hyphal tips from the advancing mycelium were transferred aseptically into the sterilized culture tube containing 2% PDA medium.

The culture was purified by single spore method and growth on PDA slants incubated for a week at 250C. On appearance of the colony, the slants were examined under compound microscope and identification of pathogen was established by comparing with authentic description as given by Ellis (1971), Breda de Hann, (1990).

Pure culture of *D. oryzae* was multiplied in sterilized Petri plates containing PDA. The inoculation plates were kept for 7 days in an incubator at 25 ± 1 °C. The culture was revived from time to time and stored at 5 °C in a refrigerator.

2.3 Field Trails

The field trial was conducted at Agriculture Research Farm of C.S. Azad University of Technology, Kanpur, India to evaluate the variability among different rice genotypes in response to disease severity of brown spot. The time of sowing of all varieties was on 15th June 2011. Recommended agronomical practices were followed. The plot size was 3 x 5 m and row to row and plant to plant distance was 30 x 15 m. The Randomized Block Design was used to conduct the experiment. The observation on disease severity was measured at vegetative, panicle initiation and milk dough stages of plant growth. The leaf samples from different rice genotypes were also collected separately at vegetative, panicle initiation and milk dough stages of plant growth for estimation of total soluble protein and total phenol content.

2.4 Measurement of Disease Severity

Disease severity was recorded at three stages of plant growth on the basis of formation of brown spots. Fifty leaves of paddy plants were randomly selected and number of lesions per leaf of each variety was counted. Disease severity was recorded using a score chart consisting of five (0, I, II, III, IV) different grades of infection was prepared on the basis of percentage leaf infection (Nayak & Padmnabhan, 1970). The leaf with no sign of infection received a score of 0 while those with highest infection i.e. with the 76 per cent or above leaf blighted received a score of IV. Similarly leaf with 1-25, 26-50 and 51-75 per cent area covered with spots received a score of I, II, and III, respectively. The disease severity of individual plant was calculated by the following formula.

$$\text{Disease severity (PDI)} = \frac{\text{Class rating} \times \text{Class frequency}}{\text{Total no.of leaves} \times \text{Maximum class rating}} \times 100$$

2.5 Phenol Estimation

The accumulation of phenols in different rice varieties was estimated following Bray and Thrope (1954) procedure. In this method, the total phenol estimation was carried out with Folin Ciocaltu Reagent (FCR) which was measured at 650 nm calorimetrically.

Exactly, 1gm of leaf sample of different rice varieties was ground in pestle and mortar sequently by adding in 10 times volume of 80% ethanol. It was then centrifuged to homogenate the suspensions at 10,000 rpm for 20 minutes. Supernatant was separated and the residue was re-extracted five times volume with of 80% ethanol. Again it was centrifuged and the supernatants were pooled. The supernatant was evaporated near to dryness and residue was dissolved in 5ml of distilled water. Different aliquots 0.2, 0.4, and 0.6, 1.0 and 1.5ml were pipette out into test tubes and the volume in each tube was make up to 3 ml with distilled water. Subsequently, 0.5ml of Folin Ciocaltu Reagent was added and after 3 minutes, 2 ml of 20% Na_2CO_3 solution in each tube was thoroughly mixed. The tubes were placed in boiling water for one minute and then cooled. Then absorbance of different concentrations at 650nm against a reagent blank was measured using UltraViolet Visible (UV –VIS) Spectrophotometer and the standard curve using different concentration on catechol was prepared. From the standard curve the concentration of phenol in the test sample was determined and expressed as mg phenols per gm. of fresh sample material.

2.6 Estimation of Total Soluble Protein

2.6.1 Protein Extraction

Rice leaves from different genotypes were harvested at vegetative stage, panicle formation stage and milk dough stage and washed with distilled water several times and blotter dried before protein extraction. A quantity of 1 gm of each leaf sample was cut into small pieces and grinded in pestle and mortar as 1: 5 ratio of leaves: extraction buffer. The extract was then centrifuged at 12000 rpm for 30 minutes at 4 °C. The supernatant was collected and used for quantification and profiling of protein.

2.6.2 Quantification of Protein

The method developed by Lowry et al. (1951) was used with slight modification for quantification of the total soluble protein content. The working standard solution was pipette out 0.2, 0.4, 0.6 and 1.0 ml and was put into series of test tubes. A quantity of 0.2, 0.4, 0.6 and 1.0 ml of the sample extract was also pipette out and kept into another series of test tube. Then volume in all the tubes was made up to 1ml with distilled water. Two tubes with 1ml of water each was served as the blank. Later on, 5ml of solution C was mixed well and incubated at room temperature for 10 min. Thereafter, 0.5 ml of FCR was mixed well immediately and incubated at room temperature in dark for 30 minute .The absorbance at 660 nm against the blank was read and standard graph was drawn to calculate the amount of protein in sample. The concentration of soluble protein was expressed as mg per gm. of fresh leaf material.

2.6.3 Protein Profiling

Profiling of soluble proteins was also done in different rice genotypes. Analysis of total soluble proteins through Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis was carried out for the study of variable response of resistance to brown leaf spot. SDS PAGE was done to get soluble protein pattern. Soluble protein was electrophorised by 12 per cent SDS polyacrylamide gel, based on the method of Laemmli (1970).

2.6.3.1 Gel preparation

In order to prepare stacking and resolving gel, the following quantities of different chemicals are used

Chemicals	Quantity	
	Stacking gel	Resolving gel
Acrylamide-bisacrylamide 30 %	2.6 ml	20.0 ml
Tris-Hcl	5.0 ml (0.5 M, pH 6.8)	12.5 ml (1.5 M, pH 8.8)
10% SDS	0.20 ml	0.50 ml
TEMED	10.0µl	25 µl
10% APS	100.00 µl	250 µl
Distilled water	12.1 ml	16.7 ml
Total	20.0 ml	50.0 ml

All the chemicals were mixed well and poured into vertical cassette leaving behind 3-4 cm from upper side. Subsequently, stacking gel solution was poured over the resolving gel. A comb was inserted into the gel mould to create wells for sample loading.

2.6.3.2 Sample loading

Take 75 ml of extracted soluble protein in an Eppendoff and mixed with 25 µl of sample buffer and 5 µl of tracking dye (Bromo phenol blue). Before loading the sample, it was boiled for 1 minute at 1000 °C to activate the protein molecules. Exactly 20 µl of sample was poured in each well. Then electrophoresis was carried out in Tris-glycine buffer at 30 mA current in stacking gel and 40 mA in separating gel. The electrophoresis was stopped after the tracking dye reached the bottom of the gel. The gel was then separated gently from the electrophoresis unit and placed in staining solution. After destaining, gel was illuminated with diffused fluorescent light and photographed.

2.7 Correlation Coefficient and Regression Equation

The biochemical analysis of rice leaves under different growth stages and disease severity of the corresponding value under the experiment was done to determine the level of correlation coefficients (r) between soluble protein and disease severity as well as between total phenol and disease severity. Simple regression equations (Y = a + bx) were also developed for both the variables (Protein and Phenol) separately to understand their relation with disease severity.

3. Results

3.1 Severity of Brown Leaf Spot on Different Rice Genotypes

The resistance could be assumed to be one factor for the reduction of disease severity. Severity of disease was recorded in the field trial under natural condition. It has observed that all the genotypes give variable reaction on disease severity even though at different stages of plant growth of the same variety (Table 1). The maximum disease severity with the value of 20.16, 30.31 and 38.71% at vegetative stage, panicle initiation and milk dough stage, respectively was observed in genotype IRRON M2 402, followed by genotype IRRON M2204 showing 6.12, 9.25 and 11.90% disease severity at vegetative stage, panicle initiation and milk dough stage while minimum with the value of 5.10, 6.15 and 9.30% are in genotype IRRON M2 205. Thus genotype IRRON M2 205 represented comparatively resistant to brown leaf disease among the all genotypes. It is also cleared that the stages of plant growth also give the different reaction on disease development. The highest disease severity was recorded in milk dough stage and lowest in vegetative stage in all genotypes, indicating that the disease severity is increases with increase age of plants.

Table 1. Disease severity of different rice genotypes at different stages

Genotypes	Disease severity (%)		
	Vegetative phase	Panicle initiation	Milk Dough
IRRON M2 201	13.08	19.2	21.92
IRRON M2 202	9.02	12.98	16.02
IRRON M2 203	8.54	12.9	15.5
IRRON M2 204	6.12	9.25	11.9
IRRON M2 205	5.1	6.15	9.3
IRRON M2 301	8.98	10.15	12.9
IRRON M2 302	14.8	18.75	28.12
IRRON M2 303	16.15	25.5	33.12
IRRON M2 304	13.33	18.21	25.22
IRRON M2 401	10.4	15.31	17.32
IRRON M2 402	20.16	30.31	38.71
IRRON M2 501	18.39	27.38	34.24
CD(5%)	0.598	1	1.788

3.2 Biochemical Changes

3.2.1 Soluble Protein

The variable content of soluble protein in rice is one of the determining factors for resistance to brown spot caused by *D. oryzae*. Data represented in Table-1 showed that the soluble protein content in all rice genotypes are different and also varies from age of plant growth. The maximum soluble protein content was observed that 22.46 mg/g, 21.94 mg/gm and 21.27 mg/g of fresh leaf at vegetative phase, panicle initiation stage and milk dough stage, respectively in the genotype IRRON M2 205. The rice genotype IRRON M2 204 was showing second highest in respect of soluble protein content with the value of 20.25 mg/g of fresh leaf at vegetative stage which is also statistically at par with genotype IRRON M2 203, IRRON M2 and IRRON M2 202, whereas at panicle initiation stage, genotypes IRRON M2 301 and IRRON M2 202 are statistically at par and at milk dough stage, IRRON M2 202, IRRON M2 204 and IRRON M2 301 are statistically at par. It showed that soluble protein content was gradually decreased from vegetative to panicle initiation and panicle initiation to milk dough stage.

Table 2. Soluble protein content in leaves of different rice genotype at vegetative phase, panicle initiation stage and milk dough stage

S.No.	Genotypes	Soluble Protein (mg/gm of fresh leaves)		
		Vegetative phase	Panicle initiation	Milk Dough
1	IRRON M2 201	19.1	18.4	18.25
2	IRRON M2 202	20.1	19.15	19.1
3	IRRON M2 203	20.35	19.85	19.54
4	IRRON M2 204	19.72	20.25	19.13
5	IRRON M2 205	22.46	21.94	21.27
6	IRRON M2 301	20.15	19.45	19.3
7	IRRON M2 302	18.95	17.85	17.53
8	IRRON M2 303	17.36	17.25	17.1
9	IRRON M2 304	19.1	18.16	18.1
10	IRRON M2 401	18.65	18.9	18.5
11	IRRON M2 402	16.3	16.25	16.1
12	IRRON M2 501	16.53	16.33	16.4
	CD (5%)	0.847	0.882	0.533

3.2.2 Total Phenol

The total phenol content in plant varies from genotype to genotype and also depends on the age of plant. The maximum total phenol content with the value of 1.79 mg/gm, 1.68 mg/gm and 1.61 mg/gm at vegetative stage, panicle initiation stage and milk dough stage, respectively was found in the genotype IRRON M2 205, followed by genotype IRRON M2 204 which shows 1.63, 1.58 and 1.51 mg/gm of fresh leaves at vegetative stage, panicle initiation stage and milk dough stage, respectively.

The total phenol content was decreasing from vegetative to panicle initiation stage and to milk dough stage. Variety IRRON M2 402 showed minimum amount of phenol content as 1.32 g/gm of fresh leaf at vegetative stage, 1.24 mg/gm of fresh leaf at panicle initiation stage and 1.19 mg/gm fresh leaf at milk dough stage. The genotypes IRRON m2 402 and IRRON M2 501 are found statistically at par at vegetative stages.

Table 3. Total phenol content in leaves of different rice genotypes at vegetative stage, panicle initiation stage and milk dough stage

Genotypes	Total Phenol (mg/gm of fresh leaves)		
	Vegetative stage	Panicle initiation stage	Milk Dough stage
IRRON M2 201	1.48	1.37	1.35
IRRON M2 202	1.5	1.41	1.4
IRRON M2 203	1.59	1.51	1.47
IRRON M2 204	1.63	1.58	1.51
IRRON M2 205	1.79	1.68	1.61
IRRON M2 302	1.39	1.3	1.27
IRRON M2 303	1.37	1.27	1.25
IRRON M2 304	1.41	1.32	1.29
IRRON M2 401	1.48	1.39	1.35
IRRON M2 402	1.32	1.24	1.19
IRRON M2 501	1.34	1.25	1.21
CD (5%)	0.121	1.141	0.078

3.2.3 Protein Profiling

Protein profiling of soluble protein from fresh rice leaves was done to determine if any new protein was associated with resistance to brown spot in different rice genotypes. SDS PAGE was used to find out the banding pattern of proteins. The banding patterns of different genotypes are shown in Table 4. The number of protein band present in each varieties range from 9-13. The maximum number of band was found in genotypes IRRON M2 205 followed by genotype IRRON M2 204. Minimum number of protein band was found in genotype IRRON M2 402. The banding pattern of proteins (Figure 1) represents that some new protein is present in genotype IRRON M2 205 and IRRON M2 204 which were not present in any other genotypes. The total numbers of bands in genotype IRRON M2 205 are 13 whereas IRRON M2 402 is 9. The presence or absence of protein bands may be responsible factors for resistance in rice against brown spot.

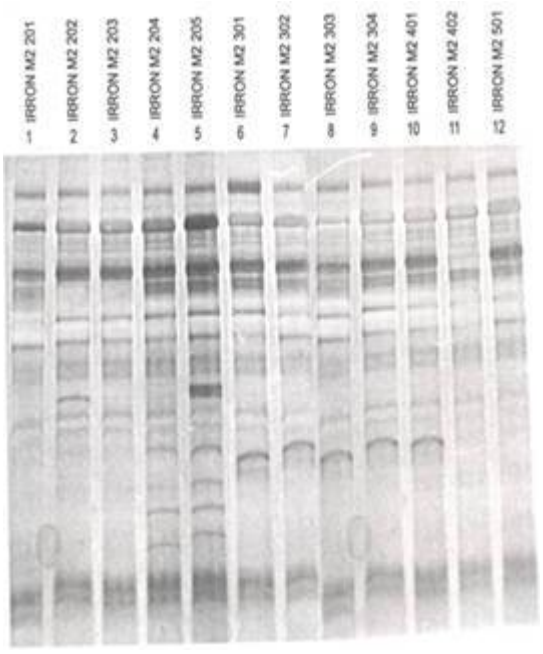

Figure 1. Banding pattern of different rice genotypes Resolved by SDS-PAGE

Table 4. Protein profiling of different rice genotypes by SDS-PAGE

S.No	Genotypes	Number of bands
1.	IRRON M2 201	11
2.	IRRON M2 202	10
3.	IRRON M2 203	12
4.	IRRON M2 204	13
5.	IRRON M2 205	13
6.	IRRON M2 301	12
7.	IRRON M2 302	10
8.	IRRON M2 303	12
9.	IRRON M2 304	11
10.	IRRON M2 401	11
11.	IRRON M2 402	9
12.	IRRON M2 501	10

3.3 Correlation of Disease Severity With Soluble Protein Content

The correlation between disease severity and soluble protein content at different stage of rice genotypes revealed that there was a negative correlation showing (r) 0.3212(vegetative), -0.3221 (panicle initiation) and -0.3592 (milk dough) in the genotype IRRON M2 201.

The regression equation of soluble protein and disease severity showed that higher regression value, lower disease incidence. The genotype IRRON M2 205 has the correlation coefficient (r) -0.3310 at vegetative stage, -0.3415 at panicle initiation stage and -0.3510 at milk dough stage, resulting resistance response to brown spot. Similar observations have also been found in case of phenol. The genotype IRRON M2 201 shows -0.2421, -0.2460 and -0.2475 correlation values at vegetative phase, panicle initiation stage and milk dough stage, respectively.

Table 5. Correlation of disease severity with soluble protein content

S. No.	Genotypes	Stages	Correlation coefficient (r) with disease severity	Regression equation
1.	IRRON M2 201	Vegetative stage	-0.3212	y=41.66-1.23x
		Panicle initiation	-0.3221	Y=41.50-1.20x
		Milk dough	-0.3592	Y=42.10-1.21x
2.	IRRON M2 202	Vegetative stage	-0.3210	Y=42.38-1.18x
		Panicle initiation	-0.3225	Y=43.35-1.22x
		Milk dough	-0.3420	Y=41.26-1.23x
3.	IRRON M2 203	Vegetative stage	-0.3110	Y=43.21-1.21x
		Panicle initiation	-0.3210	Y=43.23-1.20x
		Milk dough	-0.3250	Y=41.25-1.21x
4.	IRRON M2 204	Vegetative stage	-0.3210	Y=49.24-1.23x
		Panicle initiation	-0.3220	Y=42.56-1.21x
		Milk dough	-0.3360	Y=45.25-1.21x
5.	IRRON M2 205	Vegetative stage	-0.3310	Y=40.24-1.21x
		Panicle initiation	-0.3415	Y=41.28-1.22x
		Milk dough	-0.3510	Y=43.21-1.21x

6.	IRRON M2 301	Vegetative stage	-0.3215	Y=43.20-1.22x
		Panicle initiation	-0.3350	Y=40.20-1.20x
		Milk dough	-0.3375	Y=41.36-1.21x
7.	IRRON M2 302	Vegetative stage	-0.3010	Y=40.36-1.21x
		Panicle initiation	-0.3110	Y=41.10-1.21x
		Milk dough	-0.3215	Y=42.15-1.23x
8.	IRRON M2 303	Vegetative stage	-0.3210	Y=42.17-1.21x
		Panicle initiation	-0.3360	Y=41.35-1.23x
		Milk dough	-0.3225	Y=43.21-1.21x
9.	IRRON M2 304	Vegetative stage	-0.3375	Y=41.15-1.22x
		Panicle initiation	-0.3215	Y=41.10-1.21x
		Milk dough	-0.3592	Y=43.20-1.20x
10.	IRRON M2 401	Vegetative stage	-0.3115	Y=40.23-1.23x
		Panicle initiation	-0.3210	Y=43.25-1.20x
		Milk dough	-0.3250	Y=45.19-1.20x
11.	IRRON M2 402	Vegetative stage	0.3310	Y=43.32-1.21x
		Panicle initiation	-0.3310	Y=44.25-1.23x
		Milk dough	-0.3315	Y=41.23-1.21x
12.	IRRON M2 501	Vegetative stage	-0.3215	Y=43.31-1.21x
		Panicle initiation	-0.3215	Y=41.30-1.21x
		Milk dough	-0.3300	Y=44.36-1.23x

Table 6. Correlation of disease severity with total phenol content

S.no.	Genotypes	Stage	correlation	Regression equation
1.	IRRON M2 201	Vegetative stage	-0.2421	Y=23.03-5.24
		Panicle initiation	-0.2460	Y=23.23-5.30
		Milk dough	-0.2475	Y=24.20-5.75
2.	IRRON M2 202	Vegetative stage	-0.2326	Y=22.25-5.24
		Panicle initiation	-0.2328	Y=23.20-5.30
		Milk dough	-0.2402	Y=24.23-5.75
3.	IRRON M2 203	Vegetative stage	-0.2115	Y=20.21-5.24
		Panicle initiation	-0.2120	Y=21.23-5.30
		Milk dough	-0.2160	Y=21.22-5.75
4.	IRRON M2 204	Vegetative stage	-0.2125	Y=19.25-5.24
		Panicle initiation	-0.2150	Y=22.23-5.30
		Milk dough	-0.2330	Y=25.21-5.75
5.	IRRON M2 205	Vegetative stage	-0.2020	Y=21.20-5.24
		Panicle initiation	-0.2165	Y=22.25-5.30
		Milk dough	-0.2103	Y=23.26-5.75
6.	IRRON M2 301	Vegetative stage	-0.2112	Y=20.35-5.24
		Panicle initiation	-0.2150	Y=23.30-5.30
		Milk dough	-0.2265	Y=23.31-5.75

7.	IRRON M2 302	Vegetative stage	-0.2165	Y=21.26-5.24
		Panicle initiation	-0.2170	Y=22.23-5.30
		Milk dough	-0.2225	Y=23.21-5.75
8.	IRRON M2 303	Vegetative stage	-0.2250	Y=20.20-5.24
		Panicle initiation	-0.2158	Y=21.25-5.30
		Milk dough	-0.2345	Y=2315-5.75
9.	IRRON M2 304	Vegetative stage	-0.2475	Y=21.15-5.24
		Panicle initiation	-0.2350	Y=20.10-5.30
		Milk dough	-0.2300	Y=21.14-5.75
10.	IRRON M2 401	Vegetative stage	-0.2425	Y=20.15-5.24
		Panicle initiation	-0.2450	Y=21.15-5.30
		Milk dough	-0.2470	Y=21.13-5.75
11.	IRRON M2 402	Vegetative stage	-0.2320	Y=23.20-5.24
		Panicle initiation	-0.2528	Y=25.21-5.30
		Milk dough	-0.2125	Y=25.20-5.75
12.	IRRON M2 501	Vegetative stage	-0.2160	Y=23.25-5.24
		Panicle initiation	-0.2300	Y=24.23-5.30
		Milk dough	-0.2260	Y=23.15-5.75

The genotype IRRON M2 205 shows correlation coefficient value -0.2020 at vegetative phase, -0.2150 at panicle initiation phase and -0.2165 at milk dough stage. The genotype IRRON M2 205 is statistically non-significant at per at vegetative stage, panicle initiation phase and milk dough stage.

From the above two parameters, it is clear that among the three different stages of plant growth, the minimum co-relation value was found at vegetative stage, followed by panicle and milk dough stage, representing disease severity is decreases with increase of plant age.

4. Discussion

The resistance could be assumed to be one factor for the reduction of disease severity. All selected genotypes of rice showed different reaction to the brown spot. Disease reaction is one of the key factors to differentiate a variety from other. The data presented in Table-1 showed that all the genotypes give variable reaction on disease severity in three different stages of plant growth. The maximum disease severity with the value of 20.16, 30.31 and 38.71% was observed at vegetative stage, panicle initiation and milk dough stage, respectively. Mishra et al. (2011) reported that disease severity of Alternaria blight of wheat gradually increases from flower to dough and hard dough stage. Sahu and Biswas (2010) also found that the morphological, pathogenic and biochemical variations among popular varieties of wheat. Association of protein with plant defense against fungi and bacteria was earlier reported by several workers in graminaceous hosts, such as wheat (Sock et al., 1990; Biswas et al., 2003), rice (Kumawat et al., 2008; Biswas et al., 2010) oat (Fink et al., 1988), maize (Nasser et al., 1990) and barley (Hoj et al., 1989), Rajik and Biswas (2012) in tomato.

Accumulation of phenols is considered the expression of defence response in plants was reported by several workers (Matern & Kneusal, 1988, Kumar & Biswas, 2010, Arzoo et al., 2012, Biswas et al., 2012). Matern and Kneusal (1988) suggested that the first stage of the defense mechanism involves a rapid accumulation of phenols at the infection site which restrict or slower the growth of the pathogen. Nicholson and Hammerschmidt (1962) reported that the involvement of phenol in expression of disease resistances occurs in many ways like hypersensitive cell death or lignification of cell walls. Vidhyasekaran (1974) observed that Ragi *(Eleusine caracana)* resistant to *Helminthoshorium tetramera* contained more phenols than susceptible leaves. In the present study, total phenol content in twelve rice genotypes at vegetative stage, panicle formation and milk dough stage was also found inversely proportionate with increasing disease severity.

Protein is another important compound involved in the expression of disease resistances. During the course of present study, level of soluble proteins were also accumulated and enhanced in different rice genotypes. The

protein profiling by SDS-PAGE revealed the qualitative and quantitative differences on comparing the pattern of soluble proteins among rice genotypes. Successful electrophoresis profiles of total soluble proteins or a specific fraction for varietal identification and plant defense mechanism in crop plants was also done by several workers such as in wheat (Shewrey et al., 1978; Cooke, 1989; Cooke, 1993), in rice (Guo et al., 1986), in tomato (Biswas et al., 2012). Chen and Chang (1986) recommended SDS-PAGE as a useful technique for grouping of rice varieties.

Mishra et al. (2011) also reported that the correlation co-efficient between disease severity and soluble protein and total phenol content at different stages of wheat varieties revealed that there was negative correlation. The negative correlation co-efficient between disease severity and soluble protein and total phenol content have also been reported by several workers in different crops against different diseases such as in tomato against Fusarium wilt (Rajik et al., 2012; Biswas et al., 2012), in wheat against spot blotch (Biswas et al., 2003; Singh, 2010), in rice against brown leaf spot (Kumawat et al., 2008; Biswas et al., 2010)

It may be concluded from the present finding that among the three different stages of plant growth, the minimum co-relation value was found at vegetative stage, followed by panicle and milk dough stage, representing disease severity is decreases with increase of plant age.

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Effects of Water Depth and Seedling Rate on Weed Control and Yield of Late Season Lowland Rice (*Oryza sativa* L)

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Abstract

Three-year late season field experiment was conducted between 2011 and 2013 on the irrigated lowland experimental field at Edozhigi (9º04N, 6º7E) in the Southern Guinea savannah ecological zone of Nigeria, to determine the effects of different water depths and seedling rates on weed control, yield and yield components of lowland rice. The trial was laid out using a split plot design with six water depths (5 cm, 10 cm, 15 cm, 20 cm, saturated soil and continuous flow of water at 3 cm depth) as the main plots while seedling rates of 2, 4, and 6) per stand constituted the sub-plots. The treatments were replicated three times. The results indicated that the growth of weed species was significantly affected as water depth increased while rice yield was significantly enhanced as water depth increased to 20 cm. The 20 cm water depth gave weed control efficiency (WCE) of 57.6, 94.1 and 93.3% at 60 days after transplanting (DAT) in 2011, 2012 and 2013 respectively which was about 87% better than that obtained from saturated plots. At water depths of 10 and 20 cm, the growth of grasses and sedges were reduced by 60 and 100% respectively, while saturated and continuous flow of water encouraged their growth. Water depths of 10, 15 and 20 cm gave grain yield of 5052, 4700 and 4066 kg ha^{-1} which were 84, 85 and 85.5% higher than yields obtained from saturated plot in 2011, 2012 and 2013 respectively. Transplanting of 4 to 6 seedlings significantly suppressed weed growth and enhanced rice grain yield than 2 seedlings per stand. It is therefore concluded that maintaining water depths of 15 and 20 cm and seedling rates of 4 and 6 significantly suppressed weed growth and enhanced rice yield.

Keywords: water depth, seedling rate, weed control, rice yield

1. Introduction

The major impediment to the cultivation of rice is the heavy weed infestation which competes with the crop to such an extent that the crop gets smothered by the weeds. The weeds share not only plant nutrients but transpire a lot of valuable conserved water from the soil. The weeds also serve as alternative hosts for certain diseases and pests. Weed infestation can also interfere with operations at harvest and significantly increase harvesting and drying costs.

Weed competition is the most important yield reducing factor followed by drought, blast, soil acidity and general soil infertility (Johnson et al., 1997). Pandey (2009) reported that weeds are at present the major biotic constraint to increased rice production worldwide. Weeds constitute a big constraint to the production of rice in Nigeria. Ukwungwu and Abo (2004) reported that weeds constitute the greatest bottleneck to increased yield and quality of rice in Nigeria.

Management systems to meet the challenges of weeds are reflected in the varied rice production systems worldwide. Rice is characterized by its adaptability which allows it to grow in almost any biophysical environment in West and Central Africa. Rice is grown in a whole range of agro-ecological zones and five main rice-based systems can be distinguished with respect to water supply and topography in sub-Saharan Africa. These are rainfed upland, rainfed lowland, irrigated lowland, deep water and mangrove swamp. All these systems are increasingly threatened by weeds. Losses to weeds tend to be “chronic” in nature rather than sporadic and, as a result, are often underestimated (Johnson et al., 2010).

Management of weeds is an important component of production systems as elimination of weeds is expensive and hard to achieve. Presence of weeds is a constraint and their improper management further accentuates their effect. Among eco-friendly techniques for weed control in rice fields is effective water management which is among the oldest and cheapest cultural weed control methods.

Previous studies have shown that weed occurrence is a constant component of the ecosystem in comparison to the epidemic nature of other pests which makes farmers unaware of the significant losses they incur from their infestation (Johnson et al., 1999). The author also observed that a major impediment in the cultivation of rice is heavy weed infestation particularly in upland ecology, which competes with the crop to such extent that it could get smothered. Thus, farmers spend over US \$400 ha^{-1}, or 20% of their production costs to control weeds in rice fields (Islam et al., 2005). Improving weed control in farmers' fields was shown to increase rice yields by 15-23%, depending on the agro-ecosystem, and it is estimated that weeds may account for annual rice yield losses in sub-Saharan Africa of at least 2.2 million tonnes equating to US \$1.45 billion (Rodenburg and Johnson, 2009). The authors noted that rice yield losses due to uncontrolled weed growth was 28-74% in transplanted lowland rice, 28–89% in direct-seeded lowland rice and 48-100% in upland ecosystems.

Weeds pose one of the greatest challenges in lowland rice production systems. Grain yield losses due to weeds in lowland rice fields range from 20% to 60% in transplanted crops and from 30% to 80% in direct-seeded rice (Janiya, 2002).

The report of FAO (1997) indicated that the adoption of economically viable and environmentally friendly cropping systems is the key to successful weed management. Water management is a major component of any weed control programme in rice production, whether herbicide is used or not. The experiment was hence conducted in order to determine the water depth and seedling rate that effectively suppress weeds and enhance rice yield in lowland ecology.

2. Materials and Methods

The experiment was conducted in late seasons of 2011, 2012 and 2013 at Edozhigi lowland rice research field of National Cereals Research Institute, Badeggi, Nigeria, (Latitude 09º 45' N and Longitude 6º 07' E at an elevation of 75 meters above sea level) in Niger State in the southern Guinea savannah ecological zone of Nigeria. The average annual rainfall was 1287.5, 1158.3 and 1158.6 mm in 2011, 2012 and 2013 respectively, while the peak rainfall was between July to September each year (Table 1). During the three-yearfield experimentation, the rainfall season began in April (Table 1).

Table 1. Rainfall data for three years (mm)

Months	2011	2012	2013
January	0	0	0
February	0	9.8	0
March	0	0	0
April	36.7	53.3	58.6
May	173	101.6	253.9
June	106.1	259.4	144.8
July	336.3	206.4	236
August	264.9	146.7	181.9
September	130.2	289.7	199.7
October	224.2	101.2	83.7
November	14.1	0	0
December	0	0	0
Total	1285.5	1168.1	1158.8

Source: NCRI meteorological station.

The trial was laid out using split plot design with six levels of water (5 cm, 10 cm, 15 cm, 20 cm, saturated soil and continuous flow of water at 3 cm depth) as main plots while three seedling rates (2, 4, and 6 seedlings per stand) constituted the sub-plots. Main plot size was 10 m × 4 m and sub-plot size was 3 m × 4 m. The experiment was conducted from September to December in each year, being the late cropping season in Nigeria.

Irrigation water was supplied through a channel that had its source from River Kaduna. The water was let into the field through the alley way and a 3-inch PVC pipe was connected from the alley way to each plot to serve as water inlet pipe. White plastic indicator was fixed at the middle of each plot to monitor the water depth while 10 cm plastic hose of 3-inch diameter was connected to each plot to drain excess water when the maximum water level was attained.

2.1 Agronomic Practices

The land was mechanically ploughed, harrowed and leveled but the bunds round the perimeter of the plots were manually constructed using hoe. The rice seed used for the study was obtained from the Seed Unit of National Cereals Research Institute, Badeggi. A nursery was established in August each year. The rice seedlings were transplanted 30 days after sowing (DAS) according to the treatments at the spacing of 20 × 20 cm. Each sub-plot received a uniform application of 40 kg/ha N, 40 kg/ha P_2O_5 and 40 kg/ha K_2O one week before transplanting. Additional 40 kg/ha N was applied at panicle initiation stage. The source of N, P_2O_5 and K_2O was urea (46% N), single super phosphate (18% P_2O_5) and muriate of potash (60% K_2O) respectively. The field was flooded to various heights as dictated by the treatments at 15 days after transplanting (DAT).

Fertilizer were applied by broadcasting after proper drainage of water from the field. The field was then flooded immediately after fertilizer application. The field was finally drained one week before harvesting and harvesting was done when the grains were hard and had turned yellow/brown, which occurred 30-45 days after flowering or one month after 50% flowering.

2.2 Weed Identification

Weeds were identified and classified into three classes as grass, broad leaved weeds and sedges and there occurrence was determined,

2.3 Percentage Weed Control Efficiency (%WCE)

Weed control efficiency was determined using the following formula by Das (2011).

$$\% \text{ WCE} = \frac{(\text{WDc} - \text{WDr})}{\text{WDc}} \times 100 \quad (1)$$

where:

% WCE = percentage weed control efficiency

WDc = weed density (m^{-2}) in control plot

WDr = weed density (m^{-2}) in treated plot

2.4 Percentage Weed control Index (% WCI)

Weed control index was determined using the following formula by Das (2011).

$$\% \text{ WCI} = \frac{(\text{WDMc} - \text{WDMr})}{\text{WDMc}} \times 100 \quad (2)$$

where:

WDMc = weed dry weight (m^{-2}) in control plot

WDMr = weed dry weight (m^{-2}) in treated plot

2.5 Percentage Weed Composition

This was carried out by counting the weeds within 1 m^2 quadrant in each plot and the weeds found were then classified into broad leaf, grasses and sedges and expressed in percentage.

2.6 Rice Yield Parameters

Rice grain yield was obtained from a net plot of 2.8 m × 4 m. The chaff was separated from the grains by shocking in water for two minutes. After proper stirring, the floating chaff and the grains were collected and both dried separately and weighed using weighing balance. Percentage chaff was determined using the following formula:

$$\% \text{ Chaff} = \frac{\text{Chaff weight}}{\text{Total harvest}} \times 100 \quad (3)$$

The weight of 1000 grains was determined by taking the measurement of 100 grains using an electrical digital weighing balance and the result was multiplied by 10 to give 1000 grains weight.

2.7 Data Analysis

Data collected were subjected to analysis of variance (ANOVA) using the M-Stat-C version 1.3 (Snedecor & Cochran, 1967) statistic package and significant means were separated using LSD at 5% probability.

3. Result

Morphologically, three classes of weeds were identified; broad leaved weed, grasses and sedges. The dominant broad leaved weeds were *Hyptis lanceolata, Sphenoclea zeylanica, Ludwigia decurrens* and *Merremia aegyptia* while grasses with high occurrence were *Echinochloa stagnina, Paspalum polystachyum* and *Pennisetum polystachion Fimbristylis littoralis* was the dominant sedge (Table 2).

Table 2. The dominant weeds found on the experimental site

Weed species	Families	Life span	Occurrence		
Broad leaf			2011	2012	2013
Hyptis lanceolata (Poir)	*Lamiaceae*	A	+	-	-
Ipomoea aquatica (Forsk)	*Convolvulaceae*	A	++	+	+
Indigofera hirsuta	*Rubiaceae*	A	+	-	-
Ludwigia abyssinica (Rich)	*Onagraceae*	A	++	++	++
Ludwigia decurrens(Walk)	*Onagraceae*	A	++	++	++
Merremia aegyptia (Linn)	*Convolvulaceae*	A	+	-	-
Oldenlandia corymbosa (Linn)	*Rubiaceae*	A	-	-	-
Phyllathus amarus (Schum)	*Euphorbiaceae*	A	-	-	-
Sphenoclea zeylanica (Gaertn)	*Sphenocleaceae*	A	+++	+++	+++
Commelina benghalensis		A	+	-	-
Ageratum conyzoides		A	++	-	-
Grass					
Echinochloa colona (Gaertn)	*Poaceae*	A	++	+	+
Echinochloa stagnina (Beauv)	*Poaceae*	A	+	+	+
Lepotochloa caerulescens (Steud)	*Poaceae*	A	+	-	-
Paspalum polystachyum (Linn)	*Poaceae*	A	+		+
Paspalum conjugatum (Berg)	*Poaceae*	A	+	+	-
Paspalum vaginatum (Sw)	*Poaceae*	A	-	-	-
Pennisetum polystachion (Linn)	*Poaceae*	A	+	-	-
Oryza barthii (Chev)	*Poaceae*	A	+	+	+
Leersia hexandra	*poaceae*	A		-	-
Panicum laxum	*poaceae*	A	-	-	+
Sedge					
Cyperus esculentus (Linn)	*Cyperaceae*	P	+	+	+
Cyperus haspan (Linn)	*Cyperaceae*	P	+	+	+
Fimbristylis littoralis (Gaudet)	*Cyperaceae*	A	+++	+++	+++
Cyperus iria	Cyperaceae	P	-	+	++

A = annual, P = perennial, - = absent + = few, ++ = many and +++ very many.

