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ARTICLES

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Full Length Research Paper

Congruence of random amplification of polymorphic deoxyribonucleic acid (RAPD) and simple sequence repeats (SSR) markers in genetic characterization of willow (*Salix* spp.)

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Willow (*Salix* spp.) includes closely related dioecious polyploid species, which are obligate outcrossers. Natural populations of willows and their hybrids are represented by a mixture of highly heterozygous genotypes sharing a common gene pool. Random amplified polymorphism DNAs (RAPD) and microsatellites (simple sequence repeats, SSRs) are useful methods for studying genetic diversity of willow (*Salix* spp.). RAPD delivers a large number of data points from a single experiment and is a useful method for distinguishing between closely related individuals. SSR markers are very robust tools, and we have identified several markers that show high levels of polymorphism in willow. Genetic characterization of 94 genotypes (four female, ten male, and 80 half sibs) of *Salix* collected from Naganji Nursery of University of Horticulture and Forestry, Solan, Himachal Pradesh, India were analyzed using 10 SSRs and 15 RAPDs PCR-based molecular markers. RAPD analysis yielded 87 polymorphic fragments (98.9%), with an average of 5.8 polymorphic fragments per primer. Similarly, SSR analysis produced 33 bands, out of which 26 were polymorphic (78.8%) with an average of 2.6 polymorphic fragments per primer. The genetic diversity was high among the genotypes (Nei's genetic diversity = 0.354 and Shannon's information index = 0.536) as measured by combination of both RAPD and SSR markers. The mean coefficient of gene differentiation (Gst) was 0.037, indicating 96.6% of the genetic diversity resided within the genotypes. It was found that the genetic diversity among genotypes was broader, suggesting the importance and feasibility of introducing elite genotypes from different hybrids for willows germplasm conservation and breeding programs.

Key words: *Salix sp.*, female, male, half sibs, molecular markers**,** genetic diversity, Random amplified polymorphism DNAs (RAPD), simple sequence repeats (SSRs).

INTRODUCTION

There are more than 300 *Salix* species, and they are widespread in both the Northern and the Southern hemispheres, excluding Australasia and New Guinea.

Willows are dioecious but may reproduce as well sexually as by vegetative propagation. The willows are typical tree-forming pioneer species in alluvial plains and riparian

zones. The delimitation between these polyploid taxa relies on relatively few diagnostic features in the morphology. Consequently, large overlaps exist which make it difficult to identify samples from the field unambiguously (Berlin et al., 2011). In many cases the two species coexist in mixed stands.

Artificial hybridization is possible, but the taxonomic identity as well as the identification of their metapopulations remains difficult. Different elements such as the lack of qualitative diagnostic characters, the frequent occurrence of intermediate morphological forms and the successful interspecific controlled crosses support the hypothesis that *S. alba* and *S. fragilis* may hybridize frequently in nature (Triest et al., 2000). *S. viminalis L.* and *S. schwerinii* E. *Wolf* are dioecious willows that are phenotypically very similar. Both are multi-stemmed shrubs with long and slender leaves and are commonly found along streams and rivers and in other wet areas. As other *Salix species*, the sex-ratio is often female biased (Ueno et al., 2007). In *S. sachalinensis*, for example, clonal propagation was less important than expected (Ueno et al., 2007). *S. viminalis* has a vast natural distribution ranging from Ireland and United Kingdom in the west to Siberia in the east. The exact boundaries of the natural range in Western Europe are uncertain due to extensive cultivation in the past.

A number of problems have been highlighted, including a largely undefined genetic pool of clonal lines which can be used as progenitors in a breeding programme and limited information on the genetic basis of many agronomically important traits. Within the last 20 years, molecular biology has revolutionized conventional breeding techniques in all areas. Biochemical and Molecular techniques have shortened the duration of breeding programs from years to months, weeks, or eliminated the need for them all together.

The use of molecular markers in conventional breeding techniques has also improved the accuracy of crosses and allowed breeders to produce strains with combined traits that were impossible before the advent of DNA technology. Many of the willow cultivars exploited for biomass production are closely related genetically and can be difficult to distinguish using traditional morphological criteria (Ngantcha, 2010). The objective of this study was to compare the effectiveness of both the PCR-based molecular approaches to determine the genetic relationships among several genotypes of willows parents and their hybrids.