There was shift in weed types during the three year trials. Broad leaved weeds like *Commelina benghalensis* and *Ageratum conyzoides*, grasses like *Lepotochloa caerulescens* and *Pennisetum polystachion* that were available in 2011 disappeared in the subsequent years while broad leaved weeds like *Merremia aegyptia* and *Oldenlandia corymbosa* and grasses like *Panicum laxum* appeared at later years (2012-2013). Also sedges like *Cyperus iria* that were not available in 2011 appeared in 2012 and 2013 (Table 2).

The percentage weed control efficiency was significantly affected by both water depth and number of seedlings transplanted in the three-year study. The water depth of 20 cm gave significantly higher weed control efficiency

than all other water depths which was consistent across the periods the sampling was taken. The weed control efficiency generally increased as the rice growth progressed between 60-75 DAT (Table 3). The number of seedlings transplanted equally had a significant effect on the weed control efficiency in the three-year study. Higher weed control efficiency was recorded in the plots with six seedlings per stand while two seedlings per stand gave significant lower weed control efficiency (Table 3).

Table 3. Effect of water depth and seedling rate on percentage weed control efficiency between 2011-2013

	2011				2012				2013			
	Days after transplanting											
Treatments	30	45	60	75	30	45	60	75	30	45	60	75
Water level cm (W)												
5	25.3	17.4	34.1	35.5	45.8	4.3	72.1	74.3	42.6	46.3	63.8	70.5
10	45.6	35.8	32.2	40.2	50.9	28.6	78.4	78.1	63.5	67.7	77.2	80.0
15	57.0	44.3	43.6	62.2	67.8	76.5	90.9	85.3	75.5	87.8	92.1	92.2
20	80.7	67.8	57.6	77.7	79.5	82.7	94.1	94.3	84.2	91.3	93.3	93.7
Continuous flow	2.8	5.8	23.5	8.9	14.3	4.4	34.8	35.9	26.5	21.4	28.3	25.5
Saturated (check)	0	0	0	0	0	0	0	0	0	0	0	0
CV	10.6	6.9	7.4	2.5	15.7	5.4	8.5	5.7	5.1	5.0	3.4	4.5
SE±	3.0	2.3	1.9	0.4	6.2	3.2	2.8	3.6	2.0	4.3	1.8	1.7
Seedling rate per stand (S)												
2	38.4	32.4	40.7	40.2	20.1	20.4	53.9	52.4	32.8	43.7	52.5	56.3
4	44.6	38.2	42.4	44.6	56.7	44.8	65.1	65.3	52.0	53.5	59.9	60.9
6	65.8	70.4	70.9	70.7	55.3	46.6	66.1	66.3	61.5	60.0	65.0	65.7
CV	10.6	6.9	7.4	2.5	15.7	5.4	8.5	5.7	5.1	5.0	3.4	4.5
SE±	5.10	3.41	4.44	7.80	4.60	7.21	4.21	3.40	10.90	7.20	6.80	6.01
W X S	*	**	*	*	*	**	*	*	*	*	*	*

* = significant at 5 % and ** = significant at 1 %.

Weed control index followed the same trend as percentage weed control efficiency. Water depth of 20 cm gave significantly higher weed control index than any other treatment (Table 4). The weed control index increased as the rice growth progressed between 60-75 DAT. Weed control index was generally higher in 2013 than other years of the study. The effect of seedling rate on weed control index was similar to that of percentage weed control efficiency. The highest weed control index was recorded under six seedlings per stand which was significantly higher than either two of four seedlings per stand (Table 4).

The growth of the three weed types (grasses, broad leaved and sedges) was only affected by different water depths. The number of seedlings transplanted had no significantly ($p < 0.05$) effects on percentage weed composition in the three-year study. Generally, increased in water depth significantly reduced percentage weed composition of all the weed species, although grasses and sedges were better controlled by deeper water of 15 and 20 cm depth. The results of percentage weed composition as influenced by various water depth are shown in Figures 1-9.

Table 4. Effect of water depth and seedling rate on percentage weed control index between 2011-2013

	2011				2012				2013			
	Days after transplanting											
Treatment	30	45	60	75	30	45	60	75	30	45	60	75
Water level cm (W)												
5	44.1	35.6	34.2	69.1	54.6	38.6	47.4	50.6	63.9	65.5	96.8	98.3
10	58.5	52.1	54.6	70.7	60.3	48.0	61.1	65.4	71.2	71.2	98.3	98.5
15	74.7	71.5	72.0	82.5	72.8	72.5	78.2	80.9	82.5	81.2	99.1	99.5
20	82.4	83.4	83.9	73.0	74.9	29.0	81.1	82.8	82.5	81.2	99.1	99.2
Continuous flow	20.6	7.2	18.3	34.0	29.1	17.5	28.9	22.6	40.0	19.1	40.0	12.9
Saturated	0	0	0	0	0	0	0	0	0	0	0	0
CV	2.6	7.1	54.8	11.2	11.5	4.1	14.7	5.9	3.4	5.9	5.9	4.4
SE±	0.7	1.7	12.6	3.4	2.7	0.7	3.9	1.0	1.6	4.8	36.1	47.2
Seedling rate per stand (S)												
2	42.6	47.1	56.7	69.8	30.6	46.2	56.7	58.2	43.2	38.9	67.8	65.6
4	55.8	50.2	55.2	68.0	54.6	53.1	57.5	59.7	61.5	54.6	70.7	68.2
6	76.4	78.2	64.8	81.5	62.7	66.3	70.4	66.5	66.9	67.0	73.8	70.2
CV	2.6	7.1	14.8	11.2	11.5	4.1	14.7	5.9	3.4	5.9	5.9	4.4
SE±	5.61	4.32	4.01	3.23	8.10	5.63	3.42	2.34	6.73	4.70	1.72	3.57
W X S	*	*	*	*	NS	*	*	*	*	*	*	*

* = significant at 5 %, ** = significant at 1 % and NS = not significant.

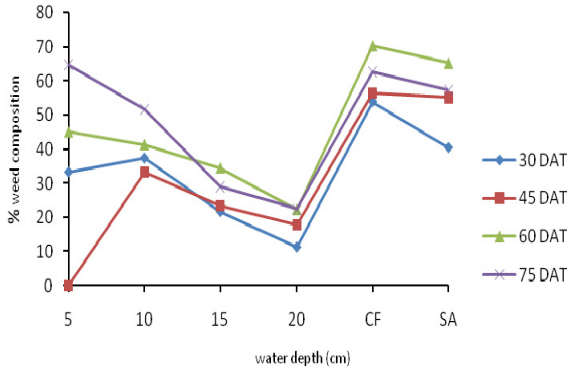

Figure 1. Percentage grass weeds as affected by various water depths in 2011

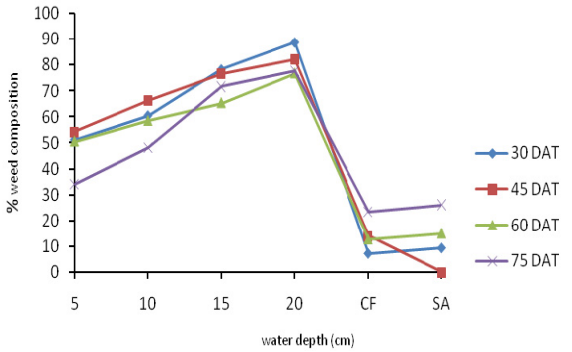

Figure 2. Percentage broad leaved weeds as affected by various water depths in 2011

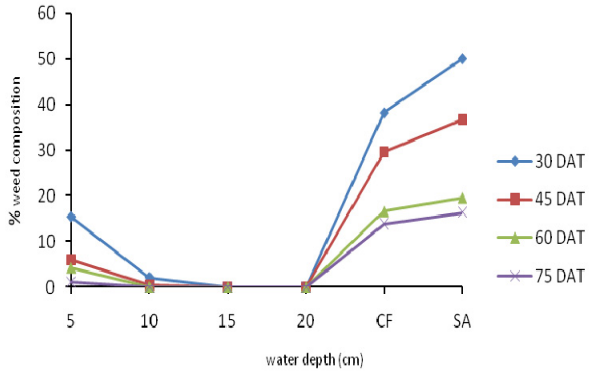

Figure 3. Percentage sedge weeds as affected by various water depths in 2011

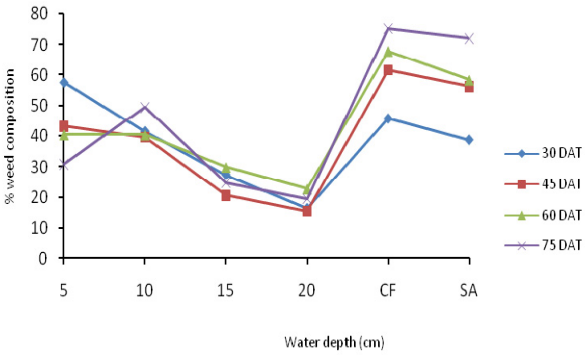

Figure 4. Percentage grass weeds as affected by various water depths in 2012

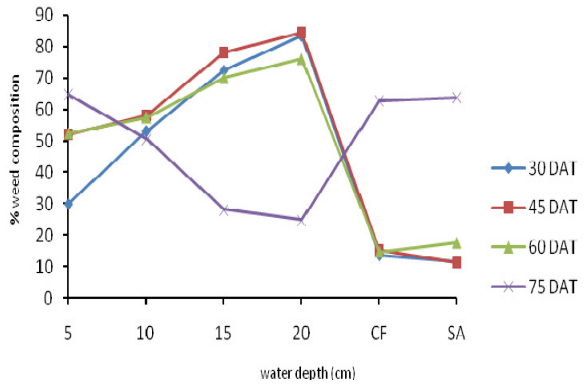

Figure 5. Percentage broad leaved weeds as affected by various water depths in 2012

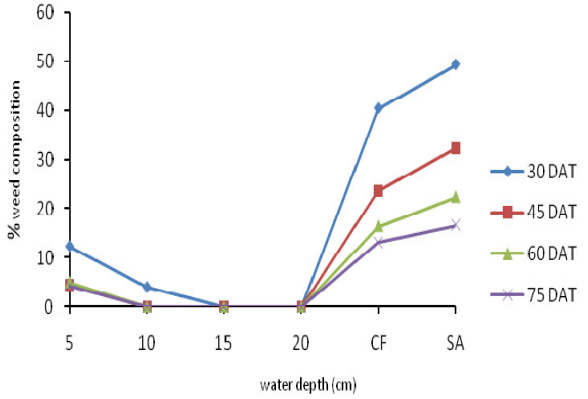

Figure 6. Percentage sedge weeds weeds as affected by various water depths in 2012

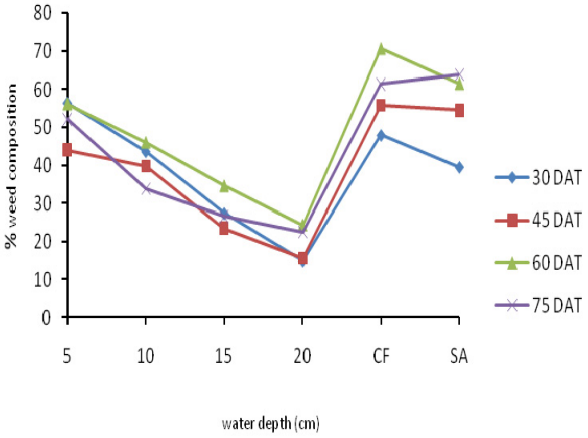

Figure 7. Percentagegrass weeds as affected by various water depths in 2013

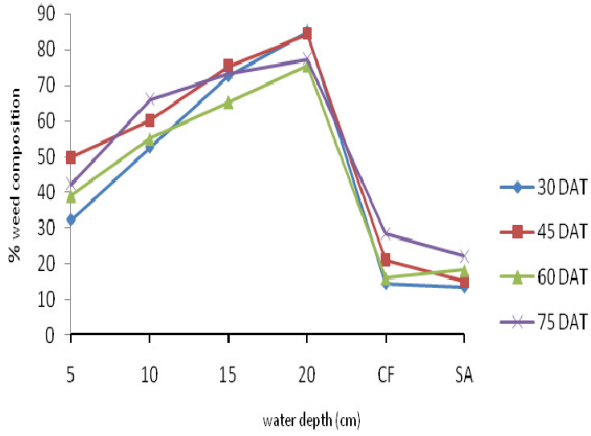

Figure 8. Percentage broad leaved weeds as affected by various water depths in 2013

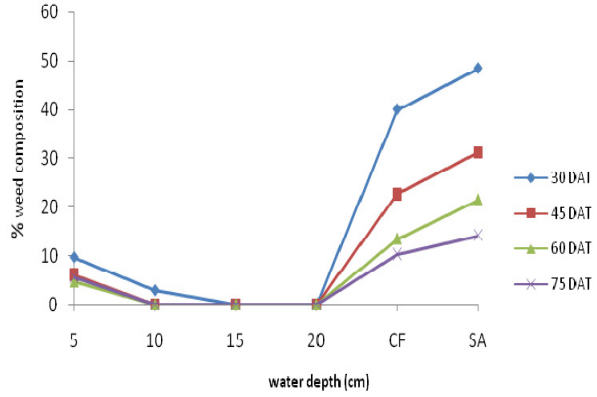

Figure 9. Percentage sedge weeds weeds as affected by various water depths in 2013

The 20 cm water depth gave significantly lower percentage grasses in the three-years study although grasses like *Oryza barthii* and *Leersia hexandra* were less affected by the various water depths. The lower percentage grass composition recorded in 20 cm water was similar to 15 cm water depth at all periods in 2013 while continuous water flow and saturated plots gave higher composition grass weed (Figures 1, 4 and 7).

The broad leaved weeds were less affected by various water depths where some of the species like *Sphenoclea zeylanica* were able to endure the various water depths. Broad leaved weeds were the common weeds found in the deep water of between 10-20 cm. The water depth of 20 cm therefore gave significantly higher number of

broad leaved weeds (Figures 2, 5 and 8) than other water depths. The result of this study revealed that sedges were more sensitive to various water depths. The growth of sedges was completely suppressed at 10-20 cm water depth. While saturated soil gave significantly higher percentage of sedges at all periods the sample was taken (Figures 3, 6 and 9).

Panicle production responded differently to different water depths and seedling rate in the three years of study. There was direct linear relationship between water depth and seedling rate, that is increase in water depth resulted in corresponding increase in panicles m^{-2}. Panicles per m^{-2} were significantly higher in 20 cm water depth while seedling rate of six gave higher number of panicles per m^{-2} (Table 5).

The percentage rice chaff was significantly influenced by both water depth and seedling rate. Generally, the higher the depths of water the lower % chaff produced. The 20 cm water depth consistently gave significantly lower percentage chaff while higher % chaff was recorded in saturated plot. Two seedling/stand gave significant lower % chaff than others (Table 5). The % chaff was generally lower in 2011 than the other years which could be attributed to an abnormal extension of rainfall in this year (Table 1).

The 20 cm water depth recorded significantly heavier grains which were statistically similar with 15 cm water depth throughout the three-year study while lower grain weight was recorded in the continuous flow of water which was at par with saturated soil water condition. Two seedling/stand gave significantly higher grain weight while six seedling rates produced consistently lower grain weight.

Table 5. Effect of water depth and seedling rate on yield components and yield in 2011-2013

	Panicle m^{-2}			**% chaff**			**1000 grain weight (g)**			**Grain yield (kg h^{-1}**		
Treatments	2011	2012	2013	2011	2012	2013	2011	2012	2013	2011	2012	2013
Water depth cm (W)												
5	191.0	187.8	185.3	15.8	16.4	16.3	29.1	29.10	27.2	3289.1	3245.2	3141.0
10	290.3	285.7	269.0	15.7	15.7	17.1	30.0	30.01	28.2	3551.7	3550.0	3311.3
15	398.1	384.8	368.7	13.5	10.0	8.5	31.3	32.02	31.8	4702.3	4493.1	4066.0
20	464.7	441.7	406.6	6.9	8.7	7.8	31.8	32.46[a]	31.7	50517.8	4700.4	4033.1
Continuous flow	121.8	110.7	90.6	27.3	28.4	24.2	28.2	28.24	26.4	2812.3	2534.3	2103.6
Saturated	101.8	53.1	34.3	29.3	33.1	35.2	27.6	27.60	25.8	990.0	696.2	607.2
CV	5.57	1.89	2.99	7.90	4.45	4.57	2.4	1.79	1.8	3.0	1.3	2.4
SE±	13.93	9.37	5.42	1.37	1.11	1.25	0.6	0.87	0.30	98.0	39.4	60.7
Seedlingrate per stand (S)												
2	200.3	193.6	184.8	11.4	12.2	13.4	31.3	31.11	29.5	3128.3	3245.2	2616.9
4	267.7	255.3	237.7	18.8	20.1	19.3	28.9	29.34	27.9	3468.9	3550.0	2956.2
6	315.9	282.9	254.7	24.1	23.9	21.9	28.8	29.27	27.9	3601.4	4493.1	3058.0
CV	5.57	1.89	2.99	7.90	4.45	4.57	2.4	1.79	29.5	3.0	1.3	2.4
SE±	9.85	3.18	4.64	0.97	0.57	0.57	0.10	0.13	1.8	69.3	28.8	48.5
W X S	*	**	*	*	*	*	*	NS	NS	*	*	*

* = significant at 5 %, ** = significant at 1% and NS = not significant.

Rice grain yield was significantly affected by both water depth and seedling rate and the highest grain yield was recorded when the field was under continuous flood of 20 cm water depth from 15 days after transplanting till maturity while the six seedling/stand gave significantly higher grain yield (Table 5). The saturated plot had the lowest grain yield across the three years of study.

4. Discussion

The prominent weeds found in the experimental plots in the three years of study included all categories of weeds. Cumulatively, broad leaved weeds were 31%, grasses 51% and sedges 18%. The presence of these weeds in the experimental plots agreed with the work of Mirza et al. (2007) that major weeds of rice include all categories. Similarly, Florez et al. (1999) observed that weed population in rice field consisted of grasses, sedges and broad leave weed species.

The higher percentage weed control efficiency recorded in 20 cm water depth and six seedlings /stand could be ascribed to inability of some weed seeds to germinate under anaerobic condition created by impounded water and suppression of already germinated weed seedlings. The combined effect of 20 cm water depth and six seedlings/stand gave 89-95.5% weed control efficiency. Similar result was recorded by Sangay et al. (2013) who observed that flooding of soil retarded the germination of weed seeds and once seedlings have established, soil may be flooded to suppress weed growth.

David (1992) observed that weed population density and total dry weight per unit area decreased as water depth increased and the experiment under lowland conditions, showed that at 8 cm height of water, the number of grasses and sedges declined and at about 16 cm water height many of them disappeared. The author concluded that the decline was explained by many weed seeds failing to germinate under anaerobic condition, and the effect of standing water in suppressing growth and development in the early stages.

The six seedlings/stand created a dense stand of rice that disallowed some weeds from growing and compete with rice. Odero and Rainbolt (2011) observed that poor stand of rice encouraged infestation by some weeds such *Commelina spp.* and *Ludwigia* spp. but in denced stand of rice, these weeds cannot compete for essential sunlight and do not become a problem. Tabbal et al. (2002) reported that maintaining continuous shallow submergence, especially during vegetative growth, effectively suppressed weed growth while poor water management often contributed to increase in biomass of weed species (Bouman et al., 2007)

The experiment conducted by Juraimi et al. (2009) indicated that submergence of rice fields hindered weed germination and suppressed the population of most germinated weeds. Similarly, Leeper 2010 observed that soon after flood were established, an anaerobic condition established at the soil surface and most weeds will not germinate.Under anaerobic conditions, water acts similar to a pre-emergent herbicide.

The six seedlings/stand created a solid (dense) stand of rice that disallowed some weeds from proliferate and compete with rice. Odero and Rainbolt (2011) observed that poor stand of rice encouraged infestation by some weeds such *Commelina spp*. and *Ludwigia spp*. but in solid stand of rice, these weeds cannot compete for essential sunlight and do not become a problem. The work of Parvez et al. (2011) to determine the seedling method and rate on weed at Malaysia reported higher weed density and dry matter in lower seedling rate.

The result of this study indicated that water depth and seedling rate had significant effect on weed control index. The result is similar to that obtained in percentage weed control efficiency where higher water depth of between 1-20 cm gave significant better control index. This is an indication that the flood depth of 15-20 cm drastically suppressed the growth of most weeds. The result was consistent for the three-year study. The analysis of interaction between weed infestation and water management in lowland rice by Jabber and Orr (2002) indicated that good water management helped in reducing weed infestation as both the seeds and growth of most weeds are suppressed by standing water. Wopereis et al. (2009) also reported that good water management in rice usually helps to reduce the weed population, as flooding prevents most weeds from germinating.

The variation of weed species composition as influenced by various water depth is an evidence that weed species differ in their response to different water depths. The lower percentage of grasses and sedges in the deeper water depth of 10-20 cm might be the consequences of the deep water on their growth. The progressive decrease of sedges to zero percent at 45-75 DAT in deeper water could be due to the fact that this class of weeds cannot survive in deep water for a long time. This is similar to the result obtained by Venkataraman and Gopalan (1995), that continuous submergence of rice field to 5 cm depth resulted in a minimum number of grassy weeds while maintaining a water depth of 6 to 8 inches for 21 to 28 days after planting can provide partial control of *Echinochloa crus-galli* (Monaco et al., 2002). In Indonesia, Haden et al. (2007) observed an increased incidence of sedges due to reduced periods of flooding.

Dominance of grasses such as *Echinochloa* species and *Leptochloa chinensis* is favoured by saturated and below saturation conditions (Bhagat et al., 1999), while increase in flooding depth and flooding duration encourages broad leaved weeds and sedges (Kent & Johnson, 2001). Grasses such as *Echinochloa crus-galli* grow at field capacity or saturation, whereas a high water table favors aquatic broadleaved weeds and sedges (Bhagat et al., 1996). Bhagat et al. (1999) reported that broadleaved weeds produced higher weed biomass than sedges and grasses in flooding regimes, while in saturated condition the opposite result was obtained. Rodenburg et al. (2011) reported that in irrigated, non-flooded rice systems, weeds are expected to become more serious specifically perennial rhizomatous weeds and species adapted to hydromorphic conditions are expected to increase in prevalence.

In deep water, the surviving weeds were mostly the C3 grass such as *Oryza barthii, Leersia hexandra* while *Sphenoclea zeylanica and Ludwigia abyssinica* were the C3 broad leave weeds that survived in the deep water of

10-20 cm. Most of the weeds that were found in those plots that were not impounded with water were mostly C4 species which might be the reason why yield attributes and yield of rice in those plots were highly reduce as C4 weeds are known to be higher competitor than C3 weeds. Therefore, the negative impact of the C4 weeds such as (*Echinochloa colona, Panicum repens* and *Fimbristylis littoralis*) on rice is always higher than that of C3 weeds (*Sphenoclea zeylanica, Cyperus difformis* and *Ludwigia abyssinica)* because rice itself is C3 plant.

Haden et al. (2007) observed that weed shifted to sedge under reduced flooding while Rodenburg and Johnson (2009) suggested that perennial C3 weed species such as *Oryza longistaminata, Leersia hexandra, Bolboschoenus maritimus* and *Sphenoclea zeylanica* will increase in irrigated rice production systems, but where water saving production systems are adopted, the hydromorphic conditions will favour C4 weed species such as *Echinochloa colona, Echinochloa pyramidalis, Digitaria horizontalisFimbristylis littoralis, Cyperus esculentus, Imperata cylindrica,*and *Paspalum scrobiculatum*. John (2010) also reported that in deep water, the most common weed growing was *Monochoria vaginalis* while at zero water depth (soil saturation) grasses such as *Echinochloa spp* and sedges such as *Fimbristylis miliacea* were predominant.

In rice system, whatever affect weed growth will definitely enhance the yield and yield components of rice, all things been equal. The plots that were not pounded with water recorded the higher weed growth which was mostly C4 weeds which resulted in higher competition which eventually lead to lower panicle production in this treatment. The higher seedling rate on the other hand had fast canopy cover that shaded up the weeds which reducted their growth and the possible competition.

The experiment conducted by Abdul et al. (2009) to determine the influence of flood density and duration on rice growth and yield indicated that the responses of rice panicle number m^{-2} were significantly affected by the flooding treatments and continuous flooding till maturity gave significantly higher panicle m^{-2} of 434 which was higher than that produced by either intermittent flooding till 55 or 30 DAS which produced 426 and 425 respectively. Lowest panicle production of 320 m^{-2} was recorded in saturated field in their experiment. They equally attributed the higher panicle production in continuous flooding to higher tiller production in this water condition. This is in line with the finding of this study.

Beser and Sürek (1999) also observed higher panicle number in experiment to determine the effect of water stress on grain and total biological yield of rice. Jahan (2004) and Sariam (2004) recorded higher panicle production from continuous flooding field while Sariam (2004) and Siti Mardina (2005) on the other hand reported that significant higher panicle production was recorded when rice was grown under field capacity (unflooded field).

Chaff is a negative attribute of yield and what causes stress in rice field most especially at the crucial stage will definitely result to higher percentage chaff. Therefore, the higher percentage chaff recorded in the nonflooded plots could then be attributed to higher stress caused by higher weeds infestation and water deficit at the critical period of rice growth.

Abdul et al. (2009) recorded similar result of higher spikelets from continuous flooded field and the number decreased with decreased in flood depth. The result of this work also agreement with that of Sariam (2004) who observed higher number of spikelets in continuous flooding while rice under field capacity recorded the least number of spikelets. Upadhaya (1996) reported that less biomass and number in grain production under the reduced water regimes could be caused by the lack in water availability at the anthesis (flowering) stage, which restricted rice pollination process and caused the rice to produce infertile and empty rice grain.

Weight of 1000seeds is one of the major yield components and the significant higher grain weight recorded in the deeper water at fewer seedling rates could be attributed to less competition from weeds as a result of suppression of weeds by the continuous flooding at deeper water of between 15-20 cm. Beser and Sürek (1999), recorded similar result of higher grain weight in continuous flooded field than saturated or intermittent irrigation system. Talpur et al. (2013) also recorded higher 1000 grain weight of 24.85 g in the continuous flooded field.

Rice plant needs more water during the reproductive stage particularly during the grain formation and continuous flooding provide the plant enough water at this stage of development for optimum grain development. The saturated soil might not be able to provide the plant with the required moisture for good grain formation. Abdul et al. (2009) reported higher grain weight in continuous flooded field than that recorded in continuous field capacity.

Jahan (2004), in his study on rice production under glasshouse condition, indicated contrary results where no significant difference of 1000-grain weight was observed under the different flooding regimes. Meanwhile, Sariam (2004) reported that 1000-grain weight varied significantly with water management, where lower grain

weight was observed under the field capacity condition as compared to flooded conditions. Zenolabedin et al. (2008) reported 17% 1000 grain weight reduction when water stress occurred at grain filling stage.

The grain yield of rice is dependence of yield components like tiller, panicle production, grain weight and percentage filled grain. In rice production system, whatever happened to any of these components will directly translate to total grain yield. The significant higher grain yield recorded in deeper water at six seedling rates could be as result of less competition from weeds in this treatment combination also the rice plant didn't suffer from water deficit at any stage of its growth. In those plots that received no flooding recorded higher weed growth which affected the yield components. Zinolabedin et al. (2008) also reported that water deficit during the vegetative, flowering and grain filling stage reduced grain yield by 21, 50 and 21%respectively. Evaluating the effect of different during of water stress at various growth stage of rice showed that water stress at any stage would reduced yield of rice (Salam et al., 2001; IRRI, 2002).

Tabbal et al. (1992) observed no significant yield difference between rice grown in standing water and those grown under saturated field conditions in the 1988-1989 dry seasons; however, yields under saturated soils were statistically lower in the 1990-1991 dry seasons because of more weed growth, as compared to flooded field. IRRI (2009) acknowledged that improve water management in lowland rice ecology reduced weed infestation by 40% and the labour requirement to weed one hectare is therefore reduced from 21 to 5 manday which translated to 75% decrease in labour requirement.

The negligible grain yield recorded in nonponded plots might be due to severe weed pressure in this treatment, which agreed with the work of De Datta et al. (1986) who observed that weeds are major limiting factor in rice production systems in the world and that yield reduction due to unchecked weed growth varies from 40-85% but with severe weed competition complete loss is possible. Similarly, Ahmed et al. (2005) and Alam et al. (1996) observed lower grain yield from unweeded plots due to severe weed pressure. Pandey (2009) as well reported that weeds are at present the major biotic constraint to increased rice production worldwide. Improving weed control in farmers' fields was shown to increase rice yields by 15-23%, depending on the agro-ecosystem and it is estimated that weeds may account for annual rice yield losses in Sub Saharan Africa of at least 2.2 million tones equating to US $1.45 billion (Rodenburg & Johnson, 2009).

5. Conclusion and Recommendations

Based on the result of this study, it could be concluded that flooding of lowland rice field to depth of between 15–20 cm gave better weed control and enhanced the yield and yield components of lowland rice. We therefore recommend the adoption of flooding of lowland rice field to a depth of 15-20 cm for effective weed control. Integration of hand weeding with flooding is also advisable since some weeds like *Oryza barthii*, *Echinochloa spp.* and *Sphenoclea zeylanica* which were not controled by the various water depths.

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Effect of Plant Height on Fusarium Head Blight in Spring Wheat

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Abstract

Fusarium Head Blight (FHB), caused by the fungal species *Fusarium graminearum,* is a disease affecting wheat cultivars across Canada. Recent and severe outbreaks have spurred research in developing FHB-resistant cultivars and evaluating the underlying causes of FHB susceptibility. In this study, the effect of plant height on Fusarium Head Blight in Canadian spring wheat was evaluated over a two-year period. Cultivars of spring wheat varying in origin, height, and disease susceptibility were artificially inoculated with FHB, and the subsequent disease symptoms and height data was collected. It was found that plant height is negatively correlated with FHB incidence and severity. However, varieties originating from Eastern Canada had a much stronger negative correlation between plant height and FHB, whereas the trials with Western Canada origins had a weaker correlation.

Keywords: Fusarium Head Blight, spring wheat, plant height, Canada, correlation

1. Introduction

Fusarium Head Blight (FHB), caused by the fungal species *Fusarium graminearum*, is the principal head blight pathogen in Canada and around the world (Sutton, 1982). This fungus is prevalent in areas of high precipitation, high humidity, or heavy dews (Windels, 2000) and commonly attacks wheat spikes and stems at the heading stage (Sutton, 1982). Plants infected with FHB are characterized by: dark purple, brown, or black lesions on the glume and florets, deformed awns (Yuen & Schoneweis, 2007), premature blighting, bleaching of spikes, sterile florets and poorly filled grains (Sutton, 1982). In recent years, severe FHB outbreaks have occurred in Canada, specifically in the provinces of Ontario, Quebec, the Maritimes, Manitoba and Alberta (Sutton, 1982).

The susceptibility of wheat lines to FHB is of great concern to the agri-food industry (Miller et al., 2010), because plants infected with this disease have lower yields, shriveled and discolored kernels, and lighter-weight and poorer-quality seeds (Windels, 2000). Furthermore, *F. graminearum* produces the mycotoxin deoxynivalenol, commonly referred to as DON, on the infected grain, which then becomes unsuitable for flour, cereals, or malt and too toxic for use as non-ruminant animal feed (Windels, 2000). These symptoms are the cause of wheat losses of nearly $300 million over a five year period in certain provinces (Windels, 2000). Because of the prevalence, severity, and detrimental commercial implications of FHB, the USDA ranks it as the worst plant disease since the 1950s. The damaging effect of *F. graminearum* on wheat plants has led to a demand for resistant varieties and spurred much research on the subject. Plant resistance mechanisms for FHB can be active or passive; examples include physiological processes for active mechanisms and morphological features for passive mechanisms (Mesterházy, 2006). Scientists have evaluated many factors that influence passive resistance, such as awn presence and floret density on spikes (Yuen & Schoneweis, 2007), however plant height in Canadian trials has not yet been evaluated in any depth. The purpose of this study was to examine the relationship between plant height and FHB incidence and its severity for Eastern and Western Canadian spring wheat varieties.

2. Materials and Methods

2.1 Field Preparation and Plant Materials

This study was conducted over a two-year period, in 2013 and 2014, at the Eastern Cereal and Oilseed Research Centre located in Ottawa, Ontario, Canada. The Fusarium nursery was planted in a different field each year, however both fields had been planted with corn in the previous year, and both fields had a sand–sandy loam soil type. Field preparation was consistent in both years: individual plots of spring wheat varieties varying in FHB susceptibility, plant height, and origin were planted in two parallel rows, each 1.0 m long and 0.1 m wide. Herbicides were applied two weeks after emergence: in 2013, Buctril M at 1.0 L ha^{-1} and Puma at 770 mL ha^{-1} were applied, and in 2014, Buctril M at 1.0 L ha^{-1} and Puma at 1.02 L ha^{-1} were applied. In each year, the field was sprinkler-irrigated twice daily and hand-weeded periodically.

2.2 Inoculum Preparation and Application and Fusarium Head Blight Assessment

The Fusarium inoculum was prepared using a 1:1 barley and corn mixture, which was soaked for 24 hours and autoclaved at 120 °C twice for 90 minutes each time. The autoclaved grain was seeded with 80 mL of three separate FHB strains (DAOM 178148, DAOM 212678, and DAOM 232369) and stored in covered trays for four to six weeks. After the wheat was planted and 15 days before heading occurred, fresh inoculum was hand-applied twice between the planted rows at a rate of 50 g m^{-2}, immediately after which the field was irrigated.

Percent severity and percent incidence of FHB disease were determined 24 days after 50% of the plant heads in the plot showed extended anthesis. Fusarium Head Blight occurrence on the plant head is measured by obtaining the product of the percent severity and percent incidence values and is called the FHB index. Height measurements were taken in centimeters by placing a standard measuring stick in a single row and recording the average plant height for the plot. Lodging, awn presence, and plot abnormalities were also collected.

2.3 Statistical Analysis

Analysis of variance was performed on the data sets using Stata 13 software. Pearson's correlation coefficients, probability values, means, and range values were determined.

3. Results

The results from the 2013 and 2014 replications of this study are consistent. In 2013 (Table 1), the trials originating from Western Canada did not support the hypothesis that plant height is correlated with FHB index. Given the critical value P = 0.01, only 5 of the 18 Western trials showed statistical significance between plant height and FHB index. Conversely, 9 of the 15 Eastern trials showed statistical significance between plant height and FHB index. The Western trials also had, on average, a much lower Pearson's correlation coefficient than the Eastern trials did (−0.21 vs. −0.51).

The results from the 2014 replication parallel those from 2013 (Table 2). Of the 14 Western trials, only 8 showed statistical significance between plant height and FHB index, whereas of the 28 Eastern trials, 26 showed statistical significance between plant height and FHB index. The Western trials also had, on average, a much lower Pearson's correlation coefficient than the Eastern trials did (−0.011 vs. −0.220).

In both the 2013 and 2014 replications of this study, the Eastern plant height mean is approximately 10 cm greater than the Western plant height mean. This difference in plant height is matched by a 2% to 3% difference in FHB index between the Eastern and Western trials. However, after a t test for FHB, means between the Eastern and Western trials was performed, P values of 0.6913 for the 2013 data and 0.3842 for the 2014 data were obtained. From this we can conclude that the 2% to 3% difference in FHB index between the Eastern and Western trials is not statistically significant.

To summarize, the Eastern trials showed strong statistical significance between plant height and FHB index in both 2013 and 2014, whereas the Western trials did not. Although the Western trials had both consistently shorter plants and a higher FHB index than the Eastern trials did, this difference in FHB index was not significant and can therefore not be explained by the difference in plant height. The average Pearson's correlation coefficients for the Eastern and Western trials were consistent with the general trend: there is a strong correlation with the Eastern trials whereas the Western trials show a weaker correlation.

Table 1. Pearson's correlation coefficient (r) between wheat plant height and Fusarium head blight (FHB) resistance scores in 33 trials at the Eastern Cereal and Oilseed Research Centre in 2013 using genetic materials received from several breeding programs in Eastern and Western Canada

					FHB (%)		Height (cm)	
Trial	Origin	r	Probability	n	Mean	Range	Mean	Range
1	Eastern	−0.49	0.034	36	37.1	33.1	100	76
2	Eastern	−0.69	0.000	36	31.5	94.9	91	25
3	Eastern	−0.48	0.002	42	26	91.9	91	28
4	Eastern	−0.5	0.000	60	38	91.7	93	37
5	Eastern	−0.48	0.014	27	55.7	82.4	80	27
6	Eastern	−0.42	0.081	20	21.6	91.7	87	23
7	Eastern	−0.7	0.000	32	36.5	90.1	100	44
8	Eastern	−0.49	0.005	33	41.8	88.5	83	37
9	Eastern	−0.68	0.000	56	45.7	76.8	82	35
10	Eastern	−0.64	0.000	32	36.5	85	98	36
11	Eastern	−0.13	0.500	32	30.5	91.3	100	37
12	Eastern	−0.5	0.005	32	36.1	89.1	101	37
13	Eastern	−0.77	0.000	32	43.3	63	101	38
14	Eastern	−0.38	0.102	22	36.4	81.9	86	26
15	Eastern	−0.34	0.663	6	42.5	14.9	96	27
16	Western	−0.55	0.000	60	25.1	90.4	95	35
17	Western	−0.37	0.004	90	27.8	85.9	84	23
18	Western	−0.43	0.000	90	23	82.2	85	28
19	Western	−0.71	0.000	35	25.4	94.6	85	28
20	Western	−0.06	0.654	56	29.4	73.5	80	30
21	Western	−0.08	0.563	56	39.3	63.9	85	27
22	Western	0.03	0.878	36	43	60	83	23
23	Western	−0.28	0.150	30	27.5	25.5	88	19
24	Western	−0.22	0.268	30	70.7	45	78	16
25	Western	−0.52	0.004	32	46	85.4	82	23
26	Western	−0.3	0.126	30	31.2	59.4	81	23
27	Western	0.27	0.159	30	29.6	55.9	91	20
28	Western	0.11	0.446	56	59.4	54.3	81	26
29	Western	−08	0.564	56	54.2	60.2	77	29
30	Western	−0.11	0.438	56	37.2	52.5	77	21
31	Western	0.13	0.352	56	37	67.5	81	23
32	Western	−09	0.641	32	41.1	80	81	27
33	Western	−0.5	0.011	27	52.8	92.6	86	31
	Eastern	−0.51	0.094	33.2	37.3	77.8	92	35
	Western	−0.21	0.292	47.7	38.9	68.3	83	25

Origin was defined by the origin of the varieties tested. Probability was defined by the probability, under the assumption of hypothesis H, of obtaining a result equal to or more extreme than what was actually observed. n was defined by the number of varieties tested. FHB (%) was defined by Fusarium Head Blight index in percent, obtained from the product of FHB severity in percent and FHB incidence in percent. Height (cm) was defined by the average height of a variety.

Table 2. Pearson's correlation coefficient (r) between wheat plant height and Fusarium head blight (FHB) resistance scores in 42 trials at the Eastern Cereal and Oilseed Research Centre in 2014 using genetic materials received from several breeding programs in Eastern and Western Canada

					FHB (%)		Height (cm)	
Trial	Origin	r	Probability	n	Mean	Range	Mean	Range
1	Eastern	−0.27	0.000	270	23.0	82.5	84	62
2	Eastern	−0.62	0.000	60	24.0	57.5	89	47
3	Eastern	−0.38	0.000	216	22.7	97.0	89	42
4	Eastern	−0.26	0.000	216	25.2	97.0	90	61
5	Eastern	−0.22	0.001	216	23.9	42.0	96	48
6	Eastern	−0.55	0.000	108	33.4	92.0	95	72
7	Eastern	−0.11	0.253	108	29.3	96.5	95	47
8	Eastern	−0.41	0.000	216	20.7	42.0	84	58
9	Eastern	−0.31	0.000	216	24.8	42.0	91	47
10	Eastern	−0.57	0.000	75	24.1	37.0	95	42
11	Eastern	−0.20	0.008	180	23.6	42.0	90	34
12	Eastern	−0.29	0.000	180	33.6	82.0	97	53
13	Eastern	−0.57	0.000	75	50.7	77.0	84	71
14	Eastern	−0.56	0.000	60	54.9	71.0	78	51
15	Eastern	−0.39	0.001	75	18.7	32.0	97	37
16	Eastern	−0.50	0.000	81	39.3	91.0	108	58
17	Eastern	−0.73	0.000	93	39.4	91.0	104	56
18	Eastern	−0.15	0.006	360	31.7	97.5	96	76
19	Eastern	−0.29	0.000	168	38.6	92.0	96	42
20	Eastern	−0.59	0.000	96	40.1	96.5	106	60
21	Eastern	−0.42	0.000	96	39.5	96.5	103	48
22	Eastern	−0.60	0.000	96	45.8	96.5	101	55
23	Eastern	−0.65	0.000	66	38.0	96.5	103	62
24	Eastern	−0.59	0.000	42	46.7	97.5	86	44
25	Eastern	−0.25	0.032	75	47.6	77.0	112	55
26	Eastern	−0.58	0.000	48	49.8	71.0	85	50
27	Eastern	−0.54	0.007	24	36.5	62.0	111	30
28	Eastern	−0.44	0.000	90	36.5	97.0	97	42
29	Western	−0.21	0.001	243	25.4	50.0	86	38
30	Western	−0.62	0.000	81	32.5	97.0	90	50
31	Western	−0.26	0.000	270	24.2	62.0	83	40
32	Western	−0.31	0.000	270	27.3	67.0	85	53
33	Western	−0.42	0.000	108	33.6	72.0	87	42
34	Western	−0.11	0.311	90	52.4	75.0	96	38
35	Western	−0.19	0.104	75	32.7	51.5	89	28
36	Western	−0.38	0.000	90	48.4	65.0	84	43
37	Western	0.00	0.993	75	40.4	60.0	90	44
38	Western	−0.27	0.004	112	56.4	55	83	42
39	Western	0.13	0.091	180	30.7	51	78	38
40	Western	0.03	0.683	180	32.4	45	78	44
41	Western	−0.22	0.006	163	47.5	75	84	40
42	Western	−0.01	0.892	90	38.6	77	87	47
	Eastern	−0.43	0.011	129	34.4	76.1	95	52
	Western	−0.20	0.220	145	37.3	64.5	86	42

Origin was defined by the origin of the varieties tested. Probability was defined by the probability, under the assumption of hypothesis H, of obtaining a result equal to or more extreme than what was actually observed. n was defined by the number of varieties tested. FHB (%) was defined by Fusarium Head Blight index in percent, obtained from the product of FHB severity in percent and FHB incidence in percent. Height (cm) was defined by the average height of a variety.

4. Discussion

The results of this study confirm the hypothesis of an overall negative linear correlation between plant height and FHB severity and incidence. However, there is a distinct difference between the trials with the Eastern spring wheat varieties and those with the Western spring wheat varieties.

The Eastern trials showed a strong negative relationship between plant height and FHB index, meaning that taller plants were less susceptible to FHB than shorter plants were. One possible explanation for this difference is that in taller plants, the spikes and flowering heads are farther away from the soil surface, which is where *F. graminearum* spores are found. Fusarium Head Blight is more likely to infect plants that are closer to the ground, and thus, taller plants are at an advantage. Another possible explanation is that the microclimate of taller plants is less favorable to the disease: relative humidity is lower at higher spike heights, and because FHB thrives in humid climates, taller plants are less vulnerable to infection.

Conversely, the Western trials did not show dependence between plant height and FHB index. Not only did many of the individual trials not show statistical significance, the correlation was markedly weaker than it was in the Eastern trials. The low Pearson's correlation coefficient for the Western trials signifies that factors other than plant height are causing the incidence and severity of FHB. These auxiliary factors can include morphological traits such as awn presence, floret density on spikes, or anther extrusion. Alternatively, whereas structural features such as plant height largely governed the Eastern trials, genetic differences could have governed the Western trials. Researchers have proposed pleiotropic explanations (Lu et al., 2010) for FHB in wheat. If there is a similarity in genetics in the Western varieties, pleiotropy may be the underlying reason for the inconsistency between plant height and FHB correlation in the Western trials.

Regardless of the Western and Eastern origin distinction, plant height was still a dominant factor influencing the incidence and severity of FHB, with a total of 64% of trials supporting the hypothesis. Although taller plants are significantly less susceptible to FHB, they are also known to be linked to lower yields, reduced fertilizer efficacy, and lodging problems. Further research is required to develop wheat varieties that optimize yield and disease resistance.

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The Physiology of Chilling Temperature Requirements for Dormancy Release and Bud-break in Temperate Fruit Trees Grown at Mild Winter Tropical Climate

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Abstract

It is studied that inadequate winter chilling may interfere with the normal processes of plant growth, reproductive development and subsequent yield. As much of the evidences behind these studies are subjective and region based, the available information was collated and evaluated to further investigate the impacts of winter chill that is currently an issue of fruit growers in mild winter areas. Though, the period of adequate low temperatures is insufficient in warmer regions to satisfy the chilling requirements of temperate fruit trees, this call up on many option for chill compensation; like planting low chill cultivars, use of dormancy breaking chemicals, forced defoliation, pruning and some other techniques as an alternative strategies. However, the diverse agro-climatic conditions due to major differences in altitude, rainfall as well as in slope characteristics showed the existence of different chilling temperature requirements across locations that favor many temperate fruit trees to grow: including apple, pear, grape, peach, nectarine, plum, cherry, walnut, almond and other fruit tree species. Temperate fruit production in mild winter areas now days increased rapidly and even more new industries are being developed in regions where none previously existed. To date, in tropical highlands, fruit productivity and quality have been gradually improved through introduction and selection of better adaptable varieties based on their chilling requirements. To supplement the present existing knowledge gap in relation to the cultivar-environment interactions, the use of chill models to quantify chill accumulation during winter months would help in classifying the environment (potential growing areas) according to the amount of chill hours existed in that location.

Up to date, a number of valid methods were applied for quantification of chill accumulation in tropical and sub-tropical conditions showed varying results depending on the types of species, existing warm temperature and other climatic variables. The limitation in their predictive performance from region to region is due to their designing approaches that were primarily for temperate climate. This requires a model comparison for specific location, i.e. by using more than one model to avoid the potential misleading in calculation and chill estimation. Of the chill models tested to quantify the chill accumulation in the mild winter areas, the use of dynamic model gave good estimation that it nullifies the chilling reversal by high temperature. Also, the model of Positive Chill Units (PCU, or Positive Utah) is a competent under warm climate, next to the dynamic model. The Positive Utah model, an iteration of the original Utah, excludes the negation influence of high temperatures. The procedure for PCU is the same as for the original Utah model except that, when negative, the chill unit value is set equal to zero. Therefore, the accumulated chill units are equal to zero until the temperatures drop into the effective zone and positive chill units begin to accumulate. Though, for these areas with warm climate, using the 0 - 7.2 °C model is not recommended, because of its sensitivity to changes in temperatures that represent different weighing factors recorded for other models. Other important alternatives to these classical models include, the Growing Degree Hour Model (GDH), the Mean Temperature Model, Exponential temperature response functions and others are applied as independently, or in combination with classical chill models for a better chill estimation. Winter chill should be studied like other weather dependent processes because the present trends in chill decline across locations significantly affect fruit culture in areas with mild winter. Therefore, identifying the

problems related with lack of insufficient winter chilling would help in designing possible strategies for the changing scenarios and understanding the current physiological responses of the plant against these changes.

Keywords: temperate fruits and nuts trees, chilling requirement, chill models, dormancy release, bud-break

1. Introduction

Temperate perennial crops that are growing in these seasonally restricted temperate regions require chilling temperatures that should be satisfied in order to initiate growth and flowering in spring (Saure, 1985). They undergo a cycle of dormancy requirement that inhibits growth until exposure to low winter temperatures (chilling), prior to spring bud break. The duration of the dormancy period minimizes subsequent low temperature damage to flowers by delaying bud break and flowering. For temperate fruit trees and other woody perennials in the region, chilling requirements can be adaptive whereas in areas with mild winters, fluctuating winter temperature causes highly variable ranges of chill (Seeley, 1996; Richardson et al., 1974; Samish, 1954) and short chilling requirements are evident. Failure to receive sufficient chilling can lead to serious consequences including reduction of flower quality, abscission of flower buds, protraction of the flowering process and reduced fruit set (Jackson et al., 1983; Erez & Lavee, 1971; Abbott, 1962). Also, lack of effective chilling during winters in tropical and sub-tropical areas result in prolonged dormancy leading to poor blooming, strong apical dominance, and unsynchronized growth patterns and, consequently, low yields (Cook and Jacobs, 1999). Similarly, reproductive development in perennial crops under mild winter conditions involves an extended and complex sequence of morphological, physiological and metabolic changes (Chmielewski et al., 2004; Jackson et al., 1983; Spiegel-Roy & Alston, 1979; Erez & Lavee, 1971). Moreover, the beginning of the tree blossom stage depends on annual deviations in air temperature, i.e., years with temperatures above normal in late winter and early spring are clearly showed irregularities in the date of the beginning of the blossom stage. This indicates that high winter temperature has a negative effect on accumulated winter-chill particularly in tropical and sub-tropical climates (Byrne, 2005).

Early studies by Weinberger (1950) assumed that the hourly temperatures that were accumulated over winter for temperature ranges between 0 and 7.2 °C was found successful for dormancy release and bud break. However, the chill requirements measured as chill hours (CH) by Weinberger (1950), without considering the temperature scenarios would be a poor indicator of the chill effectively accumulated by the buds. Later on, in Utah, Richardson et al. (1974) developed the chill model that estimated hourly chill exposure using a weighted step-function. The model supposes that chill accumulation occurs within a temperature range of 2.5 and 12.5 °C, outside of which, the accumulation is nil or negative. It revealed that different temperatures have different effectiveness in accumulating chilling and notably the most effective chilling occurs above 2.5 °C and below 7^{O}C. The Utah model gives good results in cool and cold temperate climates; however, it yields a large quantity of negative chill values in tropical and sub-tropical climates. A modification of Utah model, the Positive Utah Model (Positive Chill Units (PCU), has come up with better results in sub-tropical and tropical mild winter conditions (Linsley-Noakes et al., 1994). It assumed that previously accumulated chilling cannot be negated by the influence of high temperatures, but equal to zero for every raise in temperature above 12.5 °C and below 1.5^{O}C. Fishman et al. (1987) developed the Dynamic Model for warm winter conditions, which takes a different approach to quantifying winter chill. It assumes that winter chill results from a two step process, in which an intermediate product is first formed in a process promoted by cold temperatures. Warm temperatures can destroy this intermediate product. As soon as a certain quantity of intermediate has accumulated, it is irreversibly transformed into a Chill Portion (CP), which can no longer be destroyed (Fishman et al., 1987; Erez et al., 1990).

An important alternative to the classical models are the growing Degree Hour Model (GDH), the Mean Temperature Model and Exponential temperature response function for mild winter areas. These models also give good result by nullifying the negative values generated by Utah model. (Linkosalo, 2000; Hänninen, 1990; Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981). More recent work with apple and pear suggests that a wide range of chilling temperatures between 1^{o}C and 13^{o}C were equally effective in inducing dormancy release. This may explain that chill estimates derived from the classical models (Utah chill units; Richardson et al., 1974; 1975) fail to fully estimate bud dormancy progression in areas with mild winter (Jacobs et al., 2002).

In view of the widespread perception that chilling temperature requirements has a significant impact on flowering and fruit yield, these empirical modes have been widely used to determine the amount of chilling in a specific location. Also, chill models are used to predict the historical trends of weather dynamics, that how the chill accumulation increase or decline with time. Therefore, in application, all models are arbitrary and mainly dependent on temperature, day length and types of fruit tree grown. To avoid this limitation, applying more than

one model for a specific location would help in choosing the one with a better predictive performance that could reflect dormancy development and release more accurately..

2. Dormancy

Dormancy is a phenomenon when the buds remain dormant due to growth-arresting physiological conditions, as opposed to the quiescent period that the buds remain dormant due to unfavorable environmental conditions (Cesaraccio et al., 2004). This dormancy or sleeping stage protects these buds from oncoming cold weather. Once buds have entered dormancy, they will be tolerant to temperatures much below freezing and will not grow in response to mid-winter warm spells (Cannell & Smith, 1983). These buds remain dormant until they have accumulated sufficient chilling of cold weather. When enough chilling accumulates, the buds are ready to grow in response to warm temperatures during spring. As long as there have been enough chill, the flower and leaf buds develop normally. If the buds do not receive sufficient chilling temperatures during winter to completely release dormancy, trees will develop one or more of the physiological symptoms associated with insufficient chilling include delayed foliation, reduced fruit set and increased buttoning, and reduced fruit quality (Petri & Leite, 2004). Li et al. (2003a) reported that in the temperate climates of the Northern Hemisphere, most of the fruit trees and other woody plants enter into ecodormancy at the end of summer or beginning of autumn. This was followed by a shortened photoperiod which causes the cessation of growth and a change in the development of terminal shoots, which pass from leaf shoots to form primordia with protective scales, giving rise to autumn buds.

Although the term dormancy seems intuitively obvious, it is a complex term with a variety of definitions: Lang et al. (1987) defined dormancy as a state of reduced or stopped activity or development of specific plant tissues that will resume in the future. Many researchers separated dormancy into a period of rest and a period of 'quiescence' (Linkosalo, 2000; Hänninen, 1990; Cannell & Smith, 1983). For example, Sarvas (1974) defined rest as a period when buds are dormant due to physiological conditions, and he defined quiescence as a period when the buds remain dormant due to unfavorable environmental conditions. Several models described the breaking of rest and overcoming quiescence in terms of chill accumulation to abruptly break rest followed by a period of forcing temperature to overcome quiescence (Fuchigami & Nee, 1987; Cannell & Smith, 1983).

Horvath et al. (2003) reviewed three different states of dormancy in relation to the factors regulating seasonal progression from one state to another. (i) Paradormancy occurs due to the environmental factors that would be outside the bud but within the plant that affects growth and determine all the activities (Lang, 1987). Paradormancy is in similarity with apical dominance and correlative inhibition, as occurs with lateral buds; both can be overcome by physical (terminal bud removal) and chemical (growth regulators) treatments (Hillman, 1984). (ii) Winter-dormancy (endodormancy or rest) is a true dormancy that when the bud growth is prevented by an inhibitory system such as growth regulators operating within the bud and commences after leaf abscission. It strongly differs from paradormancy in that removing bud scales or leaves does not break this type of dormancy. Seasonal endodormancy in fruit trees, as well as other woody perennials, is a phase of development that allows the plant to survive periods of stress often associated with low winter temperatures or even drought. In this dormant state, resistance to low temperatures is at its highest. Overcoming endodormancy is achieved by a period of chilling exposure that can be of considerable duration in some cases. Enododormancy results from physiological changes in the bud which prevent untimely growth due to unsuitable climatic conditions. (iii) Imposed dormancy (ectodormancy) occurs when growth is prevented directly by external environmental factors and is reversible. This type of dormancy mainly occurs in late winter when buds are ready to grow and respond to increasing temperatures as evident during 'forcing' of bud break in some fruit production systems. Once the chill requirement has been met, buds grow when exposed to warm temperatures.

2.1 The Nature of Dormant State in Fruit Trees

Saure (1985) states dormancy as a period in which visible growth is not obviously apparent, but, cellular differentiation occurs in a slow and steady manner; for example, an increase in bud weight. In apple, the development of primordial during winter has been shown to continue with respect to differentiation and enlargement throughout the season with growth being greatest from February to March (Buban & Faust, 1995). Brown (1960) reported three phases of growth in apricot flowers within buds using detailed descriptive growth curves: the first in the autumn and early winter where growth rate is very slow, the second or transition phase in late winter shows a slightly greater growth rate than the first and the final phase, associated with the events that promote very rapid bud break. There is also active synthesis of RNA and protein during dormancy in pear (Zimmerman & Faust, 1969), peach (Bagni et al., 1977) and apple (Li et al., 1989). Durner and Poling (1987) states that dormancy induction in strawberry is clearly related to short days and declining temperatures. Thus,

there is a marked morphological difference between the leafless state and leafy strawberry plant as they enter dormancy. Nishizawa and Hori (1993b) reported that dormancy becomes noticeable in as strawberry leaf size declines along with leaf petiole length but, as with deciduous fruit trees, cooling the roots of strawberry is prerequisite for effective bud break. Brennan et al. (2013) states that using a system of cold storage of raspberry canes for subsequent cropping in greenhouse conditions for 6 weeks at 2 °C or below produced even bud break and eventual yields of greater than 0.9 kg per cane. Accordingly, the canes could be stored for longer periods, but the temperature should be reduced to 1 °C or below, to prevent premature bud break and poor development. Also, they found that once the chilling requirement was fulfilled, subsequent development is entirely temperature dependent and the raspberry growth cycle can be manipulated to produce fruit beyond the normal field fruiting season.

In humid tropical climates, the induction and release of seasonal dormancy are triggered by environmental signals, mainly temperature and photoperiod. In most temperate and boreal trees, dormancy is induced by the decreasing length of the photoperiod in autumn and cool temperatures, resulting in the cessation of growth and the formation of winter buds (Thomas & Vince-Prue, 1997; Vaartaja, 1959; Wareing, 1956). Photoperiod and low temperature may induce dormancy through independent pathways (Welling et al., 2002) and in a few species; low temperatures alone seem to be sufficient to induce endodormancy (Heide, 2011; Heide & Prestrud, 2005). In tree species adapted to cool climates endodormancy is generally released after sufficiently long exposure to cool, non-freezing temperatures (Sarvas, 1974; Perry, 1971). Yet, the actual range of effective temperatures for chilling is only vaguely known for forest trees, and cool, non-freezing temperatures up to 10 °C, most likely between 2 and 4 °C, are expected to be most effective (Battey, 2000). Higher temperatures may even negate previous chilling (Perry, 1971), while lower (sub-zero) temperatures are generally considered to be ineffective for the fulfillment of the chilling requirement, presumably because very low temperatures prevent a physiological integration of signals (too low metabolic activity). Once the chilling requirement is fulfilled, metabolic activity increases, hydrolytic enzymes are activated and carbohydrate reserves gradually become mobilized. As a first visually identifiable clue, the onset of bud swelling indicates that the transition from endodormancy to ecodormancy has occurred (Pallardy, 2008; Saure, 1985). The bud water content rises (Essiamah & Eschrich, 1986) and the buds become increasingly susceptible to freezing. Then the subsequent release of ecodormancy is modulated by favourable environmental conditions.

Chilling during early or late in the winter, would affect both the number of buds which break and their subsequent growth (Thompson et al., 1975). The rate of bud development in the spring was found to depend most heavily on the cold conditions of the preceding autumn or early winter (Abbott, 1977). Accordingly, the warmer autumn conditions promote further bud development (delayed onset of quiescence), which facilitates renewal and completion of growth once dormancy has ended. It consists of the following sequence: quiescence -+ preliminary rest -+ mid rest -+ after rest -+ quiescence. Here quiescence is a state of growth stoppage reversible by favorable conditions. Preliminary rest and after rest are characterized by a lack of growth, but an ability to be forced. Mid rest corresponds roughly to innate dormancy, the state in which forcing is difficult or impossible. Also, those buds which receive more chilling can leave the quiescent state at lower temperatures than those which get less chilling. Couvillon and Erez, (1985) reported that there is a correlation within the apple species between the date of bloom and the chilling requirement: those varieties which bloom late need more chilling during winter.

2.2 Physiological Changes During Dormancy Induction and Release

2.2.1 Dormancy Induction

Physiological changes that occur when plants perceive the environmental signals for the induction and release of dormancy are associated with the responses including phytohormones, phytochromes and carbohydrates (Chao et al., 2007). The gradual transitions between the different phases of dormancy involve numerous genetic, physiological biochemicals and anatomical alterations (Cooke et al., 2012; Horvath, 2010; Faust et al., 1997). Whitworth and Young (1989) found that there is a decline in starch: total carbohydrate ratios following the onset of winter chill, may be a mechanism in the preparation for winter chill by the accumulation of sugars such as sucrose and sorbitol for freezing tolerance. The rootstocks showed particularly marked changes in carbohydrate composition: hexose, and sucrose were shown to be of particular importance during early growth. However, it may be deemed that there is no enough evidence to support the suggestion that carbohydrate status may play a major role towards the completion of winter rest. Jennings and Carmichael (1975) suggesting that overwintering raspberries for observation on carbohydrate composition in the roots and canes during the onset of dormancy revealed that a genotype with prolonged leaf retention and delayed dormancy had unusually high starch concentrations in the tissues in December. It appears to be evidence that larger fruiting plants require a priming

of the plant carbohydrate status prior to bud break and flowering. One could speculate that the reasons for this may be associated with the provision of the appropriate energy substrate for the energy-intensive processes of leaf, flower and fruit development. Thus, carbohydrate stored in the root system, basal season's shoot system and resting buds of many perennial plants is used to support the next season's growth.

Thomas (1967) described the carbohydrate status of root storage organs were slowly depleted in the winter, at an increasing rate as new spring growth appeared. It was not until the shoot had sufficient leaf area to ensure photosynthetic self-support (April-May) that carbohydrate concentration no longer declined. Young et al. (1995) reported that changes in respiration rate the Q10 and the energy of activation can be used to track bud development through endo- to ectodormancy and bud breaking. For example, when comparing the respiration characteristics of stratified and non stratified apple seeds, changes in respiration rate is higher in stratified seed when the raises in temperatures promote growth prior to imbibition. While the respiratory quotient (R_Q) during early dormancy in apple indicates that the use of lipids as the primary substrate increase after 990 chilling units, suggesting a shift towards carbohydrates as the primary substrate (Young et al., 1995).

Arnold and Young (1990) states that the amount of protein increase during chilling, reaching a maximum just prior to reach their chill requirement and are then used during bud break. Recently, a number of dormancy-specific RNAs, proteins and enzymes have been identified as signaling dormancy induction and release (Rowland & Arora, 1997). They found that dormancy proteins can be categorized into two groups; either bark storage proteins or dehydrins. As the name implies, the accumulation of dehydrins occurs in after the application of a dehydration stress, including low temperatures. Taylor and May (1967) suggesting that the concentration of bark storage protein increases dramatically (200 % in apple) in the autumn, subsequently declining when used to supply spring growth. Rowland and Arora (1997) further states that the decline or breakdown of bark storage proteins is likely to be linked to a hormonal communication mechanism involving gibberellins, cytokinins and auxin. However, neither bark storage proteins nor dehydrins have yet to be shown to be clearly linked with a regulatory role in influencing endodormancy (Coleman et al., 1992).

Frewen et al. (2000) reported that several quantitative trait loci (QTLs) have been identified for ecotypes of poplar *(Populus deltoid)* with different dormancy induction thresholds. Accordingly, mapping of some of these QTLs has been linked to chromosome regions that encode for a phytochrome gene. Phytochromes are family of plant photoreceptors proteins and act as important regulators of many plant developmental responses to light. Developmental regulation is thus achieved via the perception of radiation between 700 and 800nm (far red). This enables quality changes in sunlight caused by presence of plant canopies (shade) to be detected and revealed the developmental strategies of shade avoidance induced (Salter *et al.,* 2003). Senescence can also be linked to the induction of endodormancy, and both ethylene and ABA have senescence inducing roles, suggesting a commonality of action or hormonal linkage (Horvath et al., 2003).

Freeman et al. (2003) further confirmed that dormancy breaking results in the upregulation of several genes early in cell cycle growth phase, including cyclins and histones. The transition to the next development phase ('S phase') in the growth cycle, in which DNA replication takes place, is known to be modulated by several groups of plant hormones, i.e. gibberellic acid, cytokinins and brassinosteroids, as well as sugars (Horvath et al., 2003). In a study of peach (*Prunus persica),* IAA bud concentration fell with chilling and was low during rest, while ABA concentration was high declining close to bud break, at which time zeatin riboside increased (Ramina et al., 1995; Powell, 1986; Mielke & Dennis, 1975). Regardless of this correlation, it has yet to be proved that ABA has a direct casual role in dormancy regulation (Powell, 1987). This conclusion is supported by studies that show ABA changes in the absence of any apparent alteration in bud dormancy and ABA application does not often promote dormancy (Meilke & Dennis, 1978; Singha & Powell, 1978).

2.2.2 Dormancy Release and Bud-break

Different approaches have been suggested to examine the mechanisms of dormancy release in temperate fruit trees. The first approach is the Apical Meristem Dynamics which is based on regulation within the apical meristem itself by changes in the cell-to-cell communication and plasmodesmatal connections or in the cell cycle (Rinne et al., 2001; MacDonald, 2000; Jian et al., 1997; van der Schoot, 1996). The second approach emphasizes on the regulation of super cooling of water inside the vascular connections into the bud (De Fay et al., 2000; Quamme et al., 1995; Sakai, 1979), they addressed the sequence and regulation of water uptake into the bud; and the importance of water status/availability during dormancy release. The third approach is based on the mechanism of dormancy induction and release via a metabolic or communication block, or a permeability barrier between the bud and adjacent tissues (Faust et al., 1997; Crabbe & Barnola, 1996). Gevaudant et al. (2001) confirmed that both the buds and the underlying tissue in peach *(Prunus persica)* contains a greater accumulation

of PPA (*Prunus persica* H^+-ATPase) transcripts as compared to the buds themselves at the beginning of the dormancy period (October). This group attributed increased sucrose absorption in tissues underlying the bud during October to a stimulated H^+/sucrose co-transport driven by PPA genes and suggested this to have a role in paradormancy. This study also described that chilling-induced specific decrease in certain PPA isoforms in tissues underlying the buds in November and December could be involved in the evolution from paradormancy to endodormancy. Finally, while most work to date has focused on hormonal control of dormancy release, which, when, how, and to what degree hormones are involved is still uncertain, regardless of the evidence supporting the activities of various growth regulators involved in the processes of dormancy induction and release.

Cannell (1989) states that bud break follows fulfillment of the chilling requirements and is normally initiated during ecodormancy as the temperature increases. Also, the time of bud break appears to be strongly influenced by the time when chilling is received rather than chilling intensity (Abbott, 1970). In apple, differences in the flowering time of various cultivars described as early or late blooming have markedly different heat sum requirements, with late blooming cultivars having the higher heat accumulation requirements (Swartz & Powell, 1981). In Pears (*Pyrus communis*), artificially warmed in the autumn, are known to show delayed bud break and flowering (Atkinson & Lucas, 1996; Atkinson & Taylor, 1994). The heat sum requirements for bud break depends on the amount of chilling received; such that the longer the chilling the lower the heat sum required (Sparks, 1993; Couvillon & Erez, 1985; Swartz & Powell, 1981; Campbell & Sugand, 1979; Spiegel-Roy & Alston, 1979). However, the interaction between heat sum requirements and chilling received is important, as this may provide a poor estimate of bud break and flowering (Couvillon & Erez, 1985).

With respect to time of bud break, there is a clear difference observed in response of buds from different shoot positions (Jacobs et al., 1981). Accordingly, terminal and apical buds require much less chilling than lateral or basal buds, while the high chilling requirements of some cultivars appears to be correlated with strong apical dominance (Faust et al., 1995b). These positional effects are complex because once the buds are excised these positional responses are lost (Borkowska & Powell, 1979). Endogenous cytokinins have been shown to increase in spring prior to and during bud break (Cutting et al., 1991; Young, 1989), particularly in terminal shoot buds, relative to more distally located lateral buds (Cook & Belstedt, 2001).

Young et al. (1995) suggested that in cherry, dormancy breaking is accompanied by increases in bud nucleic acids, proteins and polyamines. This study also explains the resumption of growth that is associated with an increase in maintenance of respiration rate, fatty acid saturation and a decline in membrane sterol content. A study by Sagisaka (1974) also suggested that dormant to non-dormant buds show a change in respiratory metabolism from one which provides reducing power (pentose-phosphate cycle) to one providing energy for growth (glycolytic pathway and the TCA cycle). There is evidence from a number of studies that bud break is associated with free radical removal by activated peroxide-scavenging system, but it is unclear whether this is a simple correlation or a casual relationship (Rowland & Arora, 1997).

Gianfagna and Mehlenbacher (1985) suggests that, flowering time in apple actually dependent upon the temperature for bud growth and its heat sum requirement than a cultivars chilling requirement. They found that differences in cumulative temperatures for 'Delicious' apple between February and April showed above a day-degree value of 700 ($^{\circ}$F), not only was flowering earlier but the variation in actual flowering date was much smaller compared to values at or below 300 ($^{\circ}$F). Thus, the base temperature requirements for heat accumulation vary with species, ranging from 2.5 °C for peach to 4.5 °C for pear (Spiegel-Roy & Alston, 1979). It would appear in some species, cherry and blueberry for example, that chilling and heat sum requirements can be accumulated simultaneously (Felker and Robitaille, 1985). The case is different with peach, where exposure to temperature above 21 °C has a negative influence on accumulated chill hours (Erez et al., 1979a, b).

2.3 Bud-break by Chemicals and Cultural Practices

2.3.1 Chemical Application to Top Fruit

Chemical breaking of dormancy has been achieved initially through the use of simple plant and animal oils for a long time to compensate lack of chilling (Subhadrabandhu, 1995). Their mode of action appears two-fold; firstly they coat the branch creating an oxygen shortage. As oxygen starvation develops, the products of anaerobic respiration are implicated in breaking dormancy. These mineral oils have subsequently been modified with the addition of phenolic substances to increase their efficacy under conditions where chilling is low. Later on, other dormancy breaking chemicals have been developed and many of the substances act as respiration uncouplers. Normally in the process of respiration, electron transport and oxidative phosphorylation are tightly coupled. Uncoupling of these processes allows electron transport to proceed unlimited in the absence of ATP synthesis

that producing heat. This can have the effects of inducing anaerobic conditions with the production of ethanol in the bud (Erez, 1995).

Important dormancy breaking chemicals include cyanamide, as a calcium salt, Hydrogen cyanamide, thiourea, oil-DNOC, KNO_3 winter oil and others. Hydrogen cyanamide has been shown to be very effective in dormancy breaking of apple, pears, plum, apricot, raspberry and sweet cherry, as well as, peach cultivars with high chill requirements (Erez, 1995; Snir & Erez, 1988; Erez, 1987). Cyanamide, as a calcium salt, has also become the leading dormancy breaking chemical. However, the application of cyanamides require careful measures because of its toxicity effect. Other dormancy breaking chemicals include thiourea, which is very effective in combination with KNO_3 and oil-DNOC, but also is toxicity to humans (Erez, 1987). Plant growth regulators such a gibberellic acid and cytokinins have also been shown to break dormancy, but the high concentrations required appeal significant costs (Erez, 1987). Another problem with gibberellic acid is that the shoots often appear thin and spindly (Anonymous, 1994). Responses to GA_3 appear to be more effective depending on time of application and extent of chilling (Couvillon & Hendershott, 1974). Recently, introduction of 'Armobreak' a fatty amine, which enhances cuticular penetration, may reduce the concentration required of these rest breaking chemical agents (Erez, 1995). This would impact on key environmental issues associated with the use of these types of chemicals. Erez, (1995) also suggesting that the efficacy of KNO_3 and gibberellic acid was enhanced by Armobreak.

2.3.2 Chemical Application to Soft Fruit

Snir (1986) states that application of cyanamide to low chill cultivars of raspberry (i.e. cv. Dormanred) adapted to subtropical Israeli conditions showed improved bud break. This revealed that the use of dormancy breaking agents in combination with low chill cultivars promote better blooming and fruit set, regardless of environmental influence in mild winter areas. Linsley-Noakes (1989) conducted a series of trials in South Africa involving the application of hydrogen cyanamide solutions during the late summer/early autumn to alter bud break behaviour in other raspberry cultivars. This study indicates that after determining the optimum treatment regime, the bud break was possibly raise from 22-51% in some cases, which led to improved yields.