MATERIALS AND METHODS

Plant material

Ninety four (94) genotypes (80 half sib, four females and 10 males) of

Salix sp. were collected from Naganji nursery farm of the Dr. Y.S.Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) India, (Table 1a). Although these plants showed distinctive taxonomic traits of the different willow species, they were chosen for their great variability in terms of morphological traits such as young and mature leaves, bark colour etc.

DNA extraction

The young leaf samples were collected during the period of March to October in sampling bags under aseptic conditions. The leaves were stored at -20°C for DNA extraction. Total genomic DNA was extracted from the frozen leaves (2 g) by the CTAB method (Saghai-Maroof et al., 1984) with minor modifications, which included the use of 200 mg of polyvinyl pyrollidone per sample. The extracted DNA was then treated with 20 µL of 10 mg/ml of RNase and incubated at 37°C for 60 min. After incubation with RNase, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inverting the microcentrifuge tube followed by centrifugation at 10,000 rpm for 5 min at room temperature. The supernatant was pipetted out into a fresh tube. The sample was then extracted twice with equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated by adding 0.6 volumes of isopropanol and 2.0 M NaCl. To the above, 20 µL of sodium acetate and 1 volume of 80% ethanol were added, incubated for 30 min and centrifuged at 5,000 rpm for 3 min to pelleted the DNA. The pellet was then washed with 70% ethanol twice, air-dried and finally suspended in 40 to 50 µL of TE buffer. The yield of the extracted DNA and purity was checked by running the sample on 0.8% agarose gel along with standard (non restriction enzyme digested) lamda DNA marker (Biogene, USA). The extracted genomic DNA was tested for purity index (A_{260}/A_{280} absorbance ratio) on Nano drop spectrophotometer. A value of 1.8 of extracted DNA samples indicate high purity, whereas the value <1.8 or >1.8 denotes the contamination of proteins and RNA respectively (Sambrook et al., 1989).

RAPD markers

PCR amplification was carried out in a 25 µL total reaction volume containing 30 ng genomic DNA, 1.5 mM MgCl$_2$, 1 µM of primers and 1 unit of Taq DNA polymerase (Pharmacia) (Barcaccia et al., 1997). Amplification was performed in a 9700 Thermal Cycler (PerkinElmer) under the following temperature profile: initial denaturation for 5 min at 95°C was followed by 3 cycles of 2 min at 95°C, annealing temperature of 35°C for 1 min, 72°C for 2 min for extension, 37 cycles at 94°C for 15 s, 36°C for 30 s, 72°C for 1 min and 72°C for 10 min. The rates of temperature change adopted for heating and cooling were + 1°C/2.9 and -1°C/2.4 s, respectively. Amplification products were electrophoresed on 1.5% agarose gels run at constant voltage and 1X TBE buffer for approximately 2 h, visualized by staining with ethidium bromide (Sambrook et al., 1989) and photographed under UV light (using DC120 camera, Kodak).

SSR markers

A set of 10 pairs of SSR primers (Table 2) (synthesized by Life Technologies, Inc.) were used in this study. PCR reactions were performed with a protocol reported earlier (Barcaccia et al., 2003)

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Table 1a. List of the 94 *Salix* genotypes.