Also, different chemicals and growth regulators have been tested for improving bud break in fruit crops where lack of adequate winter chill may inhibit robust synchronous bud break. For instance, the application of compounds like potassium nitrate and thiourea were found to improve bud break and yield, with cyanamide again producing the better effects (Snir, 1983). Similarly, chemical defoliants such as maleic hydrazide have also been used to stimulate and synchronize flowering in fruit crops grown in mild winter areas of tropical and sub tropical regions.

Also, the application of growth regulators in late summer/early autumn has successfully affected the apparent accumulation of winter chill and bud break. Treatments with PP 333 (paclobutrazol) has inhibiting late season growth, inducing early leaf fall and therefore early dormancy, while the application of gibberelic acid (GA) would result in prolonged vegetative growth and delay leaf fall (Måge, 1986). Such a control mechanisms may be used to tailor growth and onset of dormancy to the local growing conditions by considering the local climate characteristics in order to maximize the efficiency of chill accumulation in that location. Other plant growth regulator such as ethephon is known to affect dormancy and can delay the bud break by up to 16 days (Durner 1995). Accordingly, this practice has been found to be useful particularly in continental North America where dehardening of the flower buds in late winter and early spring makes them highly susceptible to harsh frosts causing pistil damage or death.

2.4 Cultural Practices

2.4.1 Irrigation and Fertilization

Because of human health hazards and harmful environmental impacts, chemical applications are unlikely to be accepted by consumer and preferences shifts to organic alternatives. The routine agricultural practices such as watering, fertilizing, and pruning in the late season have all been shown to promote new growth and prolong succulent stage, with the consequent effect of increasing the chilling hours required to complete rest; thus delaying bloom in the following spring (Walser et al., 1981). Also some techniques like regulated deficit irrigation (RDI) or even partial root drying (PRD) which limit soil water availability could be worth examining with respect to control of dormancy and subsequent regulation of bud break. Spiers and Draper (1974) reported that in many fruit tree species, early leaf removal, either artificially or induced by late summer drought, can reduce the bud rest period, or synchronize flowering. Such trials have also demonstrated improved shoot growth and earlier spring flowering.

2.4.2 Pruning

Costes et al. (2006) studied the relationship between bud break and effect of pruning on shoot growth, source leaf area, current photosynthesis and annual building of storage reserves. They found that timing and severity of pruning can play a crucial role in the partitioning of photo assimilates during early and late summer. Thus, early summer pruning can stimulate the reallocation of reserves to renewing bud break and leaf growth, whereas late summer pruning may reduce leaf demand and has been shown to increase assimilates to the current season's growth. White et al. (1999, 1998) carried out cane-pruning experiments on northern European raspberry cultivars to see the effects on bud break. The study revealed that pruning in some cases increase the level of bud break compared to intact canes when exposed to simulated limiting winter chill conditions. The results also suggested that the phenomenon may be due to the reduction of apical dominance, and that management systems that include apical tip removal at the crucial phase of development may offer another method for improving bud break.

2.5 Plant and Environment Management

In warm climatic conditions, chilling requirements can be overcome by using evaporative cooling achieved through the use of overhead irrigation system (sprinklers) to reduce bud temperature (Erez, 1995). This approach is known to increases bud break and enhances uniformity of leafing out (Erez & Couvillon, 1983). Evaporating cooling also has the potential to be used with other physical methods, apart from low chill requiring cultivars, such as orchard/tree management practices that reduce bud chill requirement. These include reducing tree vigour by orientating branches to a more horizontal angle, preventing vegetative growth late in the season and delaying dormant season pruning. Thus deeper dormancy would be induced when stronger tree vigour, associated with vertically orientated branches (Erez, 1995).

3. Floral Initiation

Westwood (1993) suggested that flower buds formation is considered to involve signaling processes within the cells and also on a whole plant scale, resulting in a synchronous physiological event. As studied by many authors, floral initiation varies with all crops; in apple it requires the production of around 20 growth nodes (Luckwill & Silva, 1979; Abbott, 1977; Landsberg, 1974). Growth nodes or 'plastochron' are units of time recorded between successive development of primordia (i.e. morphological structures such as leaves, bud scales or flowers). An increase in apical meristem diameter signals the beginning of floral development, which is followed by differentiation and development of floral organs acropetally (Diaz et al., 1981). These events take place in late summer around the time shoot extension growth has terminated in August.

The rate at which initiation of apple primordia takes place has important implications in firstly determining the fate of the bud (reproductive versus vegetative), as well as its quality and fruit setting potential (Abbott, 1970). A short time to achieve the 20 growth node threshold promotes flower initiation, whereas too slow a production rate produces only a vegetative bud. Abbott (1977) also confirmed that the longer floral primordia has been initiated, the older it will be at the onset of dormancy when compared to the new fruit bud with a longer plastochron. The age of a primordium when entering dormancy can have important implications for subsequent development and performance. It is suggested that plastochron interval may have an optimal length that achieves the most effective fruit setting blossom characteristics. Variation in time of flower initiation has not, however, been shown to directly influence on blossom quality (Abbott, 1977). Accordingly, once formed, buds consist of bud scales, leaf and bract primordia and floral initials.

Felker et al. (1983) suggested that the reproductive bud scales having begun formation in early May remain in a dormant state due to correlative inhibition. The cessation of vegetative growth and terminal bud formation can be delayed in extreme cases until October, but it is normally finished by August. Accordingly, factors such as dwarfing rootstocks, heavy cropping and drought stress can all induce earlier termination of shoot growth and the production of 'resting' buds in apples.

The differentiation of organs in apple, such as the ovules and pollen sac, occurs at the end of the winter period (Bergh, 1985), while bud connecting xylem in some Prunus species does not appear until around 5 weeks before bloom (Hanson & Breen, 1985). There are many studies that have reported the influences of nitrogen, irrigation, light and temperature on floral initiation (Sedgley, 1990). There are also important impacts on floral abundance from management practices; one of the most prominent being pruning, particularly in the summer, removing sites for floral initiation (Webster & Shepherd, 1984; Lord et al., 1979).

Gutteridge, (1958) reported a similar situation exists in strawberry, in which flower initiation takes place in the autumn followed by a period of rest or dormancy. Floral induction is driven by changes in the plant's environment and occurs in short-day plants as day length declines and temperatures are reduced (Battey et al., 1998; Piringer & Scott, 1964). Even though in June bearing strawberry being a facultative, short day plant, its photoperiodic response is modified by temperature (Le Miere et al., 1996; Nishizawa & Hori, 1993a). They suggested that flower induction is primarily induced by photoperiodic reduction in the autumn, rather than the associated low night temperatures. However, Brown and Wareing (1965) suggest that with *Fragaria vesca*, temperature is more important than photoperiod. More recent work shows that flower induction with ever bearer strawberries may occur irrespective of daylength or temperature (Smeets, 1980).

Also, the formation of floral initials in straw berry starts over the winter from August through to the following in spring with differentiation of the initials occurring throughout this period. The change of apical meristems from vegetative to reproductive was stimulated by shortening day length (Williams, 1959a, b). Thus, floral initiation and development of dormancy occurred concurrently, but independently, in response to shorter days and lower temperatures as autumn progressed. For example, in cultivar 'Lloyd George', Williams (1959b) suggest that flowers were initiated without the occurrence of dormancy, suggesting separate processes, although other cultivars responded differently. It indicates that floral initiation had occurred after dormancy broken, subsequent development was temperature dependent, with little influence of day length. The age of the cane was found to influence initiation, with canes comprising no less than 15 nodes failing to initiate flowers even under long inductive treatments. Williams also found that initiation begins near the tips of canes and proceeds downwards.

3.1 Hormonal Control of Flowering

Hayama and Coupland (2003) described that light is mediated by phytochrome and cryptochrome photoreceptors found in the shoot meristems, and play a substantial role in the induction and timing of flowering, as well as many other plant physiological factors. This light regime information is fed into the plant clock systems (circadian clock) via biochemical pathways that are far unsynchronized. Recently, a model has been developed to describe the mechanism of the core of the circadian clock (the central oscillator) to synchronize the day/night photoperiod that provide a direct evidence in characterizing the molecular links between the central oscillator system and the timing of flowering (Suarez-Lopez et al., 2001; Onouchi et al., 2000). Also related here is the influence of light quality on flowering time. Simpson and Dean, (2002) reviewed the evidence collated from the generation of a number of phytochrome and cryptochrome mutants revealed that the wavelength of incident light can influence flowering time. Accordingly, the environmental significance of this appears to be in shade avoidance and competition: thus, shading by neighboring vegetation increases the relative abundance of far-red light since the absorption of red light by chlorophyll reduces the local red/far-red light ratio. The sensing of relative increases in far-red light abundance forms a signal of ecological competition resulting in accelerated flowering and quicker life cycle completion.

Plant hormones that have been associated with the control of flowering time include auxins, gibberellic acids (GAs) and cytokinins (Bernier, 1988). Physiological studies on the interaction of gibberellic acid in defoliation also implicate a role in the rest process. Autumn applications of gibberellic acid have been demonstrated to delay the development of autumnal leaf colour, leaf abscission, and the subsequent emergence from rest at bud break in the following spring (Walser et al., 1981; Brian et al., 1959). Molecular control studies involving phytohormones have been limited however, although a limited amount of information is now known for GA though work on Arabidopsis (e.g. Blazquez et al., 2002). Mutants in GA signalling or its synthesis show delayed flowering characteristics, though further work is required to determine the upstream factors that control GA activity.

3.2 The Role of Chilling in Bud Break and Floral Initiation

The developed floral buds of perennials (top and soft fruit) do not normally burst until they have received sufficient exposure to low temperatures. Time to flowering is therefore highly dependent on chilling, which is itself determined by the average daily temperature and the number of days at a species-specific temperature (Landsberg, 1974). Different combinations of the temperatures during the dormant phase and the duration of the chilling period satisfy the plant's chilling requirements (Jacobs et al., 2002). In strawberry for instance, increases in chilling leads to increased stimulation of stolon production, vegetative vigour, and floral capability (Battey et al., 1998; Smeets, 1982). However, reducing the chilling index below a threshold value dramatically increases the time to anthesis.

Tehranifar et al. (1998) suggested that chilling in strawberry is not a prerequisite for flower production – for example, with 'Catskill' and 'Elsanta', there was no negative effect of chilling, cold storage temperature or lifting date on flower numbers. However, fruit production is influenced by temperature and the duration of chill.

Short-day cultivars of strawberry, which are adapted to cooler winters, will have a better performance in tropical and sub tropical regions, but still require chilling if they are to achieve full production potential. With respect to the chill requirements among strawberry cultivars adapted to different climatic region, there are large differences observed in time length for chilling completion. For example, Tioga' requires between 2 to 4 weeks, while 'Glasa', 'Elsanta', 'Gorella' and 'Sequoia' have a 5 to 8 week requirement, with 'Redgaunlet' needing 8 weeks (Durner,1999). In low chill strawberry cultivar (Douglas), flower bud induction is inhibited by chilling (Kronenberg & Wassenaar, 1972), but enhanced in high-chill cultivars (Durner & Poling, 1987). Tehranifer et al. (1998) in their work with cultivar 'Elsanta' suggests that the way chilling is achieved may influence its effectiveness; i.e. field chilling may be more effective than cold storage for this cultivar. The reason for this may be due to oscillating changes in temperature in the field compared to cold-stored plants, or possible differences in radiation, although no mention is made in this work about whether the cold-stored plants where illuminated. Chilling requirements vary with the type of rootstock and bud positions (Couvillon & Werner, 1985), besides with species and cultivar for both top and soft fruits. For example, in vegetative buds of peach and apple, terminal buds have the lowest chill requirements, while laterals are higher and floral buds are generally in between (Faust et al., 1995b; Scalabrelli & Couvillon, 1986). In apple, there is a significant genetic difference between early and late flowering cultivars, that the late flowering cultivars have a greater chill requirement than early flowering cultivars, while additional chilling hours can eliminate these date differences (Swartz & Powell, 1981). Similarly, Chandler (1960) reported that the late autumn and early winter is conducive for apple leaves being maintained longer on the tree; then more chilling is required to induce bud break. However, this situation would result in insufficient chilling in mild winter areas that potentially causes a delay in bud break: where effective chilling does not occur until a degree of physiological bud maturity has been achieved (Walser et al., 1981).

Also, there is an interaction between chill requirements and the subsequent heating (forcing) to achieve bud break. For example, partially chilled apple trees are known to require more heat units before flowering, than fully chilled trees (Swartz & Powell, 1981). This indicates that in those years where cold winter temperatures were above threshold and effective for chilling resulted in lower apple yield. Beattie and Folley (1977) analyzed the yields of 'Cox's Orange Pippin' in Kent and Sussex (UK) from 18 orchards, managed in a similar manner, over the period 1971 to 1975. They found that there was very close correlation between mean 'Cox' yield and the temperature accumulated from February to April, each year. This analysis also showed that when the calculated temperature accumulation values were higher than average, yield was reduced. The higher accumulated temperatures are a reflection of years when the late winter and spring temperatures were higher. Atkinson and Lucas (1996) also produced similar reports regarding the influence of high temperature accumulation on yield reduction in their study of northern European fruit growing regions.

Chandler (1960), suggesting that in peaches, chilling temperatures in the presence of a certain amount of leaf area was not as effective as when leaves are absent. Thus, the time of fall leaf abscission could be correlated with the duration and intensity of rest in peach terminal vegetative buds (Walser et al., 1981). Also the time of blooming is influenced by different orchard management practices: For example, the application of foliar nitrogen in the autumn delayed the bloom of peaches compared to untreated controls (Reeder & Bowen, 1981). Similarly, trees treated with gibberellic acid showed delayed leaf abscission and retained their leaves (Walser et al., 1981). Leaf-derived gibberellic acid is the most likely candidate to be involved in delaying bud break and flowering, as its application temporally delays leaf senescence.

In semi-temperate locations, such as California, and in mild winter areas of tropical and sub tropical regions, lack of sufficient chilling was an issue for deciduous fruit cultivation (Cook & Jacobs, 1999). Advances have been made in response to this lack of chilling, with the breeding of low-chill cultivars and the development of chemical and orchard management practices to induce bud break (Sedgley, 1990). Under tropical conditions, defoliation after the first crop in low chill cultivars induced early bud break as in the case of low chill nectarine and peaches (Sherman & Lyrene, 1984). Similarly, defoliation prior to endodormancy of apple grown in the tropics will induce a second crop (Janick, 1974).

Thompson et al. (1975) suggested that the seasonal timing of chilling is also important, in that early chilling appears less effective in satisfying requirements, compared to chilling received in the latter part of the winter. This was in conformity with the results of Beattie and Folley (1977) where their correlative analysis of yearly apple yields with climate showed a negative influence of February to April cumulated temperatures with

cropping. This implies that variation in effective chilling temperatures for apple, peaches and pears, can also change if continuous chilling is not given. It appears in some cases where temperatures that were not initially chilling effective, if applied constantly, can become as effective when interrupted by warmer temperatures (Erez & Couvillon, 1987; Couvillon & Erez, 1985). It is equally evident that when chilling is interrupted by warmer periods, even over a short-term diurnal frequency, apparent chill accumulation and subsequent bud break can be influenced (Erez & Lavee, 1971).

3.3 Physiological Basis of Chilling Responses

(i) Phases of vernilization and chill accumulation

Couvillon and Erez (1985) suggested two phases of vernalization in chill accumulation processes: The first phase involves the conversion of a product from unchilled to chilled state by chilling temperatures, which can be reversed by high non-chilling temperatures. For example, for several fruit species, 2, 4, 6 and 8 hours of 24^{o}C within a 24 hour cycle at 4^{o}C negated the applied chilling by 14, 45, 82 and 100% respectively. This approach permits the effects of corresponding warm modulating temperatures to negate chilling to be derived. The second phase in the process of vernalization involves the conversion of the unstable product from phase one into a stable form (Erez, 1987a, b). When this reaction takes place it is not reversible and the accumulation of the product to a certain level results in dormancy completion (Erez & Couvillon, 1987).

Erez (1995) suggests that the dynamic model based on the effects of temperature on dormancy through a determination of climatic events on accumulated product (the chill). Following temperature induction, a steady-state level of intermediate is determined by production and breakdown. It states that chilling accumulation is acquired by quanta of the intermediate product as it is transferred in to stable product. A number of other events are also suggested to impact on how effective chilling accumulation is achieved. These include the influence of the level of intermediate, such as negative effects of heat and the duration of the daily temperature cycle. The process has been described as dynamic and biochemically active (Rowland & Arora, 1997).

(ii) Changes in protein levels

Protein levels of peach flower buds during chilling have been linked to the chill requirements to satisfy chill (Lang & Tao, 1991). Protein analysis in peach plants has revealed that a 61 kD protein which declines as the chilling requirement is met, while the reverse happens in blueberry with the accumulation of two proteins as the chilling requirement is met (Lang, 1985). During dormant season, there is a loss of cold hardiness that may induces protein changes independent of chilling and it has proved difficult to separate the various processes. However, Lang (1994) has characterized dormant season protein expression and suggests, with peach, that the observed decline in a specific protein (61 kD) was associated with dormancy rather than cold acclimation. Lang (1994) also noted that the effects observed may have been due to increased bud development and deacclimation. The 61 kD peach protein was also shown to decrease in response to the application of dormancy-breaking compounds such as gibberellic acid and hydrogen cyanamide. Also, Muthalif and Rowland (1994a, b) further quantified the protein changes in response to chilling phases and requirements in two blueberry cultivars with contrasting chill requirements (low and high), and found that three major proteins accumulated during chilling.

The increase in protein accumulation was greatest at the beginning of chill period and subsequently declined rapidly on forcing and resumption of bud growth. However, for both the low and high chill- requiring blueberry, the accumulation of the chill proteins was closely associated, temporally and quantitatively, with cold acclimation (Muthalif & Rowland, 1994a). These proteins have highly conserved regions which are lysine-rich, as with all dehydrins. However, further analysis by (Lang, 1994) showed that the dehydrin proteins to be closely related to cold acclimation rather than degree of dormancy (Rowland & Arora, 1997).

(iii) Activity of enzymes

The enzyme acid lipase examined in apple buds from cultivars with different chill requirements showed an increase in its activity correlated with satisfying chill (Liu & Norman, 1991). Accordingly, the hydrolysis of lipids affects the freezing point of water, which itself may be involved in dormancy breaking. Faust et al. (1991) noted from the analysis of NMR scans of the apple buds showed an increase in free water, compared to that bound with proteins and cell walls, as apple buds accumulate chill. Studies with apple shown the processes involved with satisfying chilling requirement also convert bound water in the bud into free water, implying the necessity of free water for growth (Faust et al., 1991). The conversion of bound water to free has been shown to be incremental in relation to chilling, and the rate at which a threshold value is achieved differs between high and low-chill requiring cultivars (Faust et al., 1995a). Studies of different peach cultivars indicates that Catalase

activity in flower buds has been shown to decrease during chilling and increase with bud break (Kaminski & Rom, 1974). However, in grapes catalase activity has been shown to decline on the application of bud breaking chemicals (cyanamide & thiorea) (Nir et al., 1986).

(iv) Changes in carbohydrate levels

In deciduous fruit trees, carbohydrate storage parenchyma on bud tissues has begin to accumulate starch in the autumn, and there is an evidence that roots provide an important sink for marked increase in the storage of reducing sugars and sucrose (Nishizawa, 1994). With subsequent chilling (350-400 h), the amount of starch declines and other factors must be responsible for the fact that plants remain dormant. Further chilling, not due to correlative inhibition, appears to be induced by a reduced ability to synthesize nucleotides (Robert et al., 1997). The physiological state of tissues during chilling can be detected through measurements of changes in ATP content and NTP, reflecting non-specific energy demands and protein synthesis (Robert et al., 1997).

Starch concentration has been shown to decline during apple chilling and forcing, while sorbitol changed very little during forcing (Whitworth & Young, 1992). Sucrose increased with chilling only to the point where around 50% bud break was obtained. These results most likely indicate only the pattern of carbohydrate utilization, particularly with respect to its metabolism during chilling and forcing. Winter carbohydrate accumulation for cold hardiness, freeze protection and respiratory consumption are all well-documented (Raese et al., 1978; Hansen & Grauslund, 1973). Work with June-bearing strawberry demonstrated an autumnal increase in root starch at a rate negatively correlated with temperature (Le Miere et al., 1996). Therefore, it can be concluded that amount of root starch was not an indicator of dormancy, so it is therefore unlikely to be a driver of dormancy.

(v) Changes in levels of growth regulators

Swartz and Powell (1981) worked in apple buds with known differences in chilling requirements. Chemical analysis of this experiment revealed that there is a distinct decline in abscisic acid (ABA) concentration in bud primordia collected in January through spring, while little difference was observed in ABA concentration for those collected in December irrespective of chilling requirements. Though, the rate of decline in ABA concentration was most rapid with low chill compared to high chill cultivars. The ABA concentration at bud break was similar, but the dates of bud break as expected were not, because no warm controls were used and differences in ABA concentration may simply be to other changes rather than ABA acting as a casual factor. ABA concentration declines in sour cherry buds under both cold and warm conditions (Mielke & Dennis, 1978). Conversely, the concentration of giberellins in buds increases during chilling (Eagles & Wareing, 1964; Frankland & Wareing, 1962).

(vi) Rootstock influence of chilling

Variability in chilling requirements of different apple rootstocks suggests that the chilling responses of scion, with respect to bud break, can be influenced by the rootstocks (Young & Werner, 1985; Couvillon *et al.,* 1984). This may be achieved through the transfer of a phloem- mobile element (or chemical 'signal'), which is initially synthesized in the roots. Such a hypothesis would fit with the idea of Erez (1995) that the timing requirements of chilling and bud break are controlled by the actual accumulation of as yet unidentified product.

4. Chill Accumulation and Estimation

4.1 Quantifying Winter Chill

Richardson et al. (1974) developed the concept of determining 'chill units', from measurements of temperature, to calculate the amount of chilling received by simple predictive method that revealed weight step function. Firstly, one chill unit was defined as an hour exposure at the optimum temperature required meeting the species or cultivar's chilling requirement. A cultivar's chilling requirement was therefore measured by the number of hours required, at a set temperature, below which chilling is received. For example, early peach chill models (Weinberger, 1950) cumulated the number of hours below 7.2°C (45°F) as a guide to adequacy of breaking dormancy. For this, optimal chilling temperatures were found to be 6°C for peach but are lower (2ºC) for apple (Thompson et al., 1975). Afterwards, there have been various attempts to refine the limitation of this early approach to take account that the rate of chilling process varied with temperature (Richardson et al., 1974).

Consequently, temperature readings between 7 and 10^{o}C are used in calculating a plant's chill requirement; with 6^{o}C as the optimum, while temperatures below 1.5^{o}C do not contribute and temperatures above 13^{o}C reduce accumulated chill units. Though, numerous studies shows that optimal temperatures (effective cut off temperatures), within the range (1.5^{o}C to 13^{o}C), vary with species and cultivar (Couvillon, 1995; Anderson & Richardson, 1987). Mahmood et al. (2000b) reported that for *Prunus avium,* temperatures below –4.5^{o}C and above 12^{o}C are ineffective in breaking dormancy and specific cultivars, i.e. Stella, Summit and Sunburst had

optimum chilling temperatures of 3.2°C and 3.7°C respectively. While with peach, temperatures around 8°C are the most effective in satisfying chilling requirements, and above which, 10°C and 12°C were only 46% and 33% as effective, respectively (Erez & Couvillon, 1987).

When applying these chill models, they commonly accumulate chill over time and when a threshold amount of chill has been amassed for tree species that require different threshold amounts of chill to break dormancy. Zhang and Taylor (2011) reported the chilling requirement of Sirora pistachio *(Pistacia vera L.)* in Australia by monitoring winter chill accumulation from 2006 to 2010. Three chill models (Chill Hour, Utah, and Dynamic) were compared both in the greenhouse and under field conditions to evaluate their predictive performance. The result indicates that Dynamic Model produced the best determination for fulfillment of chilling requirement with 59 chill portions in 2006 and with little variation in the following years (Table 1). However, the relationship among the level of fulfillment of chilling requirement (low, moderate or high), showed a significant variation for Chill Hours and Chill Units between years (Table 1).

Table 1. Estimated chill accumulation of Sirora pistachio *(Pistacia vera L.)* in (chill hours, chill units, and chill portions) at Dareton Research Station, New South Wales (Zhang & Taylor, 2011).

Winter	Chill fulfillment date	Chilling hours	Chill Units	Chill Portions	GDH	50% bloom date predicted	50% bloom date in the field	Chill catagorization
2006	10 Aug.	645	990	59	9633		20 Sept.	Moderate - High
2007	13 Sept.	677	919	58	17297	8 Oct.	7 Nov.	Moderate - High
2008	3 Sept.	569	1078	62	9818	9 Oct.	10 Oct.	High
2009	9 Sept.	412	752	60	9411	17 Oct.	16 Oct.	Moderate - High
2010	25 Aug	535	1123	61	10063	9 Oct.	10 Oct.	Moderate - High

4.2 Estimate of Hourly Temperatures Required for Winter Chill

The accuracy of chill models is sometimes reported to be better when hourly temperatures are used. The Utah model (Richardson et al., 1974) requires hourly temperature data, while the <7.2°C and 0-7.2°C models can use either daily or hourly data. Richardson *et al.* (1974) used a simple linear interpolation to obtain hourly temperatures from daily data: this involved segmenting a daily max/min temperature plot, connected by straight lines into equal segments (Figure 1). However, <7.2°C and 0-7.2°C models were used daily and hourly values for a site where both hourly and daily data were available. This indicates that hourly chill can be estimated simply from daily temperature data using relationships between the annual values of daily and hourly temperatures.

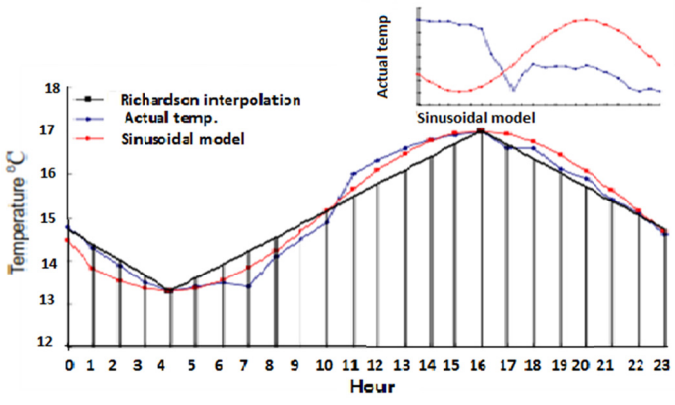

Figure 1. Method for the estimation of hourly temperature from daily maximum/minimum temperatures by simple linear interpolation (Richardson et al., 1974)

The data from the interpolation models (Richardson et al., 1974) were calculated from the maximum/minimum temperature data for the same location on the same day. Although most days follow a general sinusoidal trend (Figure 1), variability can be high due to other climatic factors: inset - plot from a winter day at the same location.

Monteith and Unsworth (1990) suggested better interpolation method is to utilize a sine function for diurnal temperature trends, so that the hourly mean temperatures can be estimated from:

Hourly temperature = (Tmin+Tmax)/2+((Tmax-Tmin)/2)* sin (radian (H* 1 5)+2 10) (Equ…1)

Where:

Tmax = Daily maximum temperature

Tmin = daily minimum temperature

H = hour of day, indexed from 0 – 23

Sanders (1975) suggest that these approaches should be further improved by considering the fact that the diurnal temperature increase tends to be more rapid than the decrease in the afternoon/evening with the time Tmin - Tmax often averaging 10-11 hours and the time between Tmax - Tmin averaging 13-14 hours. However, as illustrated by Aron (1975) in a critique of the temperature estimation methods developed by Richardson *et al.,* (1974) care must be taken in the use of these models: regions such as the Californian coastline experience regular daily changes in air mass due to coastal breezes that at times may extend well inland. Also, locations that experience the passing of a large number of fronts will also diverge from the model. The application of the method is likely to be successful in regions that generally experience sinusoidal daily temperature regimes.

4.3 Chill Calculation Methods

Calculating winter chilling can assist the fruit industry in predicting/forecasting the quality and quantity of fruit in the subsequent season. Chilling units accumulated during the cold season enable the plant to release dormancy in spring. Studies indicates that chill models used to calculate chill accumulation require different temperature ranges, above or below which the chill accumulation is negative or nil: For example, temperatures of 1.5–12.4 in the Utah Model (Richardson et al., 1974), 1.6–13^{o}C in Modified Utah Model ((Linsley-Noakes et al., 1995) and 1.8–16.9°C in North Carolina Model (Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981) were used and assigned for different weighing values of chill accumulation in a hourly basis. Gilreath and Buchanan (1981) states that, low chill requiring sungold nectarine cultivar in Florida shown faster bead break at 10°C after exposure to constant temperatures of 24 hours in the dark, while in the same cultivar at similar exposure, flower bud occurred at 7°C. Similarly, Erez et al. (1979a) reported from California that peaches require 9.6 to 9.8°C threshold temperatures which favor flower bud development.

Richardson et al. (1974) described the chilling requirements of 'Redhaven' and 'Elberta' peach trees growing in Utah, and found that the temperature at 6°C was the most effective chilling temperature and below 1.4°C did not contribute towards chilling. The same study indicates that temperatures above 16.5 °C caused the reversion of accumulated chilling for Utah model. Erez et al. (1979a) in Georgia also confirmed that, when applying Utah Model for Redhaven and Redskin peach, the temperature at 6°C is the most effective for rest completion, while the temperature above or below which may result in extended rest completion and less effective until the maximum temperature reached (12.4 ^{O}C) for chilling reversal.

All the classical models were derived from the number of hours of exposure to a given temperatures that were assigned a weighing factors. The weighing functions were mainly determined by laboratory tests and they differ because of tree species and variety (Richardson *et al.,* 1974; Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981). These weighing factors are sensitive to different temperature ranges and estimated the chill unit accumulation in an hourly basis (CU h^{-1}) as a function of temperature and plotted as a discontinuous step function that estimates different values for the model tested (Figure 2).

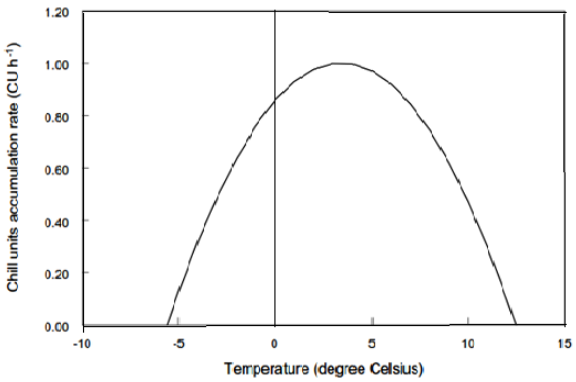

Figure 2. Chill Units accumulation rate (CU h^{-1}) as a function of temperature (Richardson et al., 1974; Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981)

In application, Chill Models all accumulate chill at hourly intervals, require summation of chill to estimate total chill exposure and operate within a defined chilling period. Furthermore, the positive section of the chilling curve becomes difficult to define in warmer locations which do not experience sharp turning points into and out of positive chill accumulation. A classical chilling model approaches can be described as:

(i) Simple temperature accumulation models.

The 0-7.2°C model (Weinberger, 1950) is a simple model first developed over 50 years ago and measures chill hours (CH) according to hourly temperature (T_t). Temperatures within the 0-7.2°C interval are allocated one chill hour, while temperatures outside this interval record zero chill hours (Equ 2 and Figure 3). To determine total chill $_{(CHtot)}$ for a given chill period, chill hours are summed from predetermined start (st) and end (en) times (Equ 2).

$$CH_{tot} = \sum_{t=st}^{en} CH \begin{cases} T_t < 0°C & ;CH_t = 0 \\ 0°C \leq T_t \leq 7.2°C & ;CH_t = 1 \\ T_t > 7.2°C & ;CH_t = 0 \end{cases} \quad \text{Equ.....}_2$$

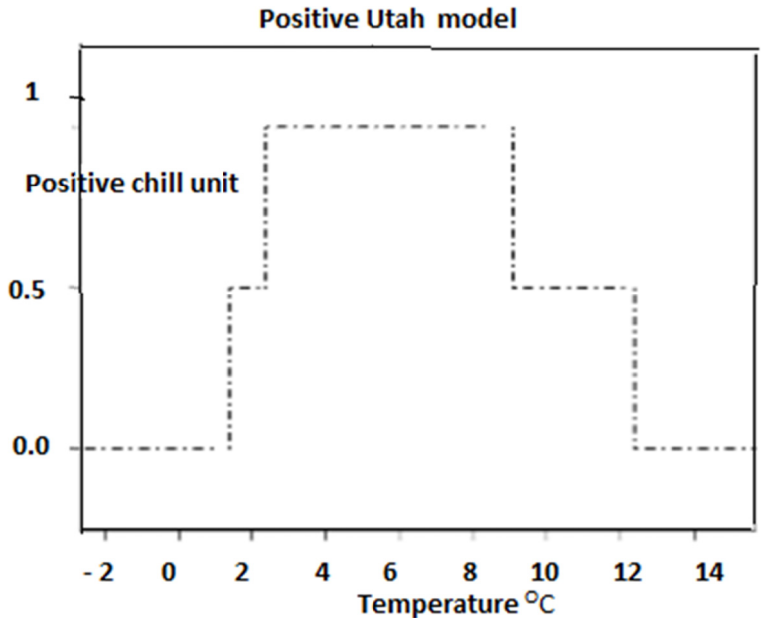

Figure 3. 0-7.2°C model as a function of temperature (Weinberger, 1950)

The attempt to quantify winter chill by Weinberger (1950) revealed that the chill requirements of species would be satisfied after exposure of trees below the threshold of 7.2°C (45°F). This was based on observations of the number of hours of chilling that were required to break dormancy in a number of peach cultivars grown at Fort Valley, Georgia, USA. This enabled the development of a ranking system for the cultivars according to whether they had high or low chilling requirements to fulfill dormancy and promote successful bud break. Further studies, however, demonstrated differential responses of species to chilling temperatures, with the largest effects often occurring above freezing (Erez & Lavee, 1971).

(ii) ***Simple Temperature weighted models***

Alternative to the >7.2°C model, a 0-7.2ºC chill model, a number of studies appeared to develop physiologically more appropriate model to estimate dormancy breaking in a number of species grown in particular regions (Byrne & Bacon, 1992). The Utah Model (Richardson et al., 1974) contains weight function assigning different chilling efficiencies to different temperature ranges, including negative contributions by high temperatures (Table 2 and Fig 3). Thus, the chill units (CU), would be considered as a duration of 1 hour period in a temperature range considered optimum (2.5-12.5 °C) to accumulate chill (Richardson et al., 1974). Temperatures < 1.4 °C have zero values and do not contributed to chill accumulation. Temperatures above 12.5 would result in a reversal of the accumulated chilling (Table 2 and Fig 4) (Richardson et al., 1974).

The chill accumulated in the Utah model for an hour at a given temperature is calculated using the weighting system (Table 2). The model takes into account the deterioration in chill accumulation efficiency above and below 6 °C (i.e. optimum chilling efficacy or effective chilling at 6 °C). It also accounts for the deductive effects of short periods of warming during winter, though does not discriminate between the differential behaviour of longer phases of warm weather (Erez & Lavee, 1971).

Table 2. Values of chill units (CU) as a function of hourly temperature for Utah model (Richardson et al., 1974)

Temperature ^{O}C	CU
<1.4	0.0
1.5 – 2.4	0.5
2. 5 – 9.1	1.0
9.2 – 12.4	0.5
12.5 – 15.9	0.0
16.0 – 18.0	-0.5
>18.0	-1.0

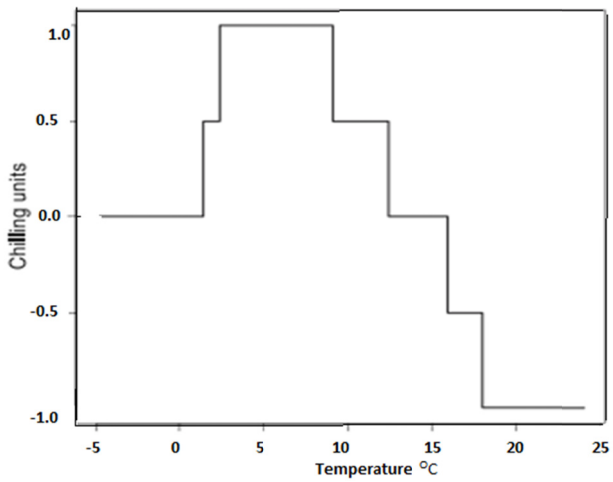

Figure 4. Utah model: Chilling units (CU) as a function of temperature (Richardson et al., 1974)

Though, the Utah model has been employed in studies to characterize the rest requirements of several fruit crops and their cultivars, it has also been used in tandem with another models to calculate growing degree hour accumulation (GDH°C) (Whitworth & Young, 1992; Couvillon, 1985), where a single GDH°C is defined as 1 hour above base temperature (4.5°C). These were applied in tandem to estimate the accumulation of energy required for the development of phenological parameters such as full bloom or fruit set. Other useful contributions made by the application of these models included the demonstration that post-rest chilling bursts beyond the chilling requirements would significantly reduce GDH°C accumulation required for development (Couvillon, 1985).

Other model following similar patterns in application with the Utah chill model is the North Carolina chill model (Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981). This model behaves some minor modification to original Utah, that it accommodates the chill accumulation until the raise in temperature reached at 16.5^{O}C, and above which, the raise in temperature would result in chilling reversal (Table 3). The corresponding temperatures and chill units (CU) values for North Carolina model showed that, because of chilling negation by high temperature, it has a limited applicability in mild winter areas (Table 3 and Figure 5).

Table 3. Values of chill units (CU) as a function of hourly temperature for North Carolina model (Gilreath & Buchanan, 1981; Shaultout & Unrath, 1983)

Temperature °C	**CU**
-1.1	0.0
1.6	0.5
7.2	1.0
13.0	0.5
16.5	0.0
19.0	-0.5
20.7	-1.0
22.1	-1.5
23.3	-2.0

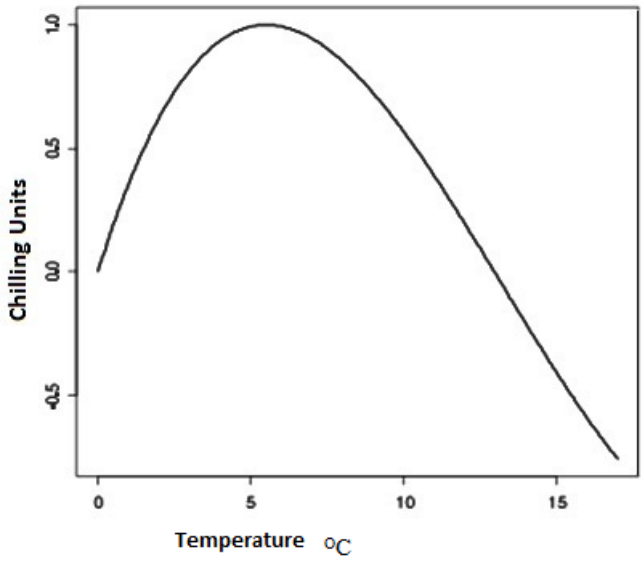

Figure 5. North Carolina chill units (CU) as a function of temperature (Shaultout and Unrath, 1983; Gilreath and Buchanan, 1981)

When considering the original Utah chill unit model (Richardson et al., 1974), it consists of three curves

showing effective bud temperature, air temperature and effective chilling temperatures (Figure 6). The outer curve represents the actual bud temperature that the tree senses and to which it responds. The inner curve is the air temperature as measured in the instrument shelter. The middle curve designated the 'Effective Bud Temperature' is an index relating shelter temperature to bud temperature.

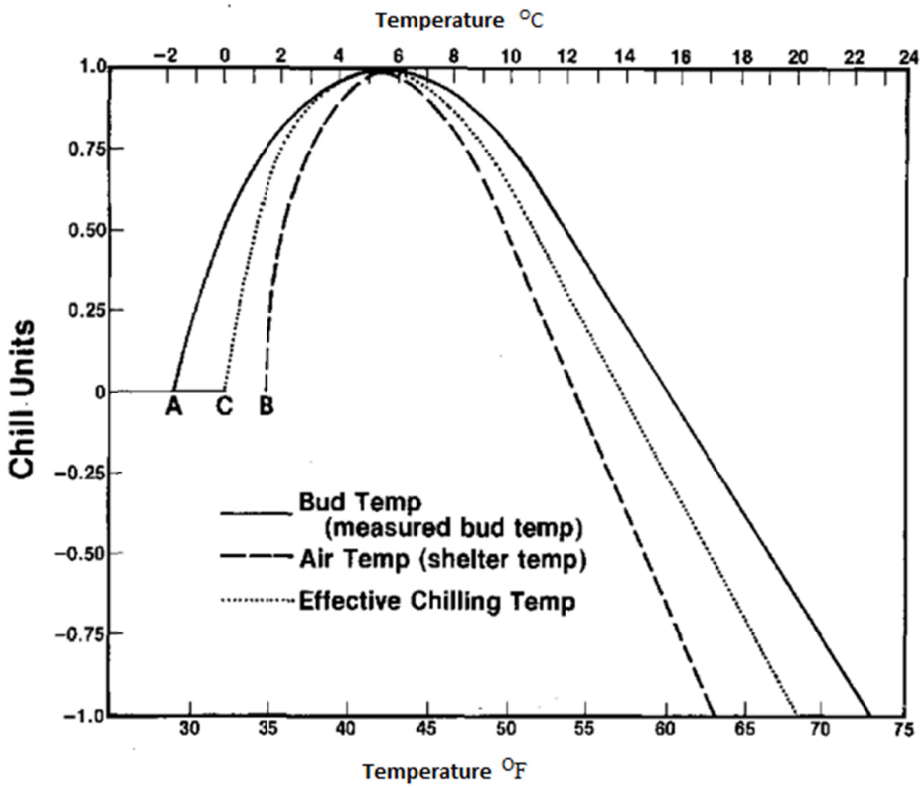

Figure 6. Curves used in estimating Utah chill units (Richardson et al. 1974)

As shown in the curves (Figure 6), the responses of several fruit tree species to their environmental temperatures indicated that, rather than following a true cosine curve, response rates to increasing temperatures often varied, depending on whether the temperature above or below the optimum temperature for the species that can severely affect chill unit accumulation. The calculation process is identical for North Carolina model (Shaultout & Unrath, 1983), because initially the cumulative chill unit curve drops, but later on it starts to increase once positive chill units begin to accumulate (Figure 5).

Gilreath and Buchanan (1981) noted that the widely used Utah model (Richardson et al., 1974) has not proven accurate when tested under climatic conditions that are milder than in the original experiment. Also, the Utah model was developed to predict bud-burst for ‘Red-haven’ and ‘Elberta’ peaches, but, in practice, it is widely used for a wide variety of crop species.

Linvill (1990) conducted experiments using the Modified Utah model for peach trees to improve accuracy in determining dormancy release. Optimum chilling temperatures ranges from 2.5-9.1°C, with temperature either side of the optimum range declining in ability to accumulate chill. Another important addition was the incorporation of the negation effect that high temperatures (~16°C) can reverse the previously accumulated chill. Thus, the Modified Utah model is more appealing than the original Utah model due to the more gentle response, that is, no solid step boundaries, which is more likely representative of the chill accumulation processes (Figure 7).

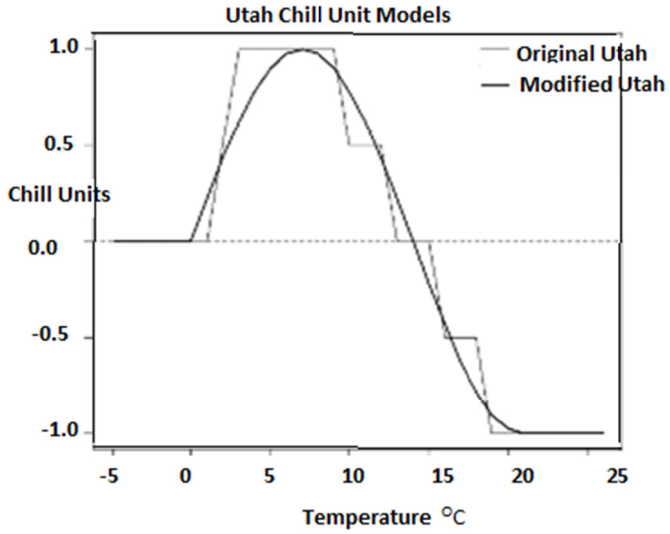

Figure 7. The Modified and original Utah model chill unit allocations for hourly temperature (°C) (Linvill, 1990)

Also, the Modified Utah model allocates chill units (CU) for hourly temperatures (Tt) which are summed over a predetermined chilling period to estimate total chill exposure (CU tot) (Equ 3).

$$PCU_{tot} = \sum_{t=st}^{en} PCU \begin{cases} T_t \leq 1.4℃ & ; PCU_t = 0 \\ 1.4℃ < T_t \leq 2.4℃ & ; PCU_t = 0.5 \\ 2.4℃ < T_t \leq 9.1℃ & ; PCU_t = 1 \\ 9.1℃ < T_t \leq 12.4℃ & ; PCU_t = 0.5 \\ T_t > 12.4℃ & ; PCU_t = 0 \end{cases} \quad \text{Equ.....3}$$

The Positive Utah model (Linsley-Noakes et al., 1994) is an iteration of the original Utah model however the chilling reversal due to high temperatures is excluded. The model defines optimal chill accumulation between 2.4 and .1°C and steps down to nil positive chill units for temperatures less than 1.4°C and greater than12.4°C (Figure 8 and Equ 4).

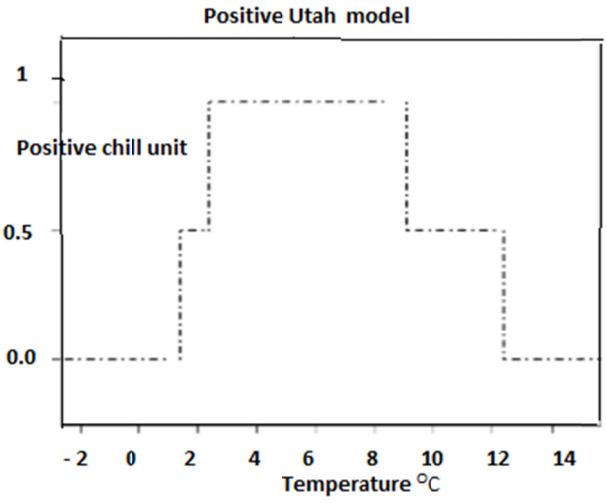

Figure 8. The Positive Utah Model chill allocations for hourly temperature (°C) (Linsley-Noakes et al., 1994)

This model also allocates positive chill units (PCU) for hourly temperatures (T_t) with total chill exposure (PCU tot) determined by summing PCU over a predetermined chilling period (Equ 3).

$$PCU_{tot} = \sum_{t=st}^{en} PCU \begin{cases} T_t \leq 1.4°C & ; PCU_t = 0 \\ 1.4°C < T_t \leq 2.4°C & ; PCU_t = 0.5 \\ 2.4°C < T_t \leq 9.1°C & ; PCU_t = 1 \\ 9.1°C < T_t \leq 12.4°C & ; PCU_t = 0.5 \\ T_t > 12.4°C & ; PCU_t = 0 \end{cases} \quad \text{Equ.. 4}$$

The Positive Chill Units model (PCU) as a modified version of the Utah, does not consider the negative values for the chill accumulation (Linsley-Noakes et al., 1995). Following the Dynamic model, its application in tropical and sub-tropical zones has shown improved results. The procedure it follows is the same as original Utah, except that when negative, the chill unit value is set equal to zero (Equ 4). Therefore, the accumulated chill units are equal to zero until the temperatures drop into the effective zone and positive chill units begin to accumulate (Figure 8). The key differences between the original Utah (Richardson et al., 1974) and the Positive Chill Unit model (Linsley-Noakes et al., 1994) were:(i) when predicting chill unit accumulation, an emphasis is given to predict the proportion of bud break rather than time of bud break; (ii) different weighing temperatures for chill accumulation was measured by a fitting procedure to exclude chilling reversal.

(iii). Dynamic Model

The Dynamic model (Fishman et al., 1987; Erez et al., 1990) determines chill exposure differently to the other models. It accumulates chill more interactively and calculates chill portions in a time dynamic two-step process. The creation of an intermediate product, promoted by cold temperatures, is initially determined. This intermediate product can then be destroyed by subsequent warm temperatures. Moderate temperatures are defined to have a positive influence on chill accumulation. Once a threshold amount of the intermediate product is created, it is irreversibly accumulated as a chill portion that cannot be destroyed regardless of subsequent temperatures (Figure 9).

An algorithm for this model was outlined by Darbyshire et al., (2011) in similar procedures with that of the original Dynamic model (Fishman et al., 1987a, b). Chill portions are calculated using hourly temperature (°K) input and chill portions are summed over a specified chill period to obtain total chill exposure.

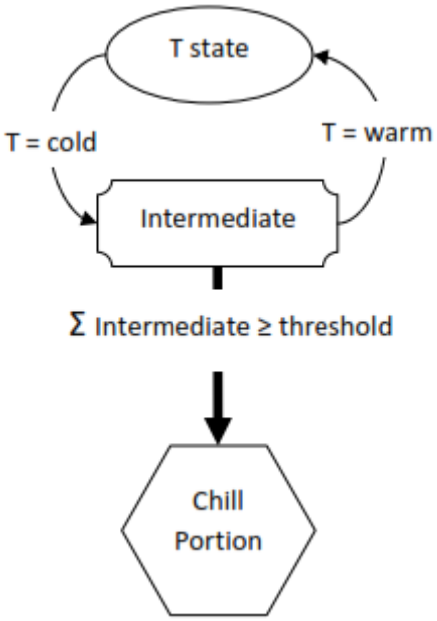

Figure 9. Dynamic model concepts for hourly temperatures, T (°K) for initial temperature (T state) followed by subsequent temperatures that can destroy the intermediary after while a threshold amount of the intermediate is amassed a chill portion is irreversibly produced (adapted from Darbyshire et al., 2011)

Fishman et al. (1987a, b) stated that the concept of Dynamic Model encompasses the following elements:

(i) The two-step system concept:

The first step builds an intermediate that is accumulated when exposed to low temperature. The intermediate level depends on following: the bell shape curve effect of chilling, the negating of chilling by high temperatures (effect of level; effect of high temperature duration; effect of cycle and the promotive effect of moderate temperatures. The dynamic curve exhibits a periodic behavior and the model incorporates a second adjustment to correct the effect of the low temperatures with the assumption that the intermediary transformed into a product cannot be reversed (i.e. Chill Portions (CP) once accumulated, cannot be nullified by high temperatures).

(ii) The concept of a fixation effect:

When a critical level of the intermediate is reached, a phase transition occurs, the intermediate level drops to zero and a quantum that is termed 'Chilling Portion' is accumulated.

(iii) The concept of a quantum:

When a portion, the size of which is a physiological measure, is accumulated, it is fixed and conserved. This follows the principle of Arrhenius equation (France & Thornley, 1984) as:

$$k = A\,x\,e^{(E/T)} \quad \text{.....Equ 5}$$

Where T is the absolute temperature expressed in Kelvin degrees (°K), E is the activation energy for the formation and destruction of the intermediary, and A is an independent coefficient of the temperature and (k), the velocity constant.

(iv) The concept of a threshold level:

A critical level of the intermediate has to be reached for effective chilling to accumulate. As long as this threshold is not reached, no matter how close the level of intermediate is to the threshold, no chilling accumulation will occur. Differences among cultivars or species are in the total portions needed for breaking dormancy, not in model parameters.

The equations for calculating chill portions are more complex than the other models (Fishman et al., 1987a, b). Although they are difficult to derive from the original publications, Luedeling et al. (2009c) extracted them from a spread-sheet commonly used by practitioners. The equations for the number of Chill Portions at time t (CPt) are:

$$x_t = \frac{e^{slp \times tetmlt \times (T_t - tetmlt)/T_t}}{1 + e^{slp \times tetmlt \times (T_t - tetmlt)/T_t}}$$

$$y_t = \frac{a_0}{a_1} \times e^{(e_1 - e_0)/T_t}$$

$$ak_t = a_1 \times e^{(-e_1/T_t)}$$

$$Inter_{Et} = y_t - (y_t - Inter_{St}) \times e^{-ak_1}$$

$$Inter_{st} = \begin{cases} t = 1, & 0 \\ \text{if} \quad t > 1 \,\&\, Inter_{Et-1} < 1, & Inter_{Et-1} \\ t > 1 \,\&\, Inter_{Et-1} \geq 1, & Inter_{Et-1} \times (1 - x_{t-1}) \end{cases} \quad \textbf{Equ.....6}$$

$$delt_t = \begin{cases} t = 1, & 0 \\ \text{if} \quad t > 1 \,\&\, Inter_{Et-1} < 1, & 0 \\ t > 1 \,\&\, Inter_{Et-1} \geq 1, & Inter_{Et} \times x_t \end{cases}$$

$$P_t = \begin{cases} \text{if} \quad t = 1, & delt_t \\ t > 1, & delt_t + P_{t-1} \end{cases}$$

$$P_{tot} = \sum_{t=st}^{en} P_t$$

The experimentally derived constants slp, tetmlt, a_0, a_1, e_0, and e_1, were set to 1.6, 277, 139,500, 2.567 x 10^{18}, 4153.5, and 12,888.8, respectively (Erez et al., 1988). T_k is the measured hourly temperature in Kelvin, whereas t denotes the time during the season (in hours) with t_0 being the starting point of chilling accumulation. As a result of the self-limiting effect of the destruction of the intermediate product, this model starts accumulation

automatically.

4.4. The concept of threshold temperatures for chill accumulation

Several factors affect whether or not tree species will accumulate sufficient chilling to release dormancy (Table 4). Threshold temperatures are important because some temperatures contribute to the chilling requirement and others do not. High temperatures do not contribute to meeting chilling requirements and generally temperatures below a lower threshold (0 °C) are not considered effective for chilling. In some models, higher temperatures counteract the positive effects of chilling and negative chill units are applied when temperatures exceed a threshold (Richardson et al., 1974; Gilreath & Buchanan, 1981; Shaultout & Unrath, 1983). In most cases, the temperature weighting factors are based on laboratory studies where plants are exposed to the same temperature for long periods of time and the hours at a specific temperature until bud-burst are observed. Chill factors vary from zero, when the temperature does not contribute to meeting the chilling requirement, to 1.0 when the temperature is the most effective at meeting the requirement (Richardson et al., 1974). Then a chill factor, as a function of temperature, can be developed and used to weight chilling for temperature effectiveness at releasing dormancy.

Table 4. Summary of the major chill models with respect to temperature effects and chill unit accumulation (adapted from E. Luedeling, 2012)

Models and Authors	Basis of Measurement	Differences in temperature weights	Continuity of weights	Negation of chill by heat	Limitation to chill negation	Enhanced by moderate temperatures	Two-step chilling
Chilling Hours Model (Bennett 1949; Weinberger, 1950)	h	-	-	-	-	-	-
Utah Model (Chili Units; Richardson et al., 1974)	h	†	-	-	-	-	-
North Carolina Model (Shaltout and Unrath, (1983)	h	†	-	†	-	-	-
Anderson and Richardson , 1987	h	†	†	†	-	-	-
+ve Utah Model (LInsley-Noakes and Allan,.(1994)	h	†	-	†	†	-	-
Modified Utah Model (Linvill, 1990)	h	†	†	†	-	-	-
Dynamic model (Chill portions); Fishman *et al.,* 1987a; 1987b)	h	†	†	†	†	†	†
Other models applied on regional basis							
Chmielewski et al. (2011)	d	-	-	-	-	-	-
Legave et al. (2008)	d	±	±	-	-	-	-
Cesaraccio et al. (2004)	d	†	†	-	-	-	-

N.B. † (plus) indicates that the characters included in the model; - (minus) indicates these characters not included; ± means different characters exists, but, include few characters; h - hourly; d - monthly; weighting - refers to different temperature ranges; continuity for continuous weighting; chilling negation indicates the reversal of chilling; limit to chill negation means how much chill can be negated by moderate temperature; two step process for chill portions and its irreversibility.

4.5 Alternative Approaches to Classical and Dynamic Models

(i) The Mean Temperature Model

The mean temperature model is one of the alternative approaches for the area requiring low-chill under tropical conditions. The Mean Temperature Model uses mean winter (October to January and/or February) monthly temperatures to estimate accumulated chilling. The model also estimates the relationship between the mean monthly temperature of the coldest month(s) and total chill unit accumulation for specific location (Linvill, 1990 ; Shaultout & Unrath, 1983; Gilreath & Buchanan, 1981).

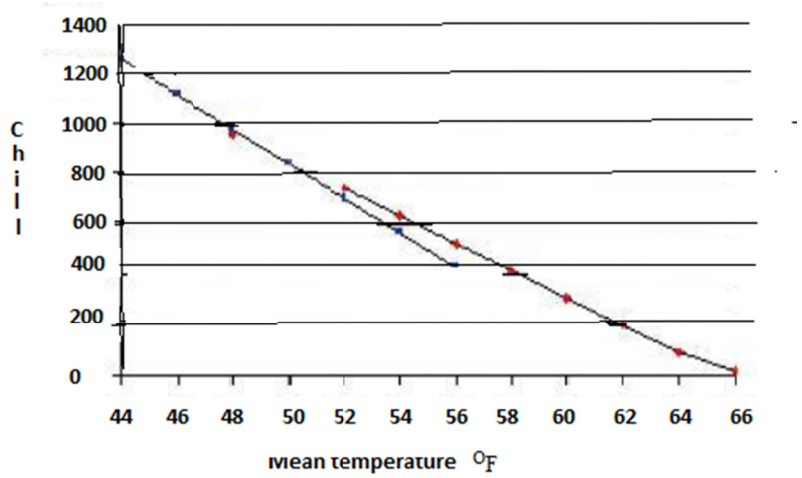

Figure 10. Total chill unit accumulation based on mean winter temperature (Linvill, 1990)

The accuracy and the simplicity of calculating chill accumulation with mean temperatures will aid fruit researchers and growers in several ways. Mean temperature data is routinely kept by cities and state climatologists and is usually easily accessible for tracking chill accumulation for a specific area over long periods of time (Shaultout & Unrath, 1983; Linvill, 1990). This will make it easy for a researcher, extension agent, or grower to more accurately match cultivars to a given locale.

(ii) Growing Degree Hour (GDH) Model

Another method useful for tropical and sub-tropical climate to estimate the chilling accumulation is the Growing Degree Hour (GDH) Model (Linkosalo, 2000; Hänninen, 1990; Cannell & Smith, 1983). The model uses degree day calculations to determine chill days (units for chilling) and anti-chill days (units for heating). The Chill days (Cd) represents a sequential accumulation of chill to break rest, while the anti-chill days (Ca) to overcoming the quiescence (i.e. occurrence of dormancy due to unfavorable environmental conditions) that contribute to chilling reversal. Rest is broken when the Cd curve falls to the chilling requirement (CR) and quiescence is overcome when the Ca curve reaches zero (Figure 11) (Linkosalo, 2000; Snyder et al., 1999; Zalom et al., 1983).

Chill days and anti-chill days are calculated from daily maximum (Tx) and minimum (Tn) temperature data and threshold temperature (Tc) Table 5. Chill days are calculated by first calculating the degree days above a 0 ^{O}C threshold temperature (Tc) and then the anti-chill days (or degree days above (Tc) are subtracted (Snyder et al., 1999; Zalom et al., 1983). The sign of the difference is changed to make Cd a negative number, and the negative (Cd) values are accumulated until they reach a pre-selected value that is identified as the chilling requirement (CR) (Figure 11 and Table 5).

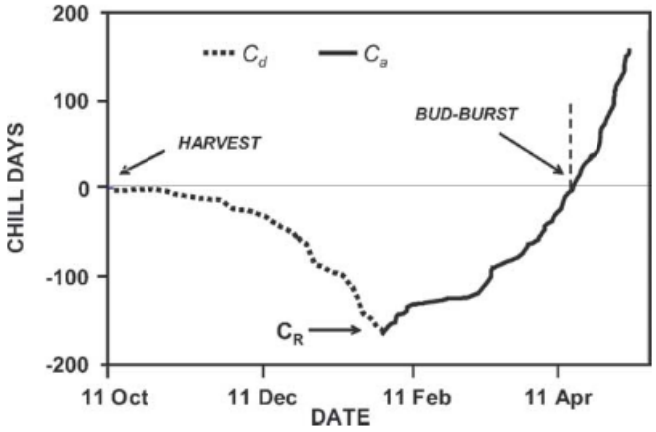

Figure 11. The single triangle degree day method showing the Chill days (Cd) and anti-chill days (Ca) accumulation from harvest, leaf fall, or fully fruit ripe to bud-burst (Zalom et al.,1983; Snyder et al., 1999)

Rest is broken when the Cd a curve falls to the chilling requirement (CR) and quiescence is overcome when the Ca curve reaches zero (Figure 11). The chilling requirement is met on the day when the $\sum$ Cd $\leq$ Cr which corresponds to breaking rest. On the following day, the model begins to add anti-chill days on each day starting at CR + $\sum$ Ca $\geq$ 0 at the predicted bud-burst (Linkosalo, 2000). The chill days and anti-chill days both depend on the selection of a temperature threshold (T_C) and CR so these parameters are iterated to find the combination that best predicts the bud-burst dates. Thus, the most important features of the Growing Degree Hour (GDH) model is a sequential dormancy model with an abrupt change from chilling to temperature forcing when the cumulative Cd reaches CR, that can potentially avoid chilling negation.

For calculating Cd and Ca, there are five possible cases, based on the single triangle degree day computation method, which depends on the relationship between Tx and Tn relative to T_C and 0 ^{O}C (Table 5) (Snyder et al., 1999; Zalom et al., 1983).

Table 5. Chill days (Cd) and anti-chill days (Ca) equations that relates the maximum (Tx) and minimum (T) temperature to the threshold temperatures TC and ^{O}C (Snyder et al., 1999; Zalom et al., 1983)

Temperature cases	Chill days	Anti-chill days
$0 \leq T_C \leq T_n \leq T_x$	$C_d = 0$	$C_a = T_M - T_C$
$0 \leq T_n \leq T_C < T_x$	$C_d = -\left[(T_M - T_n) - \left(\frac{(T_x - T_C)}{2}\right)\right]$	$C_a = \frac{T_x - T_C}{2}$
$0 \leq T_n \leq T_x \leq T_C$	$C_d = -(T_M - T_n)$	$C_a = 0$
$T_n < 0 < T_x \leq T_C$	$C_d = -\left(\frac{T_x}{T_x - T_n}\right)\left(\frac{T_x}{2}\right)$	$C_a = 0$
$T_n < 0 < T_C < T_x$	$C_d = -\left[\left(\frac{T_x}{T_x - T_n}\right)\left(\frac{T_x}{2}\right) - \left(\frac{T_x - T_C}{2}\right)\right]$	$C_a = \frac{T_x - T_C}{2}$

(iii) Exponential temperature response function

Lantin (1973, 1977) proposed an alternative approach for chill estimation based on an exponential cold action function, first described in Bidabe, (1963), summed over the period of time for which chill accumulation is being studied:

$$Af = Q_{10} - tm/10 + Q_{10} - tM/10 \ldots\ldots\ldots\ldots \text{(Equ 7)}$$

Where:

A*f* = daily chill units accumulated

tm, tM = minimum and maximum temperatures for day

Q10 = temperature coefficient (expressed as the ratio of the effect at T divided by the effect at (T-10).

Lantin (1977) selected a value of 1.4 as the one that gave the best fit to his data for a range of blackcurrant cultivars grown in UK. By raising daily max/min temperatures to a negative power function in this calculation, higher temperatures give rise to less chill-unit accumulation than do lower temperatures. The nonlinear response weights lower temperature periods, and especially extreme cold spells, even more than does the >7.2°C model. A problem with this model, however, is the fact that accumulation occurs even for temperatures well above the threshold for physiological chill effects, so that larger values of ΣAf may simply reflect longer periods of accumulation, rather than more chill. Alternative ways to use this principle could be to accumulate only daily values greater than 1.57 (equivalent to a threshold of 7.2°C), or possibly to express the chill units as a daily average over the period concerned. A second and equally important problem is that this model gives increasing weightings to lower temperatures with no lower threshold for action, even though much physiological evidence suggests that this is unrealistic.

4.6 Comparison of the Predictive Performance of Chill Models

Many studies revealed that multiple chill models are used by researchers with no consensus reached for a 'best' chill model. However, several studies have been conducted investigating chill model skill. For instance, Alburquerque et al. (2008) used the 0-7.2°C, Utah and Dynamic models to test chill model ability to predict flowering dates in seven sweet cherry varieties in Spain across several locations over two seasons. The Dynamic and Utah models were found to perform equally well but results from the 0-7.2°C model were poor in comparison. They concluded the use of the 0-7.2°C model for sweet cherry in their locations was no longer appropriate.

Viti et al. (2010) compared the skill of the Utah and Dynamic models in determining the chill requirement for apricot species in Spain and Tuscany. They found that the Dynamic model was less sensitive to temperature changes and was slightly more accurate that the Utah model. However, the author's highlight that improvement in accuracy in both models was needed. Perez et al. (2008) investigated the application of four chill models in two climatically different regions in Chile. The analysis over two seasons concluded that the 0-7.2°C model was ineffective at differentiating subtropical and temperate climates. Further, this model was not able to account for inadequate chill observations in Thompson Seedless grapes at the subtropical site. The Utah model was found to better distinguish the sites, with the Dynamic model best able to explain the regional differences.

Ruiz et al. (2007) tested the suitability of the 0-7.2°C, Utah and Dynamic models in predicting flowering in 10 apricot varieties over three years. The 0-7.2°C model was inconsistent, with the difference in recorded chill requirement between seasons as great as 30%. They found both the Utah and Dynamic models reported homogeneous chill requirements and found strong correlations between the two models. Ruiz et al. (2007) summarized that either the Utah or Dynamic model could be reliably used.

Zhang and Taylor (2011) conducted a 5 year study to estimate chill requirements of Sirora pistachio in Australia. They used the 0-7.2°C, Utah and Dynamic models to estimate chill requirements by forcing the cuttings grown in growth chambers. They found it difficult to determine a chill threshold using either the 0-7.2°C or the Utah model due to large variability in calculated chill thresholds between the seasons. The Dynamic model was found more consistent in determining threshold chilling requirement as reported in this study.

Many studies outline that the Dynamic model consistently performs similarly or better than the other chill models. The positive findings in favor of the Dynamic model are most likely due to the structure of the model. The model incorporates many observations of temperature effects on chill; including optimum chilling temperatures, and negation effects of high temperatures and the positive influence of moderate temperatures on chill accumulation. Further, the model is non-static in nature which would be expected to better reflect biological processes. The Modified Utah model similarly contains optimum chilling temperatures and negative influence of high temperatures. However, when using this model, chill that is accumulated early in the season can be negated some time later by late season high temperatures. The Positive Utah model is a derivative of the Utah model which does not include the negation aspects of high temperatures. It has been found to perform better than the original Utah model in mild locations: For example, in South Africa (Linsley-Noakes et al., 1994) and in California (Luedeling et al., 2009) in their study of walnut phenology.

The Dynamic model only considers the impact of high temperatures in influencing the production of an intermediate product, which is linked to time. Once a sufficient amount of the intermediate product is formed a chill portion is irreversibly created, and cannot be reversed by high temperatures later in the season.

The 0-7.2°C model is very simplistic and does not incorporate many of the observed effects of temperature on chill accumulation, such as the negative effect of high temperatures. The step-change structure of the model forces solid boundaries to chill accumulation, for example, 7.3°C will accumulate nil chill hours while 7.2°C will be allocated a full chill hour. Given the restricted knowledge on the chilling process this level of accuracy is unlikely to be defendable.

The 0-7.2°C model is dated and many studies have found it to perform poorly in predicting observed changes (Zhang & Taylor, 2011; Alburquerque et al., 2008; Perez et al., 2008; Ruiz et al., 2007). Nonetheless, this model has been extensively used in research and continues to be with a recent study in California using only this model to investigate future chilling conditions (Baldocchi & Wong, 2008).

Aron, (1975) compared the differences between chill models, using meteorological data recorded at HRI-East Malling research station, UK. The temperatures were extracted for the winter months over some 50 years and functions were applied to produce accumulated chill units for each of the chill models. Accordingly, <7.2°C,

0-7.2 °C and the Lantin models showed generally strong associations with the correlation coefficients between these variables being greater than 0.5. Only the Utah model was irregular and being poorly correlated with the other chill measures.

Aron (1975) also noted that the main limitation of the Utah Model resides in its specificity i.e. based on peach cultivars grown in North America. As a generic chill model it certainly conflicts with large amounts of literature that demonstrate, for example, that some species can accumulate chill below 0°C, whereas such temperatures in other species contribute negatively towards chill units (Aron, 1975). Effectively, the limitations of the Utah model varied with species, cultivar and environmental specificity. Although the Lantin, the <7.2°C and the 0-7.2 °C models all agree reasonably well and are all simple calculations that can be extracted from daily or hourly temperature data to give general assessments to the local chill conditions.

Luedeling and Brown (2010) compared chill models globally to confirm the differences among them across multiple climates using the 0-7.2°C, Utah and Dynamic models in their analysis. It was found that the chill models are not proportional and conversion factors could not be established. This global assessment was further confirmed by Darbyshire et al., (2011) in Australia who reported historical trends in chill accumulation using four different chill models.

They found that in Australian setting, trends differed in magnitude and/or direction between the chill models, with contradictory interpretation between chill models across locations. These suggesting further physiological research that is required to tailor chill models in terms of species specificity; Such tailoring is still needed for a given location where a more specific model is still required, particularly one that incorporates a more realistic model of the chill-physiology.

5. Effect of Lack of winter chilling on reproductive and vegetative development

5.1 Influence on Floral Bud and Flower Development / Reproductive Growth/

The symptoms of inadequate chilling are many, and vary with fruit species. Generally, they are recognized as a delay in flower and vegetative bud break (often described as 'delayed foliation'), which is frequently evident as an extended period of bud break (Jacobs et al., 2002; Cook & Jacobs, 2000; Couvillon, 1995; Jacobs et al., 1981). In pear *(Pyrus communis)*, autumnal warming has been shown to dramatically delay flowering time (Atkinson & Taylor, 1994; Atkinson & Lucas, 1996). This response is greater in earlier flowering cultivars than later ones. This implies that there may be two phenomena occurring, a delay in foliation and an increase in flowering irregularity. Extension of the bloom period may itself impact on the potential to crop; it may also cause large variations in crop development rate, fruit size and picking date.

The symptoms associated with lack of chilling also include the death of flower bud initials and the abscission of flowers prior to opening. Stone and pome fruit appear to generally differ in their response to lack of chilling. The stone fruit, which include almonds, apricots, peaches, plum and cherries, frequently abort entire flower buds. There are, however, studies that report anatomical abnormalities in flower buds of various apricot cultivars which could not be related to lack of chilling (Viti & Monteleone, 1991). On the other hand, pome fruits, which include apples and pears, may show dead flower clusters but vegetative buds can survive (Brown, 1952). Exposure to low temperatures has been shown to be necessary for the initiation of pistillate flowers (i.e. female and fruit bearing) in pecan (Amling & Amling, 1983).

Conversely, when chilling is inadequate, for example with peach, normal flowers are produced but they can lack stigmas and styles. Flowers of *Prunus avium* have been shown to be smaller in response to limited chilling (Mahmood et al., 2000a). Developing flowers may also fail to set fruit, and when fruits do set they may be of poor quality due to short pedicel length or insufficient leaf area development (Mahmood et al., 2000a).

Insufficiently chilled trees may not only show sparse bud break, but also maturing fruit and flowers on the same shoots. In a study carried out by Abbott (1962), trees subject to mild winters produced a larger number of flowers relative to trees exposed to average winter temperatures. However, these buds failed to break due to the sub-optimal chilling treatments so fruit bud number and yield were reduced. In years experiencing mild winters, Weinberger (1954) described "prolonged dormancy" in North American peach cultivars, characterized by irregular bud break and delayed asynchronous flowering, leading to a prolonged blossoming phase. Buds on older wood near the centre of the trees apparently had lower chill requirements, resulting in foliage clusters developing in the centre and large sections of bare stems on the newer, outer wood.