Female	Half sibs	Half sibs	Half sibs	Half sibs	Male
Salix tetrasperma	Half sib 1	Half sib 21	Half sib41	Half sib61	*Salix alba*
Salix matsudana x Salix alba (J7990)	Half sib 2	Half sib22	Half sib42	Half sib62	*Salix babylonica*
Salix matsudana (PN227)	Half sib 3	Half sib23	Half sib43	Half sib63	*Salix tetrasperma*
Salix matsudana (SE-69-002)	Half sib 4	Half sib24	Half sib44	Half sib64	*Salix matsudana* (PN722)
-	Half sib 5	Half sib25	Half sib45	Half sib65	*Salix rubence*
-	Half sib 6	Half sib26	Half sib46	Half sib66	*Salix udensis* (SX59)
-	Half sib 7	Half sib27	Half sib47	Half sib67	*Salix alba X Salix babylonica* (131/25)
-	Half sib 8	Half sib28	Half sib48	Half sib68	*Salix matsudana X Salix alba* (NZ1140)
-	Half sib 9	Half sib29	Half sib49	Half sib69	*Salix matsudana X Salix alba* (NZ1179)
-	Half sib 10	Half sib30	Half sib50	Half sib70	*S.matsudana X S.alba* (NZ1002)
-	Half sib11	Half sib31	Half sib51	Half sib71	-
-	Half sib12	Half sib32	Half sib52	Half sib72	-
-	Half sib13	Half sib33	Half sib53	Half sib73	-
-	Half sib14	Half sib34	Half sib54	Half sib74	-
-	Half sib15	Half sib35	Half sib55	Half sib75	-
-	Half sib16	Half sib36	Half sib56	Half sib76	-
-	Half sib17	Half sib37	Half sib57	Half sib77	-
-	Half sib 18	Half sib38	Half sib58	Half sib78	-
-	Half sib 19	Half sib39	Half sib59	Half sib79	-
-	Half sib20	Half sib40	Half sib60	Half sib80	-

with minor changes. The volume of PCR solution was 25 µL, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 300 µM each of dCTP, dGTP, dATP and dTTP, 800 µM of primer, 1.5 U of Taq DNA polymerase (Pharmacia Biotech) and 30 ng of genomic DNA. Amplification reactions were performed in a 9700 Thermal Cycler (PerkinElmer) using a touchdown cycling profile.

The optimized PCR amplifying conditions used were: initial denaturation at 95°C for 3 min, followed by 2 cycles of 1 min at 95°C, an annealing temperature of 1 min at 63°C and 2 min at 72°C followed by a reduction in annealing temperature by 1°C every two cycles until a final annealing temperature of 56°C was reached. The last cycle was repeated 26 times and was ended by a final step at 72°C for 10 min.

The amplified fragments were separated on 2% agarose gels with 1X TBE buffer (Sambrook et al., 1989) at 150 V for 3 h. Photographs (DC120 camera, Kodak) of the polymerized genomic fragments were taken after staining of the agarose gels with ethidium bromide.

Data collection and analysis

The genetic relationship among the entire genomic DNA under study was assessed by comparing the RAPD and SSR fragments separated according to their size. The banding pattern of each of the primer was scored as present (1) or absent (0), each of which was treated as an independent character. Only the reproducible bands were observed for scoring and the light bands were omitted as they were not reproducible. The Jaccard's dissimilarity coefficient (J) was calculated, subjected to cluster analysis by bootstrapping and neighbor-joining method using the program DARWIN (version 5.0.158). Statistically unbiased clustering of collected genotypes was performed using STRUCTURE (version 2.3.1).

POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL) across all the 94 genotypes were analyzed Nei et al. (1979). Within group diversity (Hs) and total genetic diversity (Ht) were calculated within the species and within three major groups (based on the male, female and half sib genotypes) by using POPGENE software Nei (1978). The RAPD and SSR data were subjected to a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992), using three hierarchical levels; individual, population and grouping based on their male, female and half sib genotypes. The non-parametric analysis of molecular variance (AMOVA) was done via GenAlex (Excoffier et al., 1992), where the variation component was partitioned among individuals within populations, among populations within groups and among groups. The resolving power of the RAPD and SSR primers was calculated according to (Prevost and Wilkinson, 1999). The resolving power (Rp) of a primer is: $Rp = \Sigma\ IB$ where IB (band informativeness) takes the value of: 1-[2* (0.5-P)], P being the proportion of the 94 genotypes containing the band.

In order to determine the utility of each of the marker systems, diversity index (DI), effective multiple ratio (EMR) and marker index (MI) were calculated according to Powell et al. (1996). DI for the genetic markers was calculated from the sum of squares of allele frequencies: $DI_n = 1 - \sum pi^2$ (where pi is the allele frequency of the ith allele). The arithmetic mean heterozygosity, DI_{av}, was calculated for each marker class: $DI_{av} = \sum DI_{n/n}$, (where n represents the number of the markers (loci) analyzed). The DI for the polymorphic marker is: $(DI_{av})p = \sum DI_n/n_p$ (where, n_p is the number of polymorphic loci and n is the total number of loci). EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay EMR (E) = $n_p(n_p/n)$. MI is defined as the product of the average diversity index for polymorphic bands in any assay and the EMR for that assay, $MI = DI_{avp}$ x E.

Table 1b. List of primers used for RAPD amplification, GC content, total number of loci, the level of polymorphism, resolving power and PIC value.

Primer	Primer sequence	% GC content	Annealing Temperature	Total number of loci	NPL	PPL	Number of fragments amplified	Rp	PIC
OPA-15	GGGCCACTCA	70	42	6	6	100	123	7.235	0.785
OPJ-2	GGAGAGACTC	60	41.7	6	5	83.3	144	8.471	0.795
OPJ-4	CACAGAGGGA	60	41.7	7	7	100	94	5.529	0.791
OPG-8	GGGTTTGGCA	60	38	5	5	100	116	6.824	0.783
OPJ-10	CAAGGGCAGA	60	45.5	4	4	100	93	5.471	0.713
OPJ-19	GGCAGGCTGT	70	42	6	6	100	127	7.471	0.793
OPJ-8	AACGGCGACA	60	41	6	6	100	122	7.176	0.782
OPJ-7	TTCCCCGCGA	70	41	6	6	100	118	6.941	0.779
OPG-6	AGGACTGCCA	60	45.5	6	6	100	119	7.00	0.781
OPG-11	GGTGAACGCT	60	43.8	6	6	100	123	7.235	0.785
OPH-10	CCAACGTCGT	60	42	6	6	100	123	7.235	0.785
OPH-15	GGTCGGAGAA	60	36	6	6	100	122	7.176	0.782
OPH-18	TCGGACGTGA	60	36	6	6	100	123	7.235	0.784
OPH-1	AGACGTCCAC	60	36	6	6	100	123	7.235	0.785
OPH-2	ACGCATCGCA	60	36	6	6	100	125	7.353	0.783

NPL, Number of polymorphic loci; PPL, Percentage of polymorphic loci; Rp, resolving power; PIC, polymorphism information content.

Polymorphism information content (PIC)

The frequency of the polymorphism obtained in the genotypes was calculated on the basis of presence (1) and absence (0) of the bands amplified. The PIC was calculated according to Anderson et al. (1993) based on the allele pattern of all the willow genotypes by employing the following formula:

$$PIC_i = 1 - \sum_{j=1}^{n} Pij^2$$

RESULTS AND DISCUSSION

Molecular analysis using RAPD markers

The RAPD technique had been successfully used in a variety of taxonomic and genetic diversity studies and it was found suitable for use with *Salix sp.* genotypes because of its ability to generate reproducible polymorphic markers. A total of 94 plant samples were fingerprinted using 15 RAPD makers. These primers produced multiple band profiles with a number of amplified DNA fragments varying from 4 to 7. All the amplified fragments varied in size from 100 to 2000 bp. Out of 88 amplified bands, 87 were found polymorphic (98.8%) (Table 1b). The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the *Salix sp.* The resolving power of the 15 RAPD primers ranged from 5.471 for primer OPJ-10 to a maximum of 8.471 for primer OPJ-2. Polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a population or

set of genotypes by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus. As evident, RAPD marker 'OPJ-2' showed the highest level of polymorphism with PIC value of 0.795, whereas the PIC values for the rest of the RAPD markers were in the range of 0.713 to 0.791. A dendrogram analysis based on bootstrapping and neighbor joining (NJ) method grouped all the 94 genotypes into three main clusters which are further extensively divided into mini clusters (Figure 1a). Similarly, an unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the populations clustered all the 94 genotypes into three major clusters.

Under the admixed model, STRUCTURE calculated that the estimate of likelihood of the data that is, ΔK reached its maximum value when K = 3 (Figure 1b [A]), suggesting that all the populations fell into one of the 3 clusters albeit small interference (Figure 1b [B]). This result is almost similar to the splitting in the NJ tree. Overall the cluster analysis strongly suggested that the 94 sampled genotypes can be divided into 3 clusters, however, there is no distinct clustering of genotypes based on their 4 female, 10 male, and 80 half sibs.