In a recent study of apple blossom development, cultivars subject to shallow dormancy conditions were observed to be highly apically dominant and deficient in reproductive spur density (Oukabli et al., 2003). At the differentiation stage the buds form vegetative buds rather than flower buds, resulting in poor flower indexes.

Closer anatomical investigations further showed that the flower tissues develop abnormalities that are carried on to anthesis, which is characterised by pistil abortion. Additionally, vascular disorganisation results in a failure in the establishment of the connection of the xylem vessel elements to the base of the flower buds.In raspberry, poor bud break is generally found in the lower cane, whereas the apices appear to have the lower chill requirements (White et al., 1999, 1998). Delayed bud break has been commonly observed in both raspberry and blackberry germplasm following mild winters in Californian coastal regions (Fear & Meyer, 1993).

Using the raspberry cultivar 'Autumn Bliss' as an experimental model, Carew et al., (2001) described an increase in vegetative growth and simultaneous decline in time to flowering that occurred with increasing chilling, either natural or artificial. The researchers pointed out the likely distinction between the two responses, providing experimental evidence to support the observation that cold treatment on flowering appeared to be a distinct vernalization effect. In blackcurrant, there are many symptoms associated with lack of winter chill. These include erratic or uneven bud break, leading to loss of yield and a reduction in fruit quality due to uneven ripening, delayed and protracted growth and flowering, a tendency for flower formation to precede leaf formation, and increased fruit 'run-off'.

Winter chill requirements varied with tree species and show diverse indications if it becomes inadequate in mild winter areas (Table 6). Its manifestations include a delay in vegetative bud-break and time of anthesis (Cook & Jacobs, 2000; Couvillon, 1995; Jacobs et al., 1981). For example, In *P. communis*, Atkinson and Lucas (1996) reported that autumnal warming delayed anthesis in early flowering cultivars than late flowering which showed two separate effects (i.e. a delay in bud break and an increase in irregularity of date of anthesis). They indicate that extended period in the time of anthesis causes variation in fruit development, fruit size, and harvest date and fruit quality. This was in conformity with the report of Mahmood et al. (2000) on *P. avium* flowers that inadequate chilling would resulted in failure of flower cluster to set fruit, and even when fruits do set, it may be of low quality due to short pedicel length or insufficient supportive leaf area for full fruit development.

Table 6. Summaries of the different aspects of perennial fruit crop growth, development, and production impacted by low winter chill (Atkinson et al., 2013)

Fruit trees	Aspects which are affected by low winter chilling									
	Vegetative bud break[a]	Floral bud break[a]	Bud abscission[b]	Flower abscission[c]	Flower quality[d]	Reproductive morphology[e]	Fruit set[f]	Vegetative growth[g]	Crop yield[h]	Product quality[i]
Apple	†	†		†	†			†	†	†
Pear				†		†			†	
Cherry			†		†	†	†			
Plum			†							
Peach		†	†		†	†		†		
Nectarine			†		†					
Apricots			†		†					
Almond			†					†		
Raspberry	†	†								
Blackberry	†									
Blackcurrant	†	†					†		†	†
Strawberry	†						†	†	†	†

[a] Delayed, erratic or uneven bud break (column 1 vegetative and column 2 floral); [b] Abscission of entire flower buds; [c] Abscission of single flowers within a cluster; d Reduction in flower quality; [e] Changes in reproductive morphology; [f] Reduction in fruit set or increased run-off; [g] Changes in vegetative growth, apical dominance, etc.; [h] Reduction in crop yield; and [i] Changes in crop/product quality.

In North American mild winter conditions, Weinberger (1954) stated that in *Prunus persica* cultivars ,prolonged dormancy due to lack of adequate chilling would result in irregular bud break and delayed anthesis, leading to poor fruit set and un proportional fruit development with inferior quality fruit. Accordingly, buds on older wood at the centre of the tree had lower chill requirements, resulting in a small clustering of flowers and large sections of stem devoid of flowers. Oukabli et al. (2003) also reported that when flower development in *Malus* x *domestica* cultivars subject to limited dormancy, it can not only have reduced floral buds but may also show high apically dominancy. Inadequate chilling of *Fragaria* x *ananassa* is known to result in a lack of plant vigour, reduced vegetative growth and yield (Craig & Brown, 1977; Voth & Bringhurst, 1970). Thus, plants with low vegetative vigour flower intensely and produce small fruit (Bringhurst & Galleta, 1990).

Generally, the reproductive performance of perennial trees are influenced not only by chilling temperatures based on the species requirement, but also, the post chilling cooler temperature in the area had a great impacts on vegetative growth, flower bud development, pollination, fruit setting and development. This was clearly indicated by a *Prunus avium* study, Mahmood et al. (2000) showed when chilling was limited (360 h at 4^{O}C), trees exposed to cooler post-chilling temperatures had greater fruit set. They also reported that, to achieve the same degree of fruit set as that at the lower post-chilling treatment, it required a much longer period of chilling especially in late flowering cultivars. Similar observation was made in Pyrus communis (Spiegel-Roy & Alston, 1979) showed that there was a strong relationship between chilling and the heat requirements for renewed growth and bud break. Climatic conditions during and around the time of anthesis influence crop yield by promoting or inhibiting the effective pollination periods , fertilization and fruit set, as well vegetative meristems and their growth (Williams, 1970).

5.1.1 Floral Bud Abscission and Flower Quality

In pome fruits, with their mixture of bud types, all or part of the primordial flower may abscise (Brown, 1952). This may leave flower bud clusters which are reduced in size, or they may open only as a leafy spur. Many cultivars of stone fruit grown in California are well known to shed flower buds following warm winters (Brown, 1958; Brooks & Philp, 1941). However, when these losses are small they can be beneficial because they induce a level of flower thinning which benefits subsequent fruit quality. The effect of mild winters on pears is typical of pome fruits, in that one or more of the embryonic flowers within the bud die. However, unlike the stone fruits, the entire shedding of the bud does not take place (Brown, 1952). Pear flower bud abscission has been shown to be highest shortly before bud swell and when higher temperatures are followed by sudden declines. There is also evidence of abnormalities being induced when meiosis had taken place at higher temperatures (Whelan et al., 1968).

Analysis of a wide range of different cultivars supports the notion of two distinct peaks of peach flower abscission, which can be correlated with maximum temperature values. It should also be noted that floral bud abscission and delayed bud break are more prevalent after warm winters, but bud abscission is not clearly correlated with relative chilling requirement (Brookes, 1942). Evidence with French prune and some peach cultivars shows that bud drop was less intense with cultivars with lower chilling requirements (Samish, 1954). A study carried out in California showed that in 1941 the winter orchard temperatures were higher than they had been at any time during the previous twelve winters (Brooks 1942; Brooks & Philp, 1941). The extensive descriptive study of 280 peach and 58 nectarine cultivars ranked them relative to the amount of flower bud drop. They found some 21 cultivars of peach that showed no flower bud abscission (class 1) and 78 that incurred heavy bud drop (class 4, only 0 to 15% of original numbers of flowers were left). The trees within their ‘class 2’ had sufficient flowers (50 to 85%) to enable full crops (optimal crop loads) to develop. About half the cultivars within the study lost no more flowers after the first recording date in mid-February (Brooks & Philp, 1941). In a similar survey carried out with apricots (119 cultivars) and plums (53 cultivars) after the mild winter of 1940-4 1, only one cultivar of each was found to show no bud drop (class 1). While 62 apricot and 27 plum cultivars were in the heavy bud drop, class 4, and showed correspondingly poor crops (Brooks, 1942).

The mild-winter temperatures in California in 1950-51 provided a further opportunity to describe the impacts of low chilling on fruit production. In the early 1950s, the winter of 1950- 51 was described as the ninth winter out of the past 22 where it was mild enough to cause “flower bud deterioration” (Brown, 1952). Comparing climatic records, however, showed that the winter of 1950-51 was not as mild as that in 1940-41.

Despite this, records with California almonds that have low chill requirements were not considered to be influenced by warm winters. Heavy bud drop of unopened almond flowers was apparent in early December. Apricots described in the same study where shown to be less negatively influenced than expected from previous knowledge of their vulnerability to sub-optimal chilling. The chilling received during 1950-51 was around 25%

less than the predicted requirement. Only one cultivar consistently showed severe flower abscission. There was not only site-to-site variation with data collected from the same cultivar, but also tree-to-tree and block-to-block variability. It was equally true with apples within the same geographical region, with their higher chill requirements, that they showed only an extended bloom period, but the set level was normal (Brown, 1952). Not all of these losses of flower bud can be easily attributed to mild winters; evidence exists that the decline peach flower bud quality and health can occur as early as mid-September (Brown, 1958). This implies factors other than winter chilling influence bud development.

Limitations in the amount of chilling have been shown to influence flower morphology. When the sweet cherry cultivar 'Stella' received insufficient chilling, flower size and pedicel lengths were dramatically reduced (Mahmood et al., 1999). Once a threshold value (30 days at 4 ^{o}C) had been reached there was no further change in flower size. The pattern shown with respect to flower size was very similar to that of fruit set, with chilling promoting set to an optimum after which no further increase was apparent. When the post chilling treatment was at a higher temperature, 25^{o}C compared to 19^{o}C, the amount of fruit set was much reduced and did not increase with further chilling (Mahmood et al., 1999). This has important implications for determining climate change responses where scenarios for reduced winter chilling may be linked to warmer forcing temperatures prior to bud break.

5.1.2 Fruit set and quality (size)

Observations made after a very mild winter in California in 1950-51 suggested that despite flowers of almond having aborted early in their development, subsequent cropping was, not however, limited by floral abscission but as a result of low fruit set (Brown, 1952).

Equally, it has been suggested that flower bud abscission rates in stone fruit as high as 70 to even 90% can be tolerated, providing the subsequent conditions for fruit set are favourable (Brown, 1952). Work with *Prunus avium* has shown that when plants were chilled at 4^{O}C for 360 hours, only very low levels of fruit set were achieved compared to those chilled for up to 1440 hours (Mahmood et al., 2000a). Recent work with the sweet cherry cultivar 'Stella' showed that extension of the chilling period to around 50 days (number of days at 4^{o}C) resulted in an increased number of flowers per tree. The ability of these flowers to set fruit also increased over this chilling period (Mahmood et al., 1999). Further chilling did not have any additional benefits to flower number or fruit set.

It has been suggested that forcing strawberries after short periods of dormancy can not only have an impact on the plant's vegetative growth habit, but it can also influence floral capabilities (Kronenberg & Wassenaar, 1972; Piringer & Scott, 1964). Although flower number per plant is not influenced by chilling or cold-storage temperature in cv. 'Elsanta', fruit set may well be (Tehranifar *et al.,* 1998). The interaction between vegetative vigour and fruit production suggests that fruit set is modified.

The smaller size of apples grown in regions where adequate chilling may vary has been suggested to be due to pre-anthesis differences in fruit cell number (Grebeye & Berg, 2000). The implications of this were apparent in the production of Royal Gala in the Western Cape, where small fruit size was a recurring issue compared with other fruit producing regions in South Africa. Examination of the number of cells within reproductive buds was related to winter chilling. The influence of winter chilling can be modified if cropping loads induce reduce or biennial bearing (Grebeye & Berg, 2000).

5.2 Vegetative Development

Responses can be carried over from one winter and expressed at the start of the second subsequent growing season. These symptoms include poor or late start to extension and lateral shoot growth and abnormally dominant apical shoot growth. Hoyle (1960) found that a period of 12-15 weeks at 2^{o}C was sufficient to satisfy the chilling requirement of dormant blackcurrant buds in Western Europe.

Accordingly, initial data from cultivar trials of blackcurrant in eastern England suggest that the presently available cultivars and breeding germplasm cover a wide range of chilling requirements and those cultivars can be placed in broad groupings according to the amount of chill units required for uneven bud break. Lantin (1973) suggested that differences exist between the chilling requirements of buds on the same blackcurrant plant, and that this can lead to uneven bud break, when the chilling requirement of some, but not all, buds is satisfied. Lantin (1973) also predicted that this unevenness within the bud break of a single plant was exacerbated by a high chilling requirement. Inadequate chilling of strawberry is known to result in lack of vigour, reduced vegetative growth (Voth & Bringhurst, 1970) and reduced yield (Craig & Brown, 1977). However, experiment to

induce early cropping dates of some cultivars suggests that reducing chilling exposure speeds up the initiation of fruiting in the spring (Gutteridge & Anderson, 1975).

In climatic regions, such as Kenya, where chilling is not possible and runner plants are required for plant propagation, applications of GA3 are used (Kahangi et al., 1992). Considerable development has taken place throughout the world over the last few years with the development of protected strawberry production. In France and the UK, for example, much of the cultivation occurs under plastic tunnels. It appears from work in France that, if tunnels are used too early during the winter, the inadequate chilling reduces plant vigour (Robert et al., 1997). Plants which show low vigour flower intensely and produce small fruit (Bringhurst & Galleta, 1990). It is apparent that chilling requirements differ with respect to bud type and position (Hauagge & Cummins, 1991). On vegetative shoots, the terminal bud can open prior to lateral buds, which induces strong apical dominance due to the prevention of lateral shoot growth. Branching in apple, for example, is mainly from distal buds (acrotinic) with little development of proximal buds (basitonic). This inhibition occurs via correlative influences (apical dominance) of the distal shoot (paradormancy) and can be reduced by phloem girdling (Champagnat, 1983). This suggests that developmental control is mediated via the transport of auxin (Faust et al., 1995). It is equally true that removal of distal portions of dormant shoot prior to forcing appears to remove paradormancy, allowing lateral buds to develop (Cook et al., 1998).

Application of cytokinins can also overcome the inhibition of these lateral buds from over-wintered shoots (Steffens & Stutte, 1989; Shaltout & Unrath, 1983a). The apical dominance response is well documented with peach (*Prunus persica),* which has terminal vegetative buds with the lowest chill requirements (Scalabrelli & Couvillon, 1986). This can have the effect of producing long shoots devoid of lateral side shoots capable of developing into fruit bearing spurs. Differences in bud type responses can also be detected around the point of adequate chilling when the temperature warms. Respiration rate appears to increase in relation to the switch to carbohydrate utilization and rates are higher in buds closer to the shoot apex than the shoot base (Young et al., 1995). This correlates with increased dormancy of lateral buds moving away from the apex. A quantitative study of the ontogenetic development of pear buds from a range of cultivars grown in South Africa revealed a relative high proportion reverting to a non-growing state (the 'latent phase') after being initially determined as within the growing phase (du Plooy et al., 2002). This developmental reversion has been recorded in apple, but at a much lower level (<2%) than evident with pear (Lauri et al., 1995). It is suggested that the increase in reversion (11 to 21%) was due to inadequate chilling and generally greater with the higher chill requirements of cultivars such as 'Beurre D'Anjou' and 'Golden Russet Bosc' (du Plooy et al., 2002).

6. Regional reports on winter chilling decline

Baldocchi and Wong (2008) reported that nut production in California was highly threatened by winter chill losses. They detected historic decline of winter chill for the majority of growing locations, using the chill hour model. Accordingly, the decline in winter chill ranges from 50 and 260 Chilling Hour per decade, and projected further losses in future at the rate of around 40 Chilling Hours per decade. This was further confirmed by Luedeling et al. (2009c) that the long term daily records from all weather stations in California revealed that changes in historic Safe Winter Chill by 2000 of up to -30%, compared to the 1950s baseline, according to the Chilling Hours Model. It indicates that in the future scenarios, losses were estimated at 30-60% by the middle of the 21st century, and up to 80% by the end of the century.

Luedeling et al. (2013) reported responses of three different tree species (chestnut, cherry and walnut) to temperature variation during the chilling period at different locations, using the Dynamic Model. The result (Table 7) revealed that Chestnut bloom in the cold-winter climate of Beijing was found to depend primarily on the rate of heat accumulation, while cherry bloom in the temperate climate of Germany showed dependence on both chill and heat accumulation rates. The timing of walnut leaf emergence in the mild-winter climate of California depended much more strongly on chill accumulation rates. Accordingly, spring phases of cherry in Klein-Altendorf (Germany) and especially chestnut in Beijing will likely continue to advance in response to global warming, while for walnut in California, inadequate chilling may cause delays in flowering and leaf emergence (Table 7). Such delays could serve as an early-warning indicator that future productivity may be threatened by climate change.

Table 7. Estimated start and end dates of the chill and heat accumulation periods of chestnuts *(Castanea spp.)*, cherry *(Prunus avium)* and walnut *(Juglans regia)* at different ecological zones (Luedeling et al., 2013)

Specific location	Chill period			Forcing period		
	Start	End	Requirement	Start	End	Requirement
Chestnut/ Beijing	23rd Sept	2nd Mar	79.8 ± 5.3 CP	5th Jan	11th May	13466 ± 1918 GDH
Cherry-Klein-Alterndorf	16th Sept	4th Mar	104 ± 8.9 CP	13th Feb	13th Apr	2698 ± 1183 GDH
Walnut/ Davis	25th Oct	2nd Jan	37.5 ± 5.0 CP	18th Jan	5th Apr	12245 ± 1697 GDH

Atkinson et al. (2013) reported that the amount of winter chill occurring in UK has declined and is predicted to continue to do so, as described in the UK Climate Assessment Program. This happens because of the projected climate change scenarios that are always in dynamism. They evaluated long term climatic data and linked it with the key seasonal reproductive events to describe the symptoms of lack of winter chill; these include vegetative growth and development that affects bud break, flowering and fruit setting potential of tree species. Also, the decline in chilling affects the developmental and physiological events which mainly linked with flower initiation, anthesis, dormancy and bud break.

Atkinson et al. (2013) also indicates that there is a serious lack of phenological modeling and mechanistic understanding of the physiological, molecular and genetic basis of winter chill requirement and dormancy-related environmental factors which affect tree growth and yield. This indicates future reductions in winter chill that require recognition as a potential limiting factor on fruit production in many European countries, especially in the southern parts where winter chill reduces. Possible strategies suggested for long term solutions to secure perennial fruit industries in Europe includes exploiting genotypic variability within several perennial crops, through plant breeding to develop low chill-cultivars, together with opportunities to change crop management practices and growing systems to tolerate the decline in chilling. At present a lot of evidences indicate that there is a measured decline in winter chill in UK and in other parts of the world (Baldocchi & Wong, 2008; Luedeling et al., 2009, 2011; Darbyshire et al., 2011) hypothesized that some of the recent reductions of winter chill in UK resulted in low yields of perennials crops as recorded by scientists and growers. Sunley et al. (2006) compared a range of existing chill accumulation models (data not shown) to explain the differences in date of anthesis of different fruit trees over a 50 year period. They also accessed several long-term UK geographically dispersed data sets on air temperatures and date of anthesis. Accordingly, all the chill models used to study chill accumulation show a statistically significant decline in winter chill over the last three decades.

Hennessy and Clayton-Greene (1995) used the Modified Utah Model for quantifying winter chill in Australia. The study revealed that that warm sites, and sites with wide diurnal temperature ranges, were more strongly affected by chilling decline than cooler sites with more similar temperature records. Regarding climate change scenarios, Hennessy and Clayton-Greene stated chilling declines for all sites, and for the stronger warming scenarios, they suggested that these losses have a greater impact on fruit industry. Thus, the situation requires different orchard management skills such as the use of artificial dormancy breaking, developing low chill requiring cultivars and other routine orchard management practices that can manipulate the microclimate of the area.

Darbyshire et al. (2011) in Australia evaluated four winter chill models; namely the Chilling Hours Model, the Modified Utah Model, the Positive Utah Model and the Dynamic Model for 13 locations by considering historic winter chill trends of the country from (1911-2009). Accordingly, for all 13 locations the result differs substantially and indicating that there was a limitation for all models in accuracy of estimating the chilling trends mainly for warmest locations (Figure 10). They found that the models rank the locations differently in terms of mean chill (1911-2009), with the 0-7.2°C model showing the greatest deviation (Figure 10 and Table 8). This study clearly indicates that chill model choice is important that it affects conclusions and the models are notably different when applied to multiple climates.

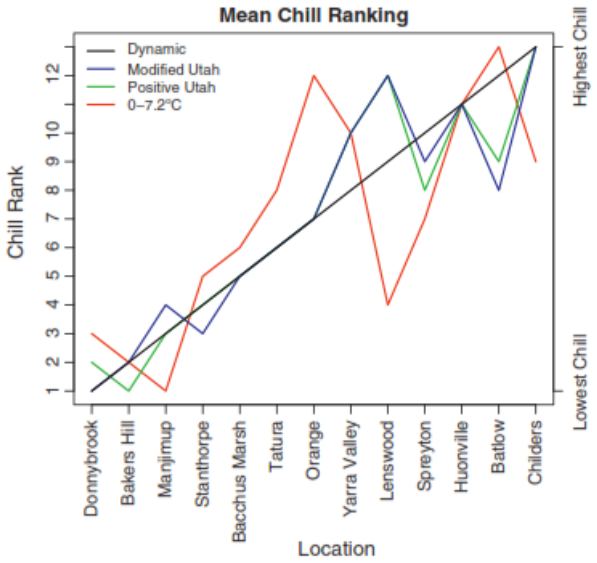

Figure 10. Chill model ranking of the 13 Australian locations from lowest to highest chill, relative to the dynamic model ordering (Darbyshire et al., 2011)

Although, the declining winter chill trends was inconsistent for almost all locations (Darbyshire et al., 2011), which varied strongly according to the model chosen (Table 8), through comparing trends at each location especially recent trends, location sensitivity to climate changes have already occurred, but many of the trends were not found to be statistically significant (Table 8), with the data showing high season-to season variability at all locations, regardless of model choice.

Table 8. Mean chill and standard deviation (in brackets) at each location for the period of 1911 – 2009 (Darbyshire et al., 2011)

	Dynamic (CP)	Modified Utah (CU)	Positive Utah (PCU)	0 -7.2 ^{O}C (CH)
Donnybrook	57 (6)	929(146)	1285(104)	576(115)
Bakers Hill	58 (7)	1011(172)	1274(128)	522(127)
Manjimup	67(6)	1247(179)	1369(134)	489(117)
Stanthorpe	72(5)	1235(141)	1443(101)	937(107)
Bacchus Marsh	78(3)	1525(94)	1690(64)	988(89)
Tatura	79(4)	1585(124)	1731(92)	1068(95)
Orange	85(3)	1671(142)	1801(137)	1395(124)
Yarra Valley	86(3)	1843(94)	1916(81)	1139(102)
Lenswood	86(4)	1978(138)	1956(117)	841(134)
Spreyton	87(3)	1840(83)	1837(78)	1016(108)
Huonville	88(2)	1882(133)	1948(81)	1377(108)
Batlow	89(2)	1785(131)	1914(144)	1624(137)
Childers	89(2)	2032(86)	2047(77)	1130(115)

Guo et al. (2013) studied the chilling and heat requirements for flowering time in two temperate fruit trees (chestnut and jujube) in Beijing, China, with daily chill and heat accumulation between 1963 and 2008. The data collected over the past fifty years was subjected to the Dynamic Model and the Growing Degree Hour Model to convert daily records of minimum and maximum temperature into physiologically meaningful metrics. The result revealed that (Table 9) over the past 50 years, heat accumulation during tree dormancy increased

significantly, while chill accumulation remained relatively stable for both species. Accordingly, heat accumulation was the main driver of bloom timing, with effects of variation in chill accumulation.

Table 9. Chilling and heat requirements estimation of Chinese chestnut *(Castanea mollissima)* and jujube *(Ziziphus jujube)* at Beijing, using the Dynamic and Growing Degree Hour (GDH) Models

Species	Chill period			Forcing period		
	Start	End	Requirement	Start	End	Requirement
Chestnut	14 Sept.	24 Mar	93±6 CP	4 Jan	23 May	17,481±1,983 GDH
Jujube	17 Sept.	19 Mar	89± 6 CP	9 Jan	13 May	13, 619±2,033 GDH

Observations on the phenology of two local peach genotypes (early and late) maturing were also conducted in Tanzania, using Utah, Dynamic and Mean Temperature models in 2010 and 2011 (Scalisi et al., 2014). The result (Table 10) showed that both Utah and Mean temperature models indicated low chilling accumulation and inconsistent with the actual phenology, suggesting that both models show some limitations under East and Central African highland conditions. This is mainly influenced by the climatic variables such as seasonal rain fall that play a role in temperature fluctuation that contribute positively or negatively for chilling accumulation. Also, chilling estimation by the Dynamic model at this location yielded a relatively low amount of Chill Portions (CP), which was not associated to CU obtained with the other two models (Table 10)

Table 10. Accumulation of Chilling Units According to the Utah and the Mean Temperature Models at Pomerini Tanzania

Year	Utah[Z]	Mean temperature[Z]	Dynamic[Y]
2001	105	303	2.02
2002	36	322	3.02
2003	118	318	3.02
2004	36	367	4.01
2005	-	-	-
2006	143	367	6.05
2007	91	472	6.04
2008	83	456	11.05
2009	131	410	4.03
2010	116	518	4.03
2011	121	462	5.05
Median	110.6	388.6	4.03
75th Percentile	85.2	333.4	3.27

Z = Chill Units (CU)

Y = Chill Portions (CP) ; 1 CP = aprx. 20 CU

This study showed that insufficient chilling is a major limiting factor in peach adaptation at the uplands of Tanzania. To further develop peach cultivation in the area, research should focus on selection of low chill cultivars and adopting the use of artificial rest breaking agents (Scalisi et al., 2014).

Luedeling et al. (2009) reported the effects of climate change to cause winter chill decline in Arabian Peninsula as they indicated in their case studies of four mountain oases of Oman. They used high-resolution record from Saiq to calibrate the long-term record of daily temperatures, especially the daily mean temperature records (Table 11). Also, in some places where the high-resolution measurements was absent, they used the mean daily

temperatures (0 – 7°C) on hour basis and/ or they are based on point measurements at a certain time of day to calculate the mean between minimum and maximum temperatures.

Table 11. Trends in monthly mean of daily minimum and maximum temperatures observed in Sayq (Oman), between 1979 and 2008, adjusted coefficient of determination (R^2) of the linear regression.

Month	Minimum temperature			Maximum temperature		
	Slope (°C a^{-1})	R^2 adjusted	P value	Slope (°C a^{-1})	R^2 adjusted	P value
January	+0.07	0.13	0.04[a]	+0.02	-0.03	0.58
February	+0.09	0.24	0.01[a]	+0.08	0.17	0.03[a]
March	+0.02	-0.02	0.50	+0.03	-0.01	0.40
April	+0.07	0.08	0.09	+0.08	0.07	0.11
May	+0.04	-0.01	0.39	+0.05	0.07	0.12
June	+0.03	-0.02	0.50	-0.01	-0.04	0.80
July	+0.05	0.17	0.03[a]	+0.03	0.02	0.25
August	+0.04	0.09	0.09	+0.07	0.22	0.01[a]
September	+0.05	0.07	0.11	+0.03	0.03	0.21
October	+0.05	0.10	0.08	+0.04	0.05	0.17
November	+0.09	0.30	0.00[a]	+0.02	-0.03	0.52
December	+0.05	0.02	0.24	-0.03	-0.01	0.42

N.B. Trends is statistically significant at $P \leq 0.05$.

Also, a historic temperature record was made between 1983 and 2008, as well as for three sets of 100-year synthetic weather records generated to represent historic conditions. A variety of temperate fruit tree crops requiring low chilling temperatures were considered in this study. These include pomegranates *(Punica granatum* L.), peaches *(Prunus persica* L.), apricots *(Prunus armeniaca* L.), walnuts *(Juglans regia* L.), and even apples *(Malus domestica* Borkh.), pears *(Pyrus communis* L.) and plums *(Prunus domestica* L.), all of which have minimum chilling requirements (Low chill) between 100 and 400 h, and unable to be cultivated in the lowlands.

The result indicates that climatic changes likely to occur within the next 30 years (temperatures elevated by 1°C and 2°C). Accordingly, in the studied areas of Northern Oman, a decrease in the numbers of chilling hours in high-elevation oases by an average of 1.2-9.5 h/year between 1983 and 2008, during this period winter chill was sufficient for most the species grown in the oasis according to the scenario analysis. For temperate fruit trees grown in oasis, a place where marginalized winter chilling, production might become impossible in the near future.

7. Adaptation Strategy Perspectives

Orchard establishment is expensive, time taking and require a lot of care and attention throughout the orchard existence. This requires special attention of scientists and growers in understanding climatic elements in a given location for successful orchard. It appears that cultivar placement in a locality must be based on the chilling requirement (Low, medium or high) as was required by the species. Accordingly, careful selection of cultivars to the particular climatic condition to meet the chilling demands against climatic scenarios is the most useful tools in areas where high temperature fluctuations happen irregularly. Inadequate chilling requirements in tropical climates forced the growers to forcedly defoliate trees after harvest to artificially induce dormancy (Edwards, 1987; Griesbach, 2007). If this is practiced, trees appear to be able to resume their annual cycle without requiring chill. This management has enabled the production of temperate fruits in the tropics and sub-tropics where the chilling requirement is incomplete, but it cannot be recommended at colder regions with pronounced seasonal cycles.

Molecular technique is another important approach in the modern breeding program to develop adaptable cultivars to a particular situation with special focus on chilling requirements. In respect of this, the past experience indicates that many low chill temperate fruit cultivars are developed for warmer regions and the present situation of climate change demands similar patterns of technology generation. Also, the use of rest breaking chemicals or growth regulator has been found to promote bud-break where the chilling requirement is insufficient especially in tropics and sub-tropical regions. For example, application of hydrogen cyanamide spray has been effective in promoting bloom in Ethiopia (Ashebir et al., 2010), Israel (Erez et al., 2008), Tunisia (Chabchoub et al., 2010), Southern United States (Dozier *et al.,* 1990) and Italy (de Salvador and di Tommaso, 2003). However, this chemical has shown phytotoxic and to cause strong yield reductions (George et al., 1992; Siller-Cepeda et al., 1992), that it has been banned in several countries. Alternative chemicals, such as plant growth regulators containing thidiazuron (Campoy et al., 2010) have a positive effect in rest breaking and less toxicity for human health as suggested by many reports.

8. Summary and Conclusion

Given the present knowledge, and the lack of conclusive evidence for any particular theory of dormancy regulation, chilling requirements and chill models used to estimate chill accumulation, it is certainly not possible to propose a general correspondence between all these phases due to diverse environmental factors that seriously influence the processes and the endogenous mechanisms of control. It is possible that observations of empirical behavior will allow one to decide between likely proposals. That is, any promising candidate among models must at least be amenable to such tests. These situations always demand exhaustive work to reconcile environmental influences across geographical regions with respect to the changing climate. Studies indicated that major chilling losses in all warm growing regions of temperate fruits, both in the past, at present and in the future. In particular the warmest growing regions, in North Africa, South Africa, the Southern United States, Northern Mexico, Southern China and Southern Australia are projected to suffer substantial losses in winter chill at present. Cold growing regions, in contrast, may experience little change, or even increases in winter chill, as increasing numbers of days become frost-free.

Many authors also indicated that cold growing regions may experience increases in winter chill. This is likely due to a geographic bias among published case studies, which have focused on growing regions where chilling is considered an important factor in temperate fruit trees. This is the case predominantly in warm growing regions, while growers in colder locations have traditionally paid little attention to winter chill. As stated in this reviews from many case studies, it clearly emerges that the Chilling Hours Model consistently detected the strongest changes in winter chill, while in particular the Dynamic Model was more moderate in the amount of change it projected. In light of the studies that have shown the Dynamic Model to be more accurate, in particular in mild winter climates than other approaches. A lot of experimentation is still needed to come to a consensus of which approach to modeling winter chill is appropriate. Until this experimental gap is closed, it appears that the Dynamic Model is preferable among the existing approaches, and it would be advantageous to determine chilling requirements in Chill Portions for many more cultivars than have been characterized to date.

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Contribution to the Knowledge of Plants Used by Bantu and Pygmy Healers in Beni and Lubero Territories (Democratic Republic of Congo)

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Abstract

Traditional medicine is largely used in Benin and Lubero Territories (D R Congo) as costs for conventional drugs increase and become unaffordable to many people, particularly living in rural areas. The aim of this work is to unlock convergences of plant species used in Bantu and Pygmy herbal medicine. An ethno botanical survey was conducted from 2010 to 2012 in Beni and Lubero Territories. Thirty- six healers, well known in villages where Nande Bantus and Mbuti Pygmy live together were interviewed concerning the plant species used in traditional medicine. One hundred and ninety seven recipes from 182 plant species were identified in Bantu ethnic group and 78 recipes from 83 plant species in pygmy ethnic group. Decoction and Carbonization have been the main modes of preparation by Bantus healers, whereas pygmy healers have mostly used triturating. Ruderal plant species were mostly used among which the Asteraceae family is preponderant (23%). Frequencies comparison with Khi squared method has shown that Bantu herbal medicine is different from pygmies' traditional medicine.

Key words: medicinal plants, healers, bantus, pygmies, beni, lubero

1. Introduction

The picking-up of medicinal and ''magic'' plants as well as in Africa and elsewhere in the world has always been considered as very important and determinant. Seeing that the traditional medicine takes in consideration the human being in its somatic and extra-material integrity, the operation of picking-up medical matters and the making of medicines is always accompanied with mysteries and rites (Amakoué, 1995; Léonardt et al., 2006).

According to Balagizi et al. (2007), developing countries are conscientious that their health systems are based on imported technologies and modern medicines, which are costly. If this state of dependence persists, the health expenses will increase and will affect the state's finances. It could be better to promote medicine by the exploitation and rational use of local knowledge found in natural local resources, particularly medicinal plants.

The WHO (2003) gives 53000 to 72000 the estimation of species of medicinal plants used through the World by different types of traditional medicine; several of the plants have medicinal properties. The same organization estimates up to 80%, the African population that uses traditional pharmacopoeia for their health care.

The DRC has at least fifty thousand vegetative species among which 10% have medicinal properties (Hans, 2006). Despite this enormous and rich flora, the vegetation of DRC is among the less known in Central Africa (CIFOR, 2007 in Ilumbe, 2010).

The use of traditional medicine to treat recurrent diseases is an indigenous practice in Africa (Hans, 2006). In fact, according to the World Health Organization (HWO, 1996) estimation of ratio noticed more than 2000 and 4000 of inhabitants per Tradi-practitioner in Uganda country. Whereas the statistics of the public health Ministry

of Burkina Faso estimates this ratio to 1/500 in Burkina Faso (Zerbo et al., 2008). In D R Congo, two third of patients do not refer their cares need to the modern system, either because they have not financial means to get access to it, or because the health services are not available. For these, medicinal plants are the key product for Congolese population. Both urban and rural populations depend on medicinal plants for their health care deed (RD Congo, 2006; Ngbolua et al., 2011). This is the case for Beni and Lubero territories.

Despite the abusive use of traditional medicine does not only advantages, but also intoxication and adulteration remains the major problems in the therapeutic management of patients. Brigham and Cocksedge (2004) attach many cases of bad identification, of adulteration of health products used wildly on belief that Tradi practitioners or healers bring to the practice of cares by plants and to the orality, which does not allow a good transfer of knowledge of generation-to-generation.

In order to frame our study, we have considered in this study the definition of Rwangabo (1993) who describes a tradi-practitioner or traditional healer as a person who is recognized by the chieftaincy in which one lives, as being competent to give health cares thanks to the employment of vegetative animal or mineral substances. In addition, he knows other methods also based on the socio- cultural religious basis than on the knowledge of mental and social behaviors as well as etiology of diseases prevalent in the collectivity.

Very often, healers use the same recipes to treat the same diseases, whereas each has a specialty for which the other members of the community come to consult. The aim of the present study is to check affinities concerning plant use and recipes in traditional medicine of ''Nande''Bantus and ''Mbuti''Pygmies healers in Beni and Lubero territories. We started from the hypothesis that the Bantus' herbal medicine could be different from the Pygmy's' specialized medicine.