The genetic diversity of 94 genotypes was calculated in terms of Na, Ne, H, I, Ht, and PPL with respect to three different groups such as 4 female, 10 male and 80 half sibs revealed higher values, indicating more variability among the genotypes (Table 3). Polymorphic loci of 100% were calculated using POPGENE among four females, 10 males, and 80 half sib's genotypes. Three

Table 2. List of primers used for SSR amplification, GC content, total number of loci, the level of polymorphism, resolving power and PIC value.

Primer	Primer sequence	% GC content	Annealing Temp.	Total number of loci	NPL	PPL	Number of fragments amplified	Rp	PIC
SB-243	FP-ACT TCA ATC TCT CTG TAT TCT	47	53	2	1	50	36	2.118	0.105
	RP-CTA TTT ATG GGT TGG TCG ATC			3	2	66.7	83	4.882	0.656
SB-38	FP-CCA CTT GAG GAG TGT AAG GAT	53	54.5	3	2	66.7	81	4.765	0.646
	RP-CTT AAA TGT AAA ACT GAA TCT			2	2	100	61	3.588	0.450
SB-199	FP-CTA TTT GGT CTC AAT CAC CTT	53	58	3	2	66.7	84	4.941	0.656
	RP-CTT TAC CTC AGA AAA TCC AGA			5	4	80	96	5.647	0.736
SB-85	FP-CTC AGC AAC TTA ATC CAA CTA	53	59	6	6	100	117	6.882	0.777
	RP-GTT TGT TAG GGG AGG TAA GAA			2	2	100	52	3.059	0.488
SB-80	FP-TAA TGG AGT TCA CAG TCC TCC	44	54.3	4	3	75	56	3.294	0.563
	RP-ATA CAG AGC CCA TTT CAT CAC			3	2	66.7	57	3.353	0.526

NPL, Number of polymorphic loci; PPL, percentage of polymorphic loci, Rp, resolving power and PIC, polymorphism information content.

groups containing genotypes with different sexes such as female, half sibs and males showed Nei's genetic diversity (H): 0.390, 0.412 and 0.398, respectively and of Shannon's information index (I): 0.563, 0.601 and 0.583 (Table 3), respectively showed a higher genetic differentiation within each of the three groups. The respective values for overall genetic variability for Na, Ne, H, I, Ht, Hs, Gst, NPL, PPL and Gene flow (Nm) across all the 94 genotypes were also given in Table 4. The rate of gene flow estimated using Gst value was found to be 70.93 which is very high. Analysis of molecular variance among genotypes based on three major groups with respect to 4 female, 10 male, and 80 half sibs plant indicated that majority of genetic variation (99.53%) occurred among genotypes, while the variation between the three groups was minimum (0.50%) (Table 5).