2. **Study Area**

The ethnobotanical investigations were conducted in Beni and Lubero Territories. This area is located in the Northern part of Democratic Republic of the Congo. Lubero borders Edward Lake and is located between 28° and 30° longitude east, and 0°34 and 0°30' latitude south. Beni is located between 29° and 30° longitude east, 0°30' latitude South and 1° latitude north (Kasay, 1988)

The two territories cover an area of 25,580 sq Km, from which 18,096 sq Km for Lubero and 7484 sq Km for Beni (Kujirakwija, 2006). The population is irregularly divided in the two territories, at one side, population density is high in the mountain zone of Lubero territory , sometimes it can reach 105 inhabitants/ sq Km(Mafikiri, 1994), on the other side, very low density in Beni area .

Beni and Lubero are located in a humid tropical climate with temperature situated between 24 to 25°C for the low lands which contain a large rain forest (2183 mm), whereas in the high lands where the average altitude is situated between 2000 and 3100m. The temperature is regulated by the equator line and altitude (15 to 17 °C) with precipitations, which varies to 1110, and 1330 mm (Vyakuno, 2007).

According to Mafikiri (1994), the ''Nande'' people is the main ethnic group in the two territories, and it is mixed to other peaples''Piri, Pakombe, Batalinga, Walese and Mbuti "Pygmies".

3. Materials and Method

The ethnobotanical investigations were conducted in Beni and Lubero territories from 2010 to 2012 in six villages where Bantus and Pygmies live together. The criteria for choosing the surveyed villages were; the distance which separates two villages (at least 80 Km), the presence of ''Nande'' Bantus and'' Mbuti'' Pygmies in the same village.

The method used is essentially based on asking questions directly to respondents, using a semi-structured questionnaire previously established according to the guide of the database of traditional Medicine and Pharmacopeia "PHARMEL 2" (Adjanohoun et al., 1994). Thirty- six healers from the two ethnic groups were interviewed. The collected data during the ethno-botanical surveys were focused on the identification of traditional healers (his ethnic group and specialty), the used plants (Vernacular and Scientific name, used parts), and the characteristics of recipes (methods of preparation, administration and pharmaceutics form) the indications on diseases and symptom (Kasali et al., 2014).

The specialists, like Professor Kamabu of Kisangani University, identified most of collected plants. Several documents have facilitated the correction and adaptation of specific names (Troupin & Bridso, 1982; Schnell, 1979; Lejoly et al., 1988; Tailfer, 1989)

The species that were not identified have been deposed and compared to the specimens of Herbarium of the Faculty of Sciences at Kisangani University.

The convergences of employment of recipes were foreseen in two ways: by comparison of lists of treated diseases and the characteristics of medicinal recipes used by the two communities, on the one part. The Jaccard index, which measures the report of double presences "a'' divided on the sum of b, c, and a, were calculated using the following formula:

$$Cj= \frac{a}{a+b+c}$$

In this formula, (a) represents the number of species or recipes commonly used by the two communities, (b), the number of species or recipes proper to Bantus healers and (c) the number of species or recipes proper to Pygmies healers). On the other part in the most analytical manner, the information citing the same plant or recipe for the same disease by members of two ethnic groups was been considered as convergence of employment. The treated data being binary and any environnemental variable was not been measured; we have referred to a method of non-parametric classification because any estimation of parameter is not necessary. The Chi-squared test which globally allows to see the links between two characters (ethnic groups- mode of preparation of recipe for example), and to compare the observed proportions to a theoretical value fixed by the method permitting to test the hypothesis. We have then referred to Chi squared of Wallis and Freidman (Cornillon et al., 2008; Husson et al., 2009). All data were analyzed using R 2.10.0 software; the cluster analysis using the Euclidian distance and the correspondence analysis were expressed to perform affinities for plants and recipes using in traditional medicine of the two ethnic groups.

4. Results

4.1 Number of Species

The results of the number of species used in traditional medicine of Bantu and Pygmy Tradi-practitioners living in the same village are summarized in Table 1.

Table 1. Comparison of list number of plant species used by Bantu and Pygmy healers of six surveyed villages in Beni and Lubero territories

Village	Number of species			
	Bantu	Pygmy	Common	Cj
Isigo	36	17	1	1.85
Kima	39	22	8	11.59
Tandandale	18	9	1	3.57
Kalibo	29	10	2	4.87
Kathundula	27	11	2	5
Maakengu	33	14	1	2.08

The coefficients of similarity drawn from the recipe following Jaccard method were inferior to 50%. This difference shows that there is not yet significant exchange between these two groups, which are claimed to possess each the secret of medicinal virtues of plants. The reticence of Tradi-practitioners of the two ethnic groups brings to a loss of much information. These ones have used medicinal plants as a profitable activity, attracting more and more people in villages for the training in the use of plants for traditional medicine.

4.2 Number of Recipes at the Level of the Same Village

Bantu and Pygmy Tradi-practitioners use several recipes in specialized traditional medicine. In general, 197 and 78 recipes have been listed respectively at the Bantu and Pygmy Tradi-practitioners; they are shared in disproportional manner trough the village as shown in Table 2.

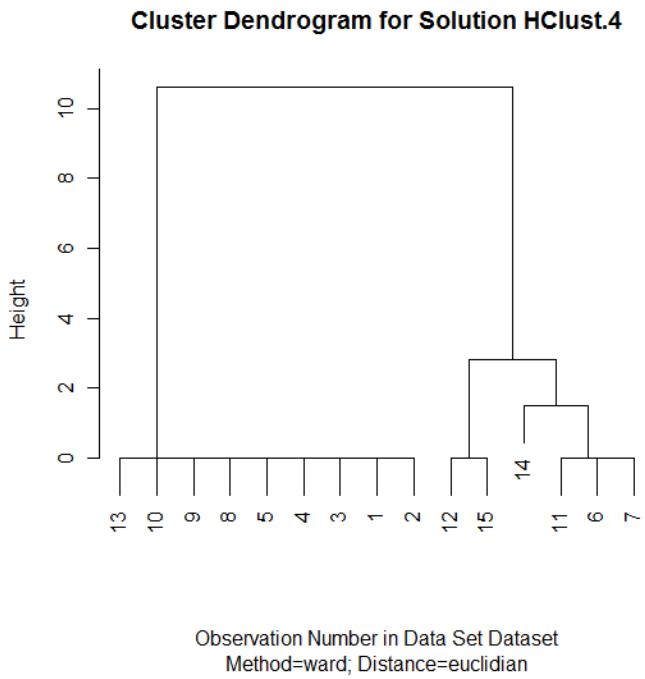
Table 2. Comparison of recipes used in traditional medicine of Bantu and pygmy healers of six surveyed villages.

Village	Number of recipes	
	Bantu	Pygmy
Isigo	78	18
Kima	36	23
Maakengu	20	11
Tandandale	15	8
Kalibo	16	8
Kathundula	31	10
Total	196	78

Bantu Tradi-practitioners have acquired much knowledge in the use of medicinal plants thanks to many exchanges during the trainings organized by associations or NGOs that are willing to promote the traditional medicine in several areas of the North Kivu Province. Thus, the number of recipes is more diversified in Bantu areas where people know how to read and write. By this, they are capable of acquiring experiences of other regions of Africa and elsewhere. Pygmies, however, refer to recipes known for years and transmit them from generation to generation without considerable addition.

In order to make it more perceptible, the affinities, which could exist, between Tradi-practitioners of the two ethnic groups, the cluster analysis model of hierarchical classification in simple link based on the recipes used by the two ethnic groups through the surveyed villages was done. We listed the illnesses according their category and based our analysis to the recurrent illnesses treated by at least 20 % of Tradi-practitioners. Among these diseases, which were evaluated to 14, three affections that the charge taking is remarkable in the two ethnic groups such as Hemorrhoid, Malaria and sexual impotence have been submitted to the cluster analysis.

4.2.1 Classification Based on "Recipes Used against Hemorrhoid- Bantu and Pygmy healers-Villages Investigated"



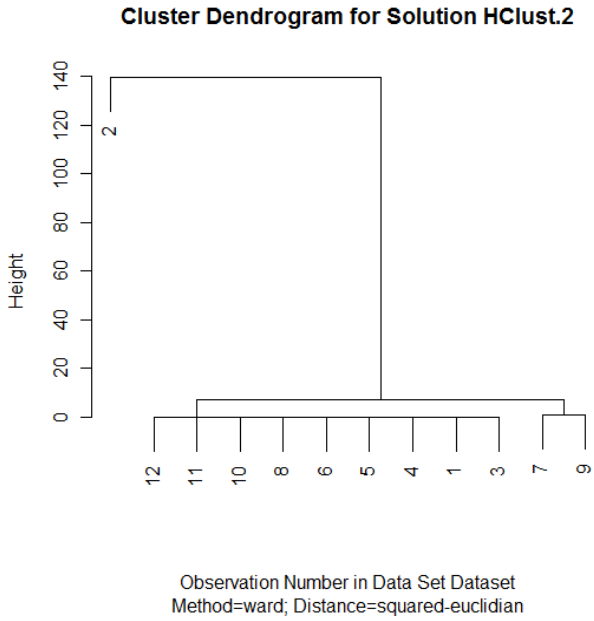
Legend: Ban= Bantus ; Pyg= Pygmées ; Isi= Village Isigo ; Kim= Village Kima ; Kat= Village Katundula ; Ma= Village Maakengu ; Ta= Village Tandandale ; Ka= Village Kalibo; 1= Decoction-Mangifera indica-BanIs, 2 =Carbonisation-Arachis hypogeae-Ricinus communis-BanIs, 3= Maceration –Phytolacca dodecandra-BanIs, 4=Pilage-*Chenopodium*-BanIs, 5= Pilage-*Canarium schweinfurthii*-Palm oil-*Tetradenia ruparia*-BanIs, 6=Pilage-*Ficus exasperata-Piper nigrum-Sorghum-Tetradenia ruparia*- BanIs, 7=Decoction-*Spathodea campanulata-Eucalyptus globolus-Harungana madagascariensis-Canarium schweinfurthii*-BanKim, 8= Preparation-*Piper capense*-BanKim, 9=Infusion-*Mangifera indica-Piper capense*- BanKim, 10= Trituration- *Artemisia annua-Ocimum gratissimum*-PygKim, 11= Decoction-*Paper capense*-BanMa, 12= Triturating-*Khaya anthotheca*-BanTa, 13= Carbonisation-*Zea mays-Ficus sp-Citrus limon-Ricinus communis*-PygTa, 14= Carbonization-*Mangifera indica*-BanKa, 15= Thinning-*Piper capense*-PygKa

Figure 1. Classification of Bantu and Pygmy specialists according to the recipes used against Hemorrhoid in Beni and Lubero Territories

At the low level of cluster, or at 100% of similarity, ten classes among the 13 repertoriated are visible. Few of convergences of recipes use against Haemorrhoids are visible at this level and constitute only 20% of found groups, whereas the classes exclusively Bantus predominate in reason of 80 % the individualized classes. This has brought us to conclude that the Haemorrhoids are more treated by the Bantu tradi-practitioners. The two cases of inter-ethnic similarity fixed at the lowest level concern the triturating of leaves of *Artimisia annua* and *Ocimum gratissimum* on the one hand and the mixture of *Piper capense* on the other hand.

At 97% of similarity, three classes of similarity exclusively formed by Bantous are visible, these concern; The carbonization of leaves of *Mangifera indica*, the decoction of leaves of *Piper nigrum*- grains of Sorghum- leaves of *Tetradenia ruparia* and then the decoction of skins of *Spathodea campanulata*- leaves of Eucalyptus globolus- skins of *Harungana madagascariensis* and *Canarium schweinfurthii*. At 85% of similarity, two classes are visible, one class exclusively Bantu and a mixed class. The observation of cluster shows a little representation of interclass's, consequently the tradi-practitioners of the two ethnic groups have different recipes to calm Hemorrhoids through the surveyed villages.

4.2.2. Classification Based on "Recipes Used against Sexual Impotence-Bantu and Pygmy Healers- Villages Investigated"

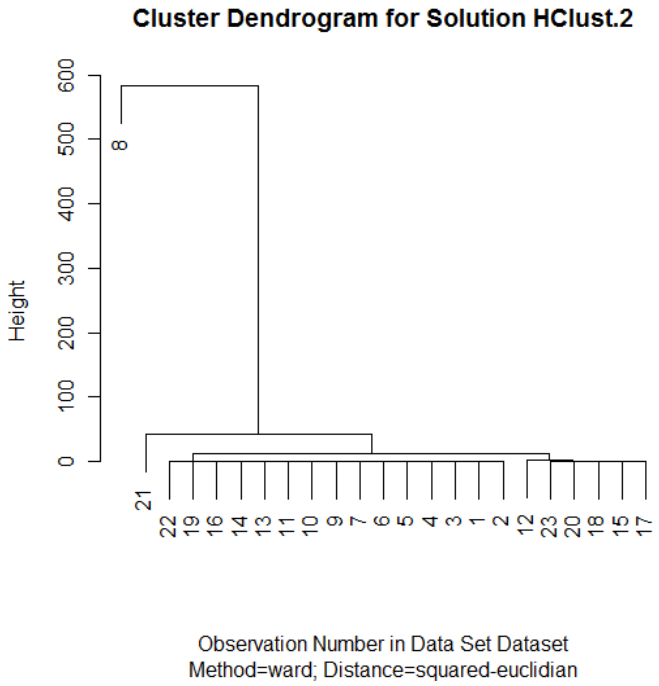


Legend: Ban= Bantus ; Pyg= Pygmées ; Isi= Village Isigo ; Kim= Village Kima ; Kat= Village Katundula ; Ma= Village Maakengu ; Ta= Village Tandandale ; Ka= Village Kalibo , 1= Maceration-*Uapaca guineensis-Cymbopogon citratus- Acacia sieberana-Piper capense-Citrus limon-Rubus rigidus*-BanIs, 2=Carbonisation-*Uapaca guineensis-Anisophelea sp-Acacia sieberana-Piper capense*-BanIs, 3=Decoction-*Tetradenia ruparia*-BanKim, 4= Pilage-*Piptadeniastrum africanum*-PygKim, 5=*Pilage-Piclanolepis bucholza*-PygKim, 6= without preparation-*Piptadeniastrum africanum*-PygKim, 7=Withaout preparation-*Garcinia kola*-BanMa, 8=Dessication-*Cola acuminata*-BanKa, 9= Infusion-*Cola acuminata-Piptadeniastrum africanum-Pterigota tracaganta*-PygKa, 10= Without preparation-*Piptadeniastrum africanum*-PygKat, 11= Without *preparation-Psidium guajava*-PygKat, 12= Without preparation-*Citrus limon*-PygKat

Figure 2. Classification of Bantu and Pygmy specialists according to the recipes used against Sexual impotence in Beni and Lubero Territories

The classification based on recipes used to treat sexual impotence clears on eleven classes of similarity. At the lowest level of Cluster Dendrogram, or at 100% of similarity, five classes are visible, among them, 2 classes, or 40% of individual classes. The recipes conjointly used by the two ethnic groups concern; the desiccation of *Cola acuminata* and the consumption of roots of *Piptadeniastrum africanum*; the consumption of *Garcinia cola* and the infusion of *Cola acuminata-Piptadeniastrum africanum and Pterigota tracagantha*. At 97% of similarity 2 mixed Bantu-Pygmy classes are drawn. At the highest level of hierarchical classification one class exclusively, Bantu forms a marginal group and concerns: the carbonization of leaves of *Uapaca guineensis- Cymbopogon citratus –Acacia sieberana-Piper capense-Citrus limon* and *Rubus rigidus*. The recipes used to treat sexual importence are in 50% similar for the two ethnic groups, whereas the Pygmy Tradi- practioners keep knowledge relatively superior to the one of Bantus for the charge taking of sexual impotence.

4.2.3. Classification on the Base "Recipes Used against Malaria - Bantu and Pygmy Healers – Villages Investigated"



Legend: Ban= Bantus ; Pyg= Pygmées ; Isi= Village Isigo ; Kim= Village Kima ; Kat= Village Katundula ; Ma= Village Maakengu ; Ta= Village Tandandale ; Ka= Village Kalibo , 1= Infusion- Aidia micrantha-Allanblackia stanerana-Dichrocephala integrifolia-BanIs, 2=Distillation-Cynodon dactylo-Carica papaya- Drymaria cordata-Achyranthes aspera-BanIs, 3=Infusion-Pigium africanum-Rauvolfia vomitoria-Ageratum conyzoïdes-Sida acuta-BanIs, 4= food-Rumex bequaertii- Drymaria cordata-Allanblackia stanerana-BanIs, 5= Decoction-*Spilanthes mauricianum-Cynodon dactyl-Pennisetum purpureum-Ageratum conyzoïdes*-BanIs, 6=Decoction-*Cynodon dactylo- Piper guineensis-Carica papaya*-BanIs, 7= Decoction-*Acacia sieberena- Piper guineensis- Anisophelea sp*-BanIs, 8=Maceration-*Acacia sieberena-Piper guineensis- Anisophelea sp*-BanIs 9= Extraction-*Fagara macrophylla*-PygIs, 10=Decoction- *Carica papaya-Ageratum conyzoïdes-Bidens pilosa*-BanKim, 11=Decoction-*Carica papaya-Allium sativum*-BanKim, 12= Carbonisation-*Fagara macrophylla-Carica papaya*-BanKim,

13=Infusion-*Morinda morindoïdes*-BanKim, 14=Infusion-*Morinda morindoïdes*-PygKat, 15= Decoction-*Khaya anthotheca*-BanMa, 16= Decoction- *Khaya anthotheca*-PygKa, 17= Decoction-*Carica papaya-Allium sativum*-BanTa, 18= Decoction-*Alstonia boonei-Fagara macrophylla*-PygTa,19= Decoction- *Carica papaya*-BanKa, 20=*Carica papaya*- BanKat, 21= Decoction- *Cymbopogon citratus*-Camomille- *Dichrocephala integrifolia-Ageratum conyzoïdes-Bidens pilosa- Conyza sumatrensis*-BanKa, 22= Decoction-*Achyrenthes aspera*-BanKat, 23= Decoction-*Alstonia boonei-Fagara macrophylla*-PygKat

Figure 3. Classification of Bantu and Pygmy specialists according to the recipes used against Malaria in Beni and Lubero Territories

Eleven classes of similarity are visible at the level of 100% of similarity, among these, the exclusive Bantus classes predominates in reason of 7 classes, or 63 6%, whereas only one pygmy class or 9% of cases is visible. This one concerns, the infusion of leaves of *Morinda morindoides* and the decoction of skins of *Khaya anthotheca*. The trained intergroup at this level represent 27 2%, or three classes on the eleven listed. These affinities concern the extraction of skins of *Fagara macrophylla*, decoction of flowers of *Carica papaya*-leaves of *Ageratum conyzoides* and leaves of *Bidens pilosa*. As well as, the carbonization of skins of *Fagara macrophylla*- flowers of *Carica papaya* and the decoction of leaves of *Alstonia boonei*- skins of *Fagara macrophylla*, then, the decoction of flowers of *Carica papaya* and the decoction of skins of *Alstonia boonei*-skins of *Fagara macrophylla*.

At the highest level of Cluster Dendrogram, two groups are visible, on the one hand, a mono-ethno pharmacological Bantu group that concerns special recipes obtained from maceration of leaves of *Acacia sieberana*-fruits of *Piper guineensis* and the *Anisophelea sp* and on the other hand, the class formed of the rest of

listed recipes. These observations show that the recipes used to treat Malaria are very different for the healers of two ethnic groups.

4.3. *Characteristics of recipes*

In general, the recipes used by Bantus and Pygmies healers in the six villages were submitted to different modes of preparation that clears to several pharmaceutics forms. As well as, the mode of administration of medicines, vary sensitively between the two groups.

4.3.1. Mode of preparation of recipes

Bantus and Pygmy Tradi-practitioners through the surveyed villages in Beni and Lubero territories prepare the recipes differently. The raised variations between the two ethnic groups living in the same villages are summarized on Figure 4.

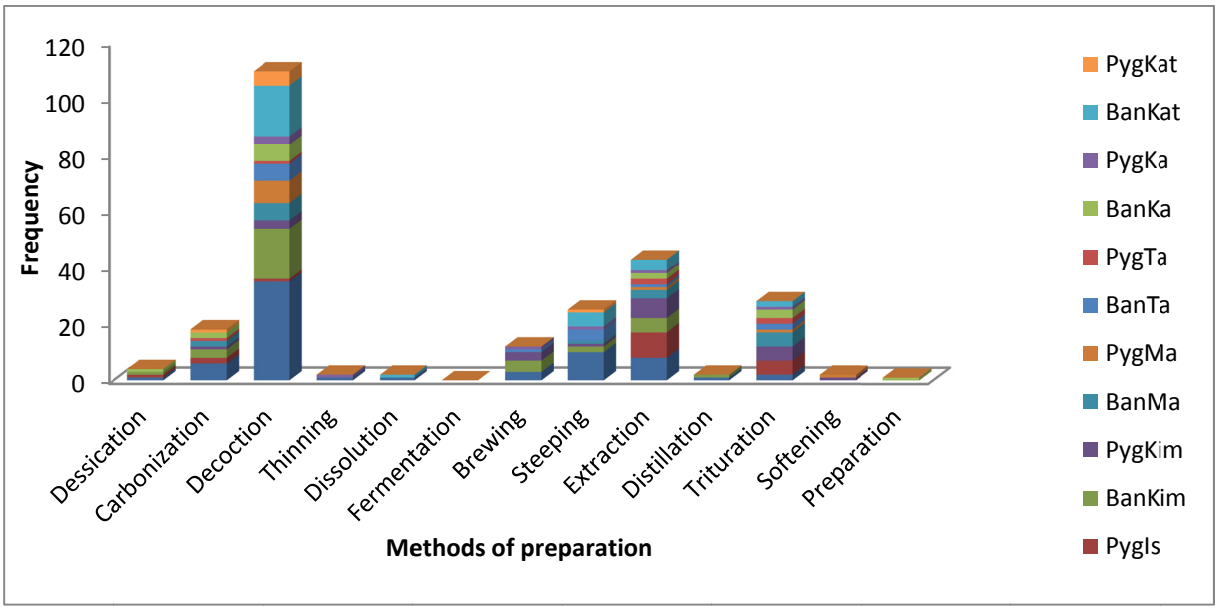


Figure 4. Variation in methods of medicine preparation between Bantus and Pygmies healers of the six villages where they live together in Beni and Lubero territories

The analysis of variations of preparation modes of the medicine shows a great divergence between the Bantu and pygmy specialists. The tendency of specialists does not differ from that of popular medicine. Bantus use mostly carbonization and decoction, whereas Pygmies roast on fire the parts of plants to be used. The Chi squared test of Wallis gives the values (χ^2= 75 26, df = 11 and p-value= $1.206e^{-11}$); the net inferiority of P-value based on the critical value which is 0.05 lets us conclude that Bantu and Pygmy Tradi-practitioners prepare very differently their medicines.

4.3.2. Pharmaceutical form

Pharmaceutical forms of drugs used in traditional medicine of Bantu and Pygmy healers depend on their ethnics' appurtenance. The Figure 5 summarizes evidence variations of pharmaceutical forms of medicines between the two groups.

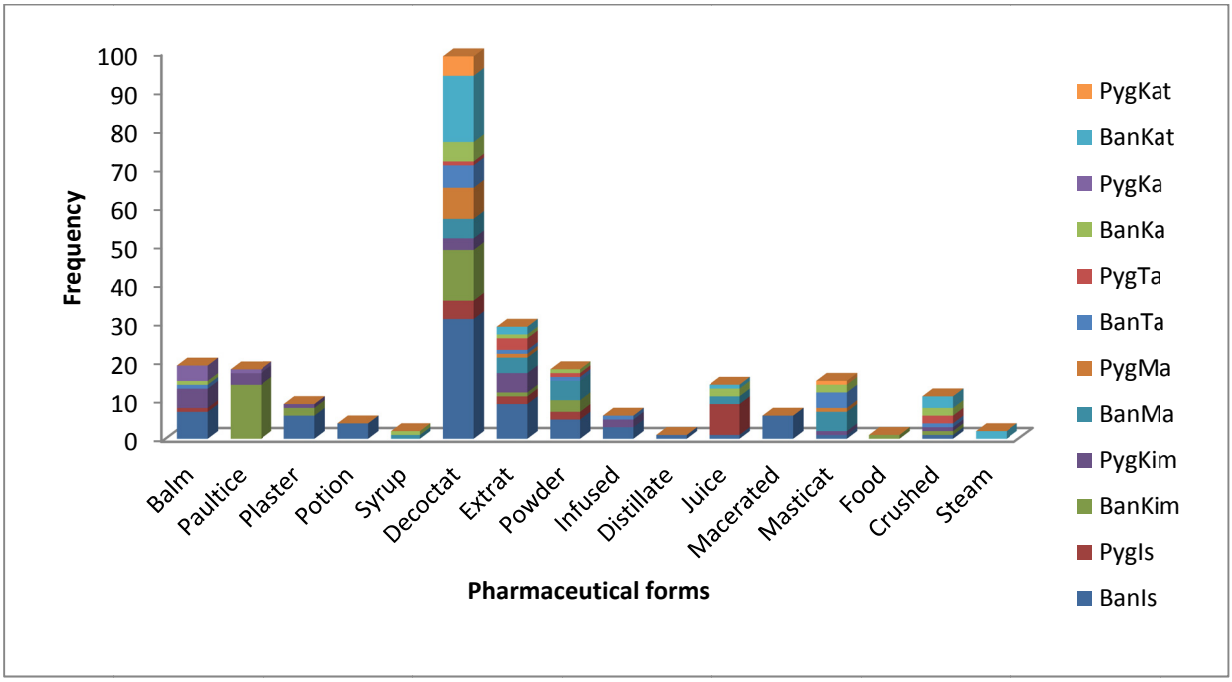


Figure 5. Variation of frequencies of pharmaceutical forms used in traditional medicine of Bantu and Pygmy healers of Beni and Lubero

Pharmaceutical forms of medicines used by Bantu specialists are very different from those preferred by Pygmy Tradi-practitioners in the same villages. Pygmies have strong values for the products used as food and cataplasm, whereas Bantu specialists prefer the extract of plants. The test of chi squared shows a highly significant difference between the two ethic groups (χ^2= 66.2997, dl = 15, p-value = 2.02e^{-08} < 0.05).

4.3.3. Mode of Administration of Medicines

The mode of administration goes in pair with the mode of preparation of medicines in the specialized traditional medicine in Beni and Lubero territories. The Figure 3 summarizes the differences, which exist between the two ethnic groups.

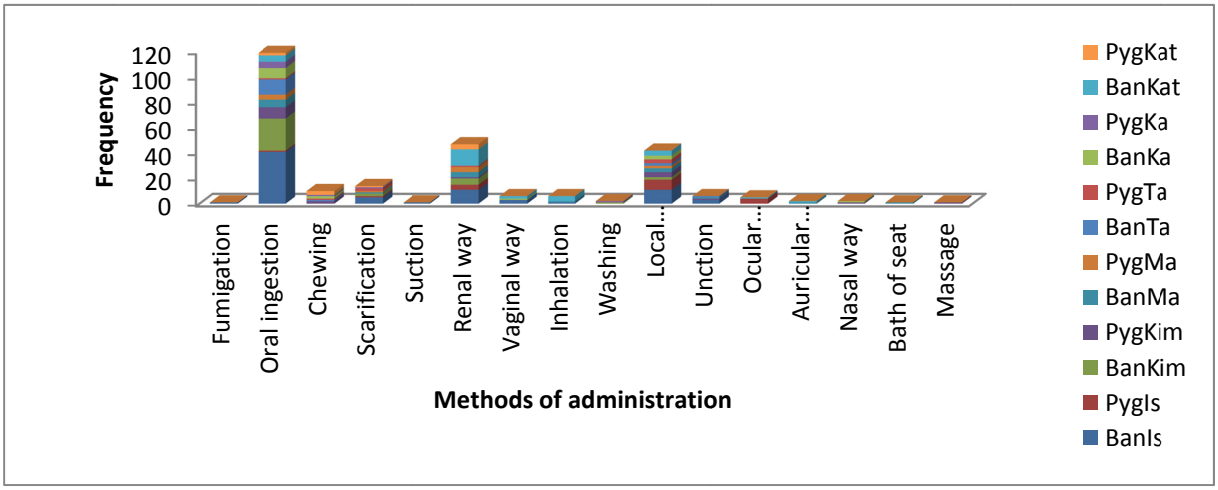


Figure 6. Ways of administering medicine to patients in investigated villages

Three modes of administration, oral ingestion and rectal way of medicines are mostly used. The oral ingestion is the most modes used by Bantu Tradi-practitioners, whereas Pygmies prefer more the products used in cataplasm and scarification. The Chi squared test of Bartlett applied on frequencies raised in the two ethnic groups has

shown a difference highly significant between the two ethnic groups (χ^2 = 479.2165, dl = 15, p-value = $2.2e^{-16}$ < 0.05). This could be in part due to the mode of life of each group. Pygmies seem to be different because of their instability, whereas Bantu prepare well their recipes by the frequency rate of patients.

4.4. *Number of Diseases*

Bantu and Pygmy Tradi-practitioners take in charge in total 72 affections, particularly 47 affections for Bantus and 7 for pygmies. The two groups take in charge in common 18 diseases, the Jaccard index taken from these (0.25) is below 50%, and this brings us to conclude that Bantu and Pygmy Tradi-practitioners treat very different diseases in Beni and Lubero Territories. In order to fear the similarities or differences in the charge taking of diseases, we listed llnesses according to the WHO classification and the following categories have been raised:

4.4.1. Diseases of Nervous System

The principal axes around which the Tradi-practitioners of two ethnic groups are regrouped in the correspondence analysis contribute to more than 70% to total inertia, so we can interpret valuably the affinities between the members of the two ethnic groups through the surveyed villages. The Figure 7 summarize the affinity that exist between the between the two ethnic groups.

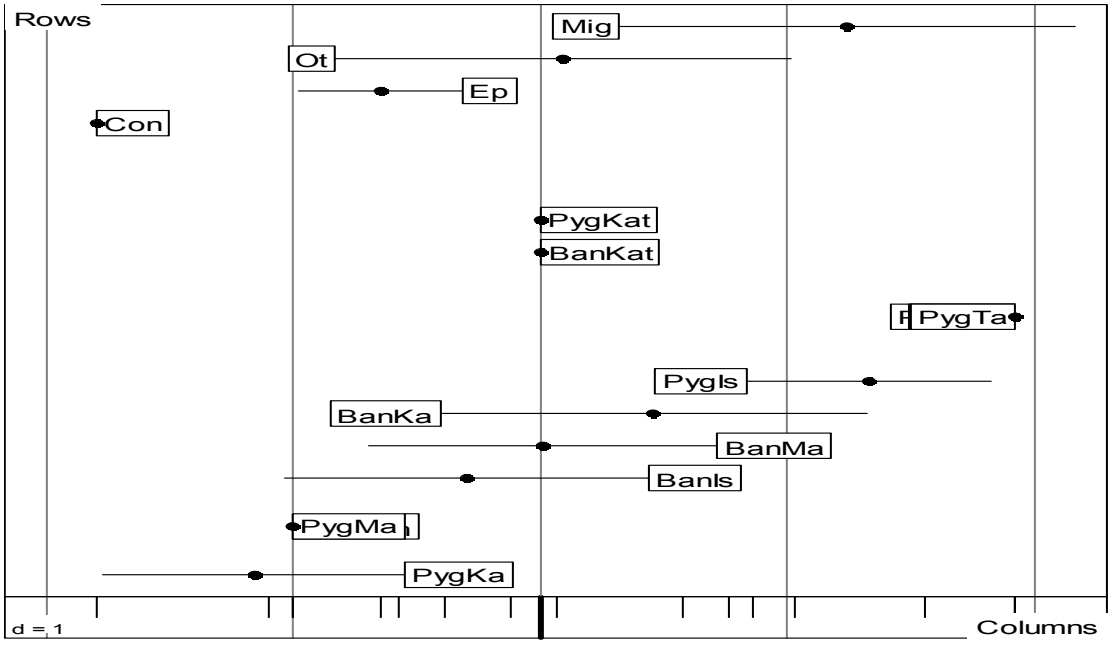


Figure 7. Representation of correspondence analysis of nervous and sense organs treated by Bantus and Pygmies healers

The correspondence analysis of charge-taking of diseases of the nervous system and sense organs shows that Bantu practitioners are more specialized in taking care of Conjunctivitis and Otitis, whereas the Pygmies Tradi-practitioners are reproached to migraine against which they apply a series of species of wild plants by scarification. Epilepsy, which obliges a skilful competence from the healer, is treated conjointly by Bantu and Pygmy practitioners. The Chi squared from Friedman test ,or (χ^2 = 12 8, dl= 11, P-value = 0,3062 > 0.05) has not raised significant differences in the charge-taking of the diseases of nervous system and sense organs by the Tradi-practitioners of the two studied ethnic groups.

4.4.2 Signs and States of Badly Defined Morbidity Traumatic Lesions and Poisoning

The interpretation of convergences of charge taking of signs of badly defined diseases has been based on the two axes of correspondence analysis, which represent more than 60% of the total inertia.

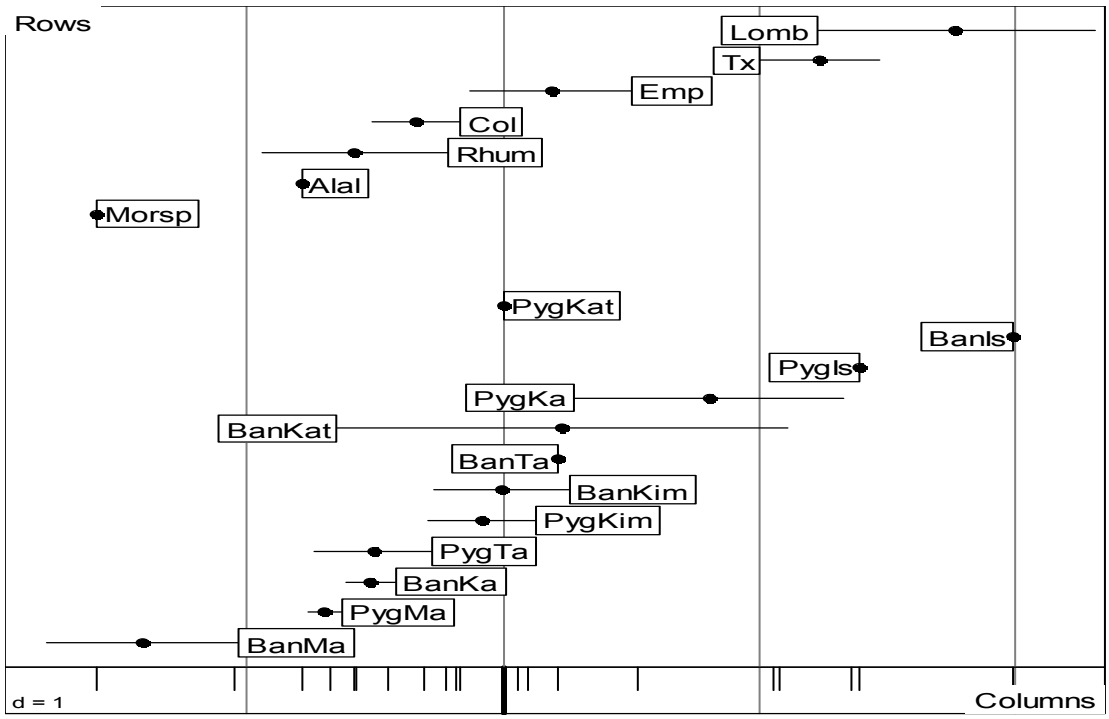


Figure 8. Representation of the correspondence analysis of morbidity signs treated by Bantus and Pygmies healers

Several badly defined signs are taken in charge in specialized medicine of Bantus and Pygmies through the surveyed villages.

The results of the correspondences analysis (Figure 8) show that Bantu and Pygmy healers present strong convergence in the charge-taking of Rheumatism, poisoning, food allergic and colic. However, pygmies specialize in the charge taking of coughing and lombalgia, yet Bantus master more the snake biting. The snake biting is rare in the pygmies' camps because of the prevention of this by scarification in young age of all member of the community.

4.4.3. Infectious and Parasitic Diseases

The two principal axes of the correspondence analysis (Figure 9) contribute in more than 70% to the total inertia and then keep the maximum of necessary information for the interpretation of convergences between the two studied ethnic groups.

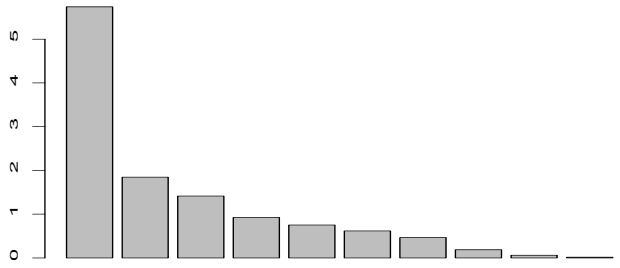


Figure 9. Value of dimensions linked to correspondence analysis of charge taking of infectious and parasitic diseases

The infections and parasitary diseases are very perceptible in the villages inhabited by Bantus and Pygmies in Beni and Lubero territories. The sharing of these diseases often linked to the state of dirtiness varies sensitively in the area inhabited by the two ethnic groups. The analysis of affinities in the charge- taking of these two groups

shows that pygmies master more the treatment of internal Candida, tuberculosis, coetaneous scabies and infectious diarrhea (Figure 10).

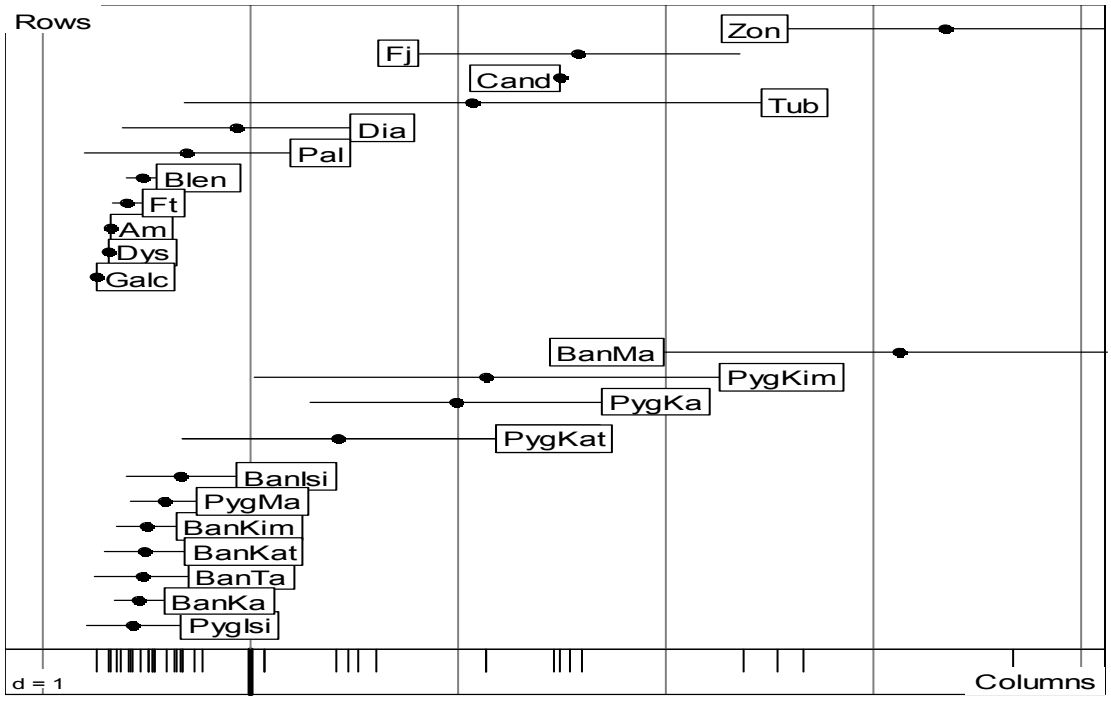


Figure 10. Representation of correspondences of infectious and parasitic illnesses across villages

The Bantus healers of Tandandale, Isigo, Kalibo, Katundula, Kima villages which represent more than 83% of the group have links with Pygmy healers of Isigo, Maakengu and Kathundula or (50%) for the charge taking of 63% of infectious and Parasitory affections. Two marginal groups are observed and concerning pygmy healers of two villages ; Kima and Kalibo(33%) for the charge-taking of Internal Candidiasis, Yellow fever, and Tuberculosis(27% of infectious and parasitory diseases). The other marginal group is formed by Bantu tradi-practitioners of Maakengu village (16%) for the charge taking of Zone or (9% infectious and Parasitory affections). The proportion of convergences between the healers of two ethnic groups which comes over 50% for more than 60% of infectious and parasitory diseases shows that they conjointly treat the infectious and parasitory affections, this shows that the exchanges of experiences are possible between the two ethnic groups despite the hidden characteristic of the traditional medicine.

4.4.4. Diseases of the Digestive System

The interpretation on convergences of charge taking of the diseases of the digestive system by Bantu and pygmy healers has been based on the two axes of correspondence analysis which participate to more than 70% of the total inertia (Figure 11).

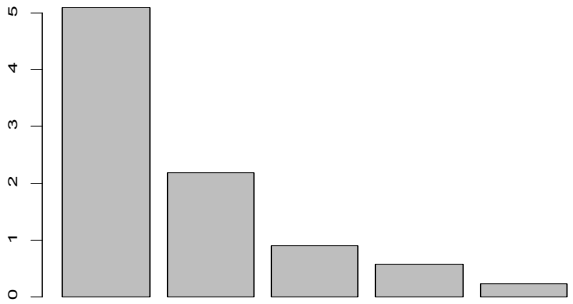


Figure 11. Value of dimensions linked to Correspondence of charge taking of diseases of the digestive system

A net duality of two categories of Pygmy and Bantu specialized traditional medicine seems to be drawn through the villages. The figure shows the correspondences, which exist between the two groups for the charge taking of diseases of the digestive system (Figure 12).

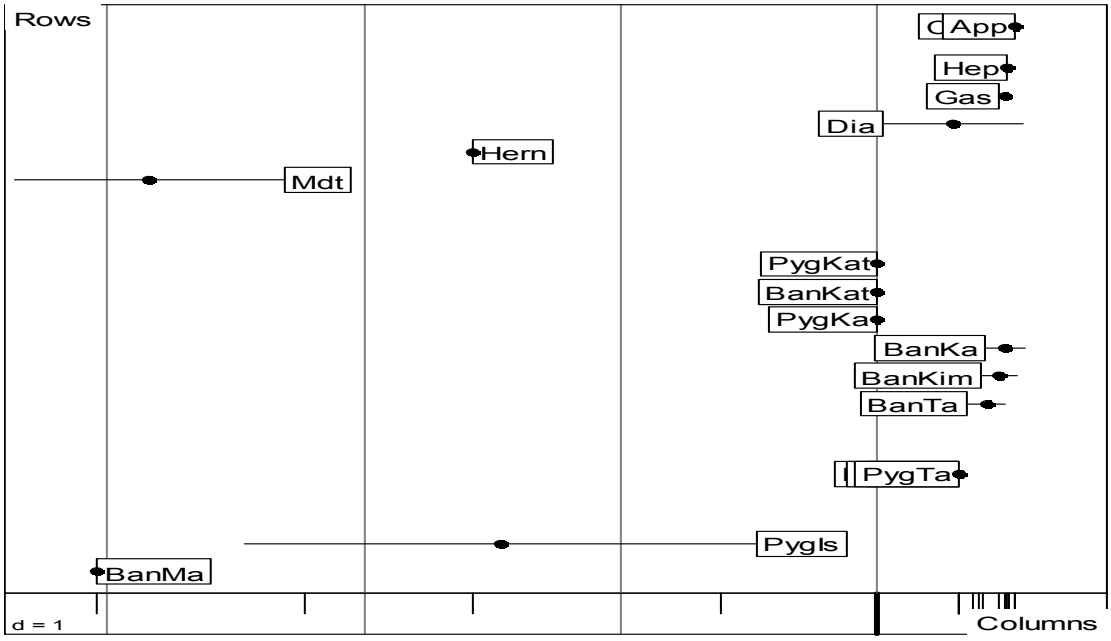


Figure 12. Representation of the Correspondence analysis of digestive system diseases treated by Pygmies and Bantus healers of Beni and Lubero areas

On the one part, Bantu healers of 3 villages; Tandandale, kima and kalibo, or (50% of Bantu healers) have strong convergences in the charge taking of; Constipation, Hepatitis, Appendicitis (57% of digestive system diseases). On the other part, Pygmy healers of three villages; Isigo, Kima and Maakengu are very linked around the charche-taking of Diarrhea (14% of the diseases of digestive system listed). Two marginal cases are observed and trained by Bantu tradi-practitioners of Isigo village who have particular practices of charge taking of hernia, whereas Bantu healers of Maakengu village have exclusivities for teeth aches. The proportions of affections taken in charge separately by healers of each ethnic group that represent 70% relatively to those conjointly treated by the two groups (more or less 30%) shows that the charge taking of digestive system diseases depend to ethnic appurtenance .

4.4.5 Diseases of Uro-Genital System

The diseases of Uro-genital system are differently tidy in the traditional medicine of Bantu and Pygmy healers (Figure 13).

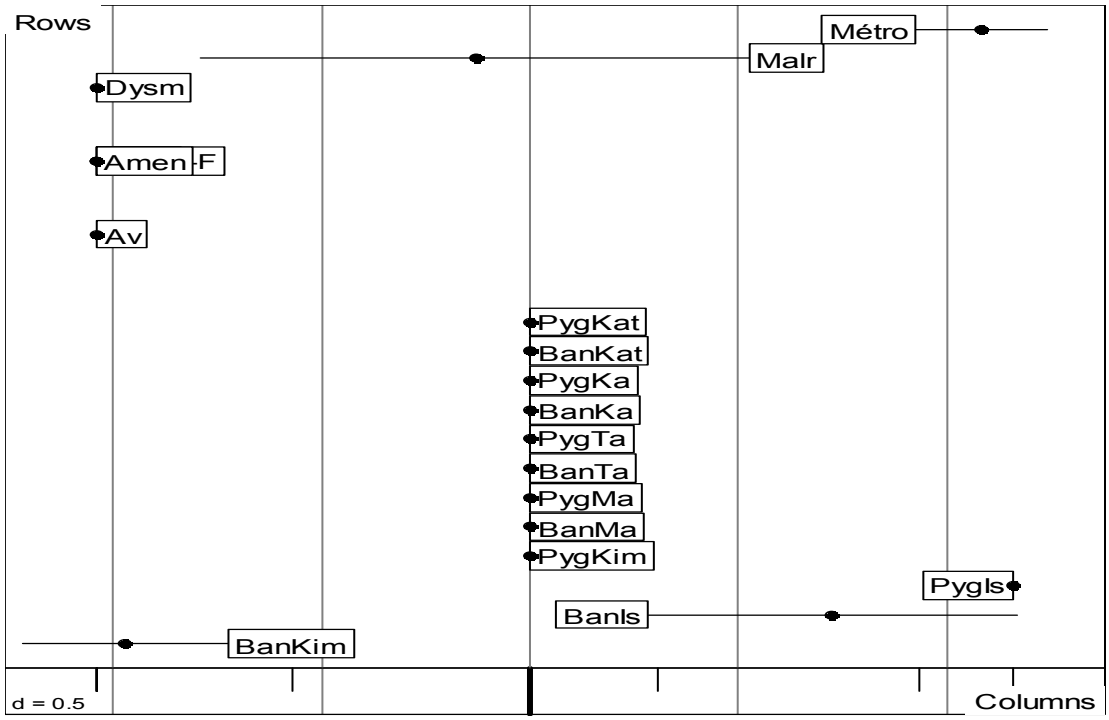


Figure 13. Representation of the Correspondence analysis of the diseases of the uro-genital system treated throughout the villages investigated

The correspondence analysis shows near nesses between pygmy healers of Katundula, Kalibo, Tandandale, Maakengu and Kima villages, or(66% of surveyed pygmy healers), on the other part Bantus healers of ; katundula Kalibo, Tandandale and Maakengu villages , who also represent 66% present strong affinities for Metroragie. Hawever individualities are visible on the one part for the tradi-practioners of Kima village(16% of surveyed Bantu healers) for charge-taking of 4 affections, Dysmenorrhea, Amenorrhea, abortions, Myomes, these ones represent 66% of affections of this category. On the other side, Pygmies of Isigo village have particular know ledges for the care of Metrorrhagy. This brings to the proportion of affections of this category taken in charge exclusively by the members of the two studied ethnic groups to more than 70%, which brings us to conclude that the two groups keep very different know ledges of recipes to treat the Uro-genital affections. Another remarkable fact is that Pygmy young girls are initiated in use of plants against the affections of this category. This increases the family self-charge –taking without referring to a specialist.

4.5 Floristic Composition

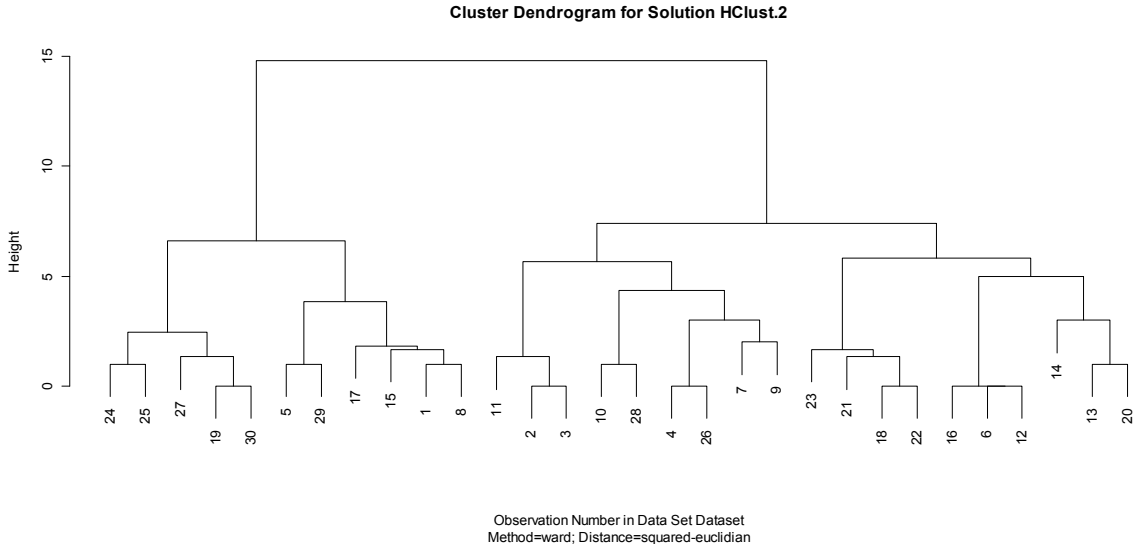
Two hundred and eighty plant species are used by Bantu and Pygmy traditional healers through the surveyed villages. One hundred and eighty two species are exclusively used by Bantus and 83 species by pygmies. The two ethnic groups use in common 15 plant species; among the used species through the survey villages, 12 enter in the preparation of at least 5 different recipes (table 3).

Table 3. Plant species mostly cited in Bantu and pygmy specialized traditional medicine

Species	Family	Biotope	Number of village where the plant has been cited	
			Bantou	Pygmée
Allanblackia stanerana	Clusiaceae	Secondary forest	1	1
Bidens pilosa	Asteraceae	Fallow	3	0
Carica papaya	Caricaceae	Fallow	2	0
Citrus limon	Rutaceae	Garden	3	0
Conyza sumatrensis	Asteraceae	Garden	2	1
Dichrocephala integrifolia	Asteraceae	Fallow	1	0
Khaya anthotheca	Meliaceae	Secondary forest	2	0
Phytolacca dodecandra	Phytolacaceae	Fallow	2	1
Piper capense	Piperaceae	Secondary forest	3	3
Rauvolfia vomitoria	Apocynaceae	Secodary forest	1	0
Sida acuta	Malvaceae	Fallow	1	1
Solanum aculeastrum	Solanaceae	Fallow	3	1

From the results of the Table 3, the healers of the two ethnic groups refer more to ruderal species to prepare recipes. The ones represent 50% of species the most cited. The progressive farness of the forest and the extinction of the species the most converted for their medicinal virtues explain the substitution by the species in great dispersion found in fallows. Plants of the family of Asteraceae are preponderant with 3 representatives or (30%) of plant species the most cited in specialized medicine. Three plant species, like: *Bidens pilosa* , *Citrus limon, Solanum aculeastrum* have been cited in 3 villages uniquely by Bantus, yet only one species(*Piper capense*) has been cited simultaneously in 3 bantu and Pygmy villages.

The hierarchical classification of plant species use by the healers of Bantu and Pygmy ethnic groups in Beni and Lubero territories by the method of Word has given the classes of similarity (Figure 14).



Legend: 1 = BanIs -*Allanblackia stanerana*; 2 = BanIs-*Allanblackia stanerana*; 3= PygIs- *Allanblackia stanerana*; 4= BanIs-*Bidens pilosa*; 5= BanKim-*Bidens pilosa*; 6= BanKa- *Bidens pilosa*; 7= BanIs-*Carica papaya*; 8= BanTa- *Carica papaya*; 9= BanIs- *Citrus limon*; 10= BanKim-*Citrus limon*; 11= BanTa-*Citrus limon*; 12= BanIs- *Conyza sumatrensis*; 13= BanKim-*Conyza sumatrensis*;14 = PygKim- *Conyza sumatrensis*;15 = BanIs- *Dichrocephala integrifolia*; 16= BanKim- *Khaya anthotheca*; 17 = BanIs- *Khaya anthotheca*; 18= PygMa-*Phytolacca dodecandra*; 19= BanKat- *Phytolacca dodecandra*; 20= BanIs-*Piper capense*; 21= PygKim- *Piper capense*; 22= BanMa- *Piper capense*; 23= PygTa- *Piper capense*; 24= BanIs- *Rauvolfia vomitoria*; 25= BanIs- *Sida acuta*; 26= PygTa- *Sida acuta*; 27= BanIs -*Solanum aculeastrum*; 28= BanKim- *Solanum aculeastrum*; 29= BanKa- *Solanum aculeastrum*; 30= PygMa- *Solanum aculeastrum*

Figure 14. Dendrogram of similarities of the usage of species in the ethnic intergroup

At the level of 100 % of similarity of use of specialized medicine 5 Bantu and pygmy mixed classes have been raised, among which, 4 mixed classes and one class exclusively bantu. At the level of 97% of similarity, five classes from which 4 exclusively Bantu and one class Bantu-Pygmy was raised. The highest level of the hierarchical tree , or 90% of similarity of use of plant species in specialized medicine, two classes are shown, one class formed by the Bantu sub-class and the other mixed sub-class of Bantu-Pygmy. It is shown that the analysis of the hierarchical tree that the mixed groups formed of Bantu and Pygmy healers predominate in reason of 60%, yet the groups exclusively, Bantus represent 40% of the whole. Any class formed exclusively by pygmies was not raised.

The predominance of Bantu classes in the hierarchical tree can explain itself by stability of Bantu healers who take in charge their patients in the pharmacy of traditional medicine and harvest most of plants used in fallows and recued forests. Concerning their colleague's pygmies, it is visible that the semi-nomad characteristic makes them instable, which does not allow homogeneous classes for the use of recipes in this ethnic group. Pygmy healers keep an experience of their own. They harvest sometimes plants very far in the forest at the same time they are hunting animals. In order to confirm these results, the test of Friedman based on the sums of rows of frequencies was used. Thus the expressed Chi-squared gave χ^2= 39 3 at dl= 13 and P-V (0.00017), very inferior value to 0.05 (Critical value). This has allowed us to conclude that Bantu and Pygmy specialists use very differently the plant species to treat diseases despite the higher exchanges between the two ethnic groups.

4.5.1. Convergence of use of species for treating recurrent diseases

The analysis of the floral composition of the lists of species used by Bantu and Pygmy specialists in the surveyed villages has shown the predominance of 12 species such as *Allanblackia stanerana, Bidens pilosa, Carica papaya, Citrus limon, Conyza sumatrensis, Dichrocephala integrifolia, Khaya anthotheca, Piper capense, Sida acuta* and *Solanum aculeastrum.* The comprehension of the distribution of species frequently used in specialized traditional medicine of Bantus and Pygmies for the control of this or other recurrent disease requires a correspondence analysis (Figure 15).

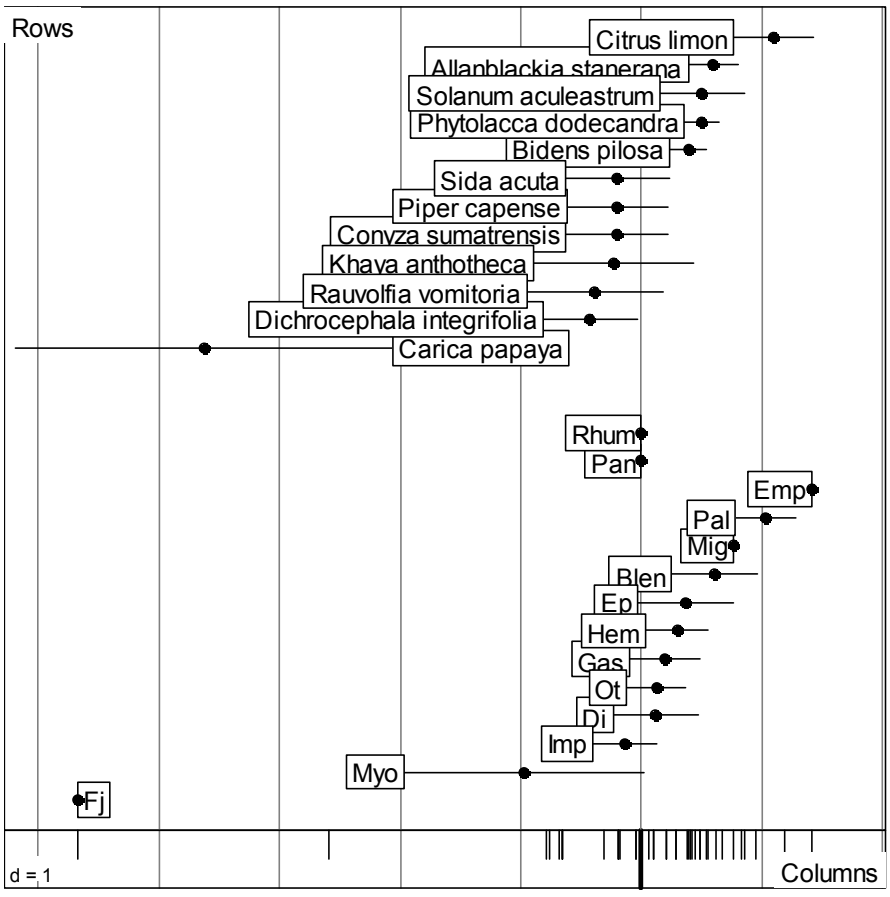


Figure 15. Representation of the Correspondence analysis of species used against recurrent diseases in Bantu and Pygmy specialized medicine

The two axes of correspondence analysis on which we have based our interpretation contribute more than 90% to the total inertia and include the necessary information for this analysis (Figure 16).

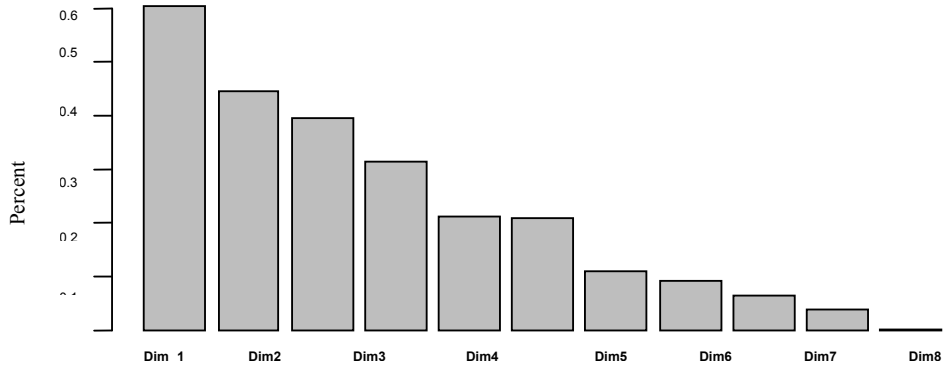
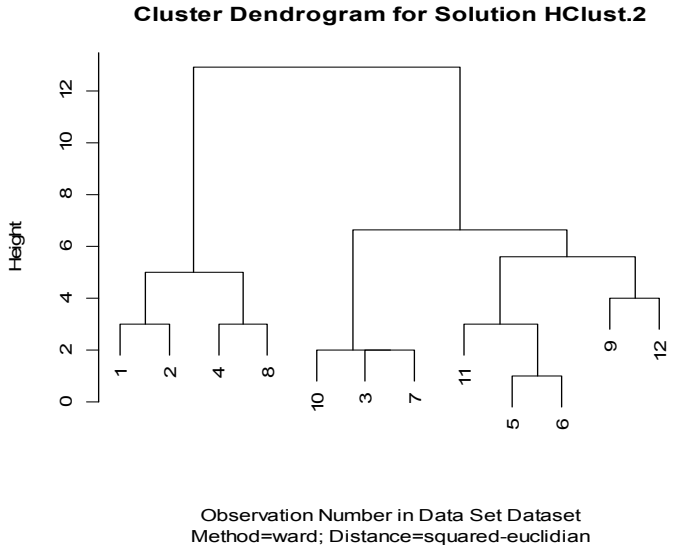


Figure 16. Proper values linked to the dimensions of Correspondence analysis of the convergence of species frequently used for the control of recurrent diseases

The analysis shows that most species frequently in Bantus and Pygmies specialized in traditional medicine in Beni and Lubero territories have strong similitude of employment against recurrent diseases; there are strong correlations between the species *K. anthotheca, R. vomitoria, D. integrifolia, C. sumatrensis, P. capense, S. acuta, C. papaya, B. pilosa, S. aculeastrum*, for treating malaria, gonorrhoea, otitis, panaris, migraine, sexual impotence, epilepsy, diarrhea and hemorrhage. The species of *Citrus limon* is mostly used in treating rheumatism, whereas *C. papaya* is used in the control of gastritis. Bantu and Pygmy herbalists independently treat yellow fever and poisoning.

4.5.2 Classification of Species Used in Treating Recurrent Diseases in Specialized Traditional Medicine

In order to raise the convergence of use of species against recurrent diseases, a hierarchical classification is seen as important. This will give an idea on the close species in charge of a given infection and those which are the closest. Figure 17 shows the classes which exist in the two types of specialized traditional medicine.



Legend: 1=*Allanblackia stanerana*; 2= *Bedens pilosa*, 3= *Carica papaya*; 4= *Citrus limon*; 5= *Conyza sumatrensis*; 6= *Dichrocephala integrifolia*; 7= *Khaya anthotheca*, 8= *Phytolacca dodecandra*; 9= *Piper capense*; 10= *Rauvolfia vomitoria*; 11= *Sida acuta*; 12= *Solanum aculeastrum*

Figure 17. Dendrogram of convergence use of plants against recurrent diseases

At the lowest level of the dendrogram or at 95% of similarity, a class regroups the species of *C. sumatrensis* and *D. integrifolia* against diarrhea, myositis and malaria. At 90% affinity of species use, a class of *P. dodeccandra, C. papaya* and *K. anthotheca* for treating gonorrheae and malaria was raised. Notice that in these classes, two sub-classes, which are the closest, should be remarked, especially one sub-class made up of *P. dodecandra* and *K. anthotheca* against gonorrhea and *C. papaya* and *K. anthotheca* against malaria. The progressive ascension in the hierarchical tree at the level of 85% of similarity brings 3 classes from which a class regroups *Allanblackia stanerana* and bidens pilosa against gonorrhea, diarrhea and epilepsy. The second class is made up of *C. limon* and *P. dodeccandra* against gonorrhea and epilepsy, whereas the third class regroups the species *S. acuta* and *C. sumatrensis* against epilepsy, myositis and malaria and the species *C. sumatrensis* and *D. integrifolia* for the control of diarrhea and malaria.

A class of affinity of using two species, *P. capense* and *S. aculeastrum* for treating epilepsy, migraine and myositis is repeated at the level of 80% of similarity; whereas, at 75%, a class of *A. stanerana, B. pilosa*, *C. limon, P. dodecandra* is used against gonorrhea and epilepsy. At the highest level of the tree, or at 70% of similarity of employment of species which is the most cited against the recurrent diseases, two classes are formed. The first is composed of *R. vomitoria*, *C. papaya* and *K. anthotheca* against malaria. The second class is subdivided into 4 sub-classes which are *S. acuta* and *C. sumatrensis* against epilepsy and myositis. *D. integrifolia* and *C. sumatrensis* were used against diarrhea and malaria, *P. capense* and *D. integrifolia* against malaria and myositis. The second sub-class is composed of *P. capense* and *S. aculeastrum* against migraine and myositis.

The Cluster analysis of use of twelve species, which are the most cited against fourteen recurrent diseases, shows that Bantus and Pygmies in Beni and Lubero territories commonly use the most diverse combinations of twelve species in nine formed classes.

5. Discussion

The medicine of traditional healers is very spread in cities as well as in several villages of Beni and Lubero territories. The obtained results of the surveys done in the mixed villages where Bantus''Nande'' and Pymies''Mbuti'' live together have shown that Bantu and Pygmy tradi –practitioners use different vegetative species for the charge taking of diseases. The classification of specialists of the two ethnic-goups has given a dendrogram in which Bantu-Pygmy classes predominate in reason of 60%, which tells about experience exchanges between the specialists of the two ethnic-groups. The similar exchanges have been signaled at Bantu and Pygmy specialists of Bikoro in Equator province (Ilumbe, 2010). However, a very high rate of classes exclusively formed of Bantu specialists or (40%) shows that there are again great secrets that the specialists of the two ethnic groups hide. Bantu specialists easily exchange the secrets among them and obtain experiences of homologue pygmies paying portions. Pygmies on the other hand do not exchange so experiences among them and great disparities are noted in their camps. This appears different from the statement of Ilumbe, op cit., who has shown the exchanges both at Bantu Tradi- practitioners of Bikoro in Equator province in the DR Congo. The situation of the exclusivity of same practices used in traditional medicine of Bantus of Beni and Lubero meets the statement of many authors in other regions of Africa, such as Pfeiffer and Butz (2005) cited by Zerbo et al(2008). Deleke koko et al., 2009 who stipulate that the geographical origin, the local culture and sex can influence the transmission of know ledges.

Great differences are observed in the number of recipes, Bantu healers use more diversified recipes because of many exchanges that they do with other specialists, whereas pygmy healers are limited to the acquired knowledges from their ancestors without much evaluation. The characteristics of recipes differ between the two ethnic groups. Bantus healers mostly refer to carbonization and decoction, whereas Pygmies prefer to soften the parts of the plants, as well as the triturating. Additionally Pygmies administer their recipes mostly by cataplasm and scarification, whereas Bantus prefer the oral ingestion. Considering the specialty of charge taking of affections between the two groups, the affinities are very numerous; however, differences should be raised for same affections. Bantus healers are specialized more in the charge taking of diseases of digestive system and the affections of Uro-genital system, less frequent in the pygmies' camps. The pygmies on their side have more experience in the charge taking of sexual impotence.

The similarities of use of same vegetative species between Bantu and Pygmy specialists of Beni and Lubero territories are in most of cases signaled in the phytotherapy of others regions of Africa. Here are some cases of similarity that we have raised:

The *Piper capense* whose all the parts are used in different manners for the charge taking of several diseases such as ; Haemororrhoids, malaria, Myositis, Migraine, Epilepsy and sexual impotence have been cited in

Cameroun in the charge taking of Epilepsy in Madagascar fresh or cooked roots are consumed as aphrodisiac tonic. These roots might contain especially α-pipene, β-pipene , Camphene, and sabinene, also extract of roots has shown a significant antibacterian activity against *Staphylococcus aureus*, *Staphylococcus pyogenes* and *Corynebacterium sclerosis* in vitro(Schmetzer et al., 2008). However, special oils extracted from fruits could be composed majoritarily of monoterpenes hydrocarbonated (58%) followed by sesquiterpenes hydrocarbonated (23,2%) which have insecticide properties vis-à-vis weevils ‘’*Callosobruchus maculates*”(Woguem, 2012). *Rauvolfia vomitoria* currently used in maceration of leaves in Bantu and Pygmy specialized medicines of Beni and Lubero for the control of Epilepsy and Malaria was cited for the similar uses in other regions of Africa. In Cameroun, the decoction of sprayed roots is taken to treat diabetes and Malaria (Schmelzer at al, 2008), in South Kivu (DRCongo) the product in basis of roots are taken currently to treat hypertensions and to calm the Epilepsies, Psychotics (Balagizi et al., 2007). The chemical composition of Rauvolfia vomitoria includes numerous alkaloids was tenor in total alkaloids varies from 7 to 10 %, numerous chemiotypes seem to exist with reserpine of antihypertensors and neuroleptic (sedative); ajmaline with antiarythmic properties neigghbouring to those of Quinidine, isolated alkaloid of Quinquina (Kabangu, 1990; Didier and Micha, 1995). Carica papaya species of which the decoction of flowers and roots are used in specialized medicine in Beni and Lubero territories for the control of gonorrhea and Malaria was signaled in South-kivu province in the DRCongo and in Congo Brazzaville for Uro-genital diseases (Balagizi et al., 2007; Adjanohoun et al., 1988). The ripe fruit of *Carica papaya* is regularly prescribed to patients who have problems of gastritis containing papaine indicated in pharmacy as in the treatment of digestive insufficiencies in internal use, whereas decoction of leaves enters in the therapy of malaria; the same usages have been cited in south-Kivu province (Defour, 1995)

Triturated leaves of *Conyza sumatrensis* is frequently used against Mycosis and several skin diseases, even in plaster against panaris, the same usages were cited for the similar ways in Congo Brazzaville (Bitsindu & Lejoly, 1992). The leaves of *Conyza sumatrensis* and *Dichrocephalla integrifolia* grinded and applied in ocular instillation against Migraine in South-Kivu province (Balagizi, op cit.) are also remarkable in the specialized medicine of the two territories. Leaves of *Bidens pilosa* used in decoction against Diarrhea in Congo Brazzaville and Ivery coast (Adjanohoun et al., 1988; TaBi, et al., 2008); were cited in the traditional medicine of healers in Beni and Lubero territories. Maceration of leaves of the same plant is used for the charge taking of gonorrhea and Epilepsy; the similar usages were cited in South-Kivu Province (DRC) (Defour, 1995).

The therapeutic vertues of *Bidens pilosa* just been mentioned in several literatures, its medicinal properties could be due to Phenylheptatryne, and other derived of Thiophene, which is antimicrobial repeated (Fotso et al., 2002).

However, the results of qualitative photochemical analysis of aqueous extraxt of leaves of *Bidens pilosa* have come to the identification of Flavonoids, Polyphenols, Terpenes (Kouakou et al., 2008).

The skins of *Harungana madagascariensis* used in decoction sometimes in maceration then administered by anal voice or again by seat bath against Haemorrhoids have been one of important in the surveyed villages.

The same plant is used against Haemorrhoid in Kisangani (Kalanda et al., 1993) and Equator province of the Democratic Repubic of Congo (Ilumbe et al., 2014)

6. Conclusion

Great differences are observed in the number of recipes used in traditional medicine of Bantus and Pygmies healers living in the same villages. Bantu healers are more informative because of several exchanges that they have with other specialists, whereas Pygmy healers limit themselves to the knowledge acquired from their ancestors without much evolution. The characteristics of formulas and the modes of administration of formulas differ between the two groups. Bantus healers mostly prefer carbonization and decoction, whereas Pygmies prefer to destroy the parts of the plants, as well as triturating. In the same way, Pygmies administer their recipes mostly by cataplasm and scarification, whereas Bantus prefer oral and rectal way. Considering the specialties of treating infections between the two groups, affinities are very numerous; however, differences are noticed for some infections. Bantus healers are mostly specialized in treating diseases of the digestive system and the infections of Uro-genital system, which are less frequent in the Pygmy camps. Pygmies on their own side have more experience in treating sexual impotencies’. Most methods used by specialists of two ethnic groups in traditional medicine are not lasting, this could attract the attention of researchers and other actors of development worked in the area to integrate the traditional medicine in lasting management of the ecosystems to preserve the species more coveted.

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