SSR analysis

The 10 SSR primers selected in the study generated a total of 33 SSR bands (an average of 3.3 bands per primer), out of which 26 were polymorphic (78.8%) (Table 2). Among the dinucleotide repeat types (AG)n and (GA)n were produced more number of bands followed by (CT)n, and (AC)n. Similarly among the tri-nucleotide repeat types, (CTC)n produced more number of bands. The primers that were based on the (GA)n, (AG)n and (CT)n motif produced more polymorphism than the primers based on any other motifs used in the present investigation. We obtained good amplification products from primers based on (AG)n and (GA)n repeats, despite the fact that (AT)n di-nucleotide repeats are thought to be the most abundant motifs in plant species (Martin and Sanchez-yelamo, 2000). Similar results were obtained in grapevine (Moreno et al., 1998), rice (Blair et al., 1999), *Vigna* (Ajibade et al., 2000), wheat (Nagaoka and Ogihara, 1979) and *Salix* (Singh et al., 2013). A possible explanation of these results is that SSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimers during PCR amplification (Blair et al., 1999) or it may be due to its-non annealing with template DNA due to its low Tm. The resolving power (Rp) of the 10 SSR primers ranged from 2.118 to 6.882 (Table 2). Similarly the PIC value
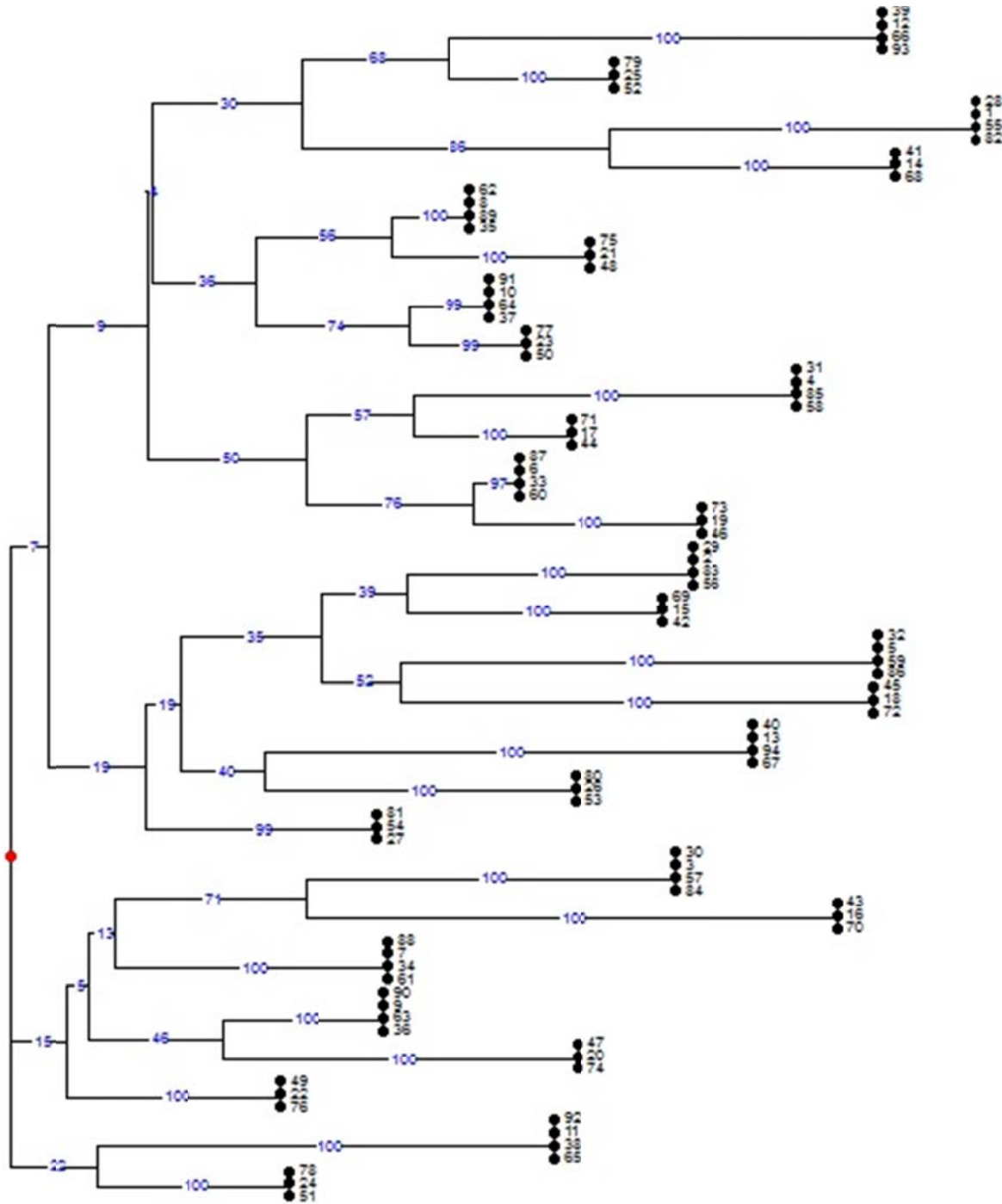
Figure 1a. Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 94 genotypes of Salix based on RAPD profiling. Number indicates bootstrap support values.

ranges from 0.105 to 0.777 demonstrating uniform polymorphism rate among all the 10 SSR primers.

The complete data set of 723 bands was used for cluster analysis based on bootstrapping and NJ method. The genotypes were clustered into three major clusters, well supported by bootstrap value of > 20 (Figure 2a). The estimated likelihood of the clustering of data using

STRUCTURE was found to be optimal that is, ΔK reached its maximum value when K = 3 (Figure 2b [A]), suggesting that all the populations were distributed with high probability into one of the 3 clusters (Figure 2b [B]). The clustering pattern of the genotypes were almost similar to the splitting in the NJ tree, however, there is no distinct clustering of genotypes based on their 4 females,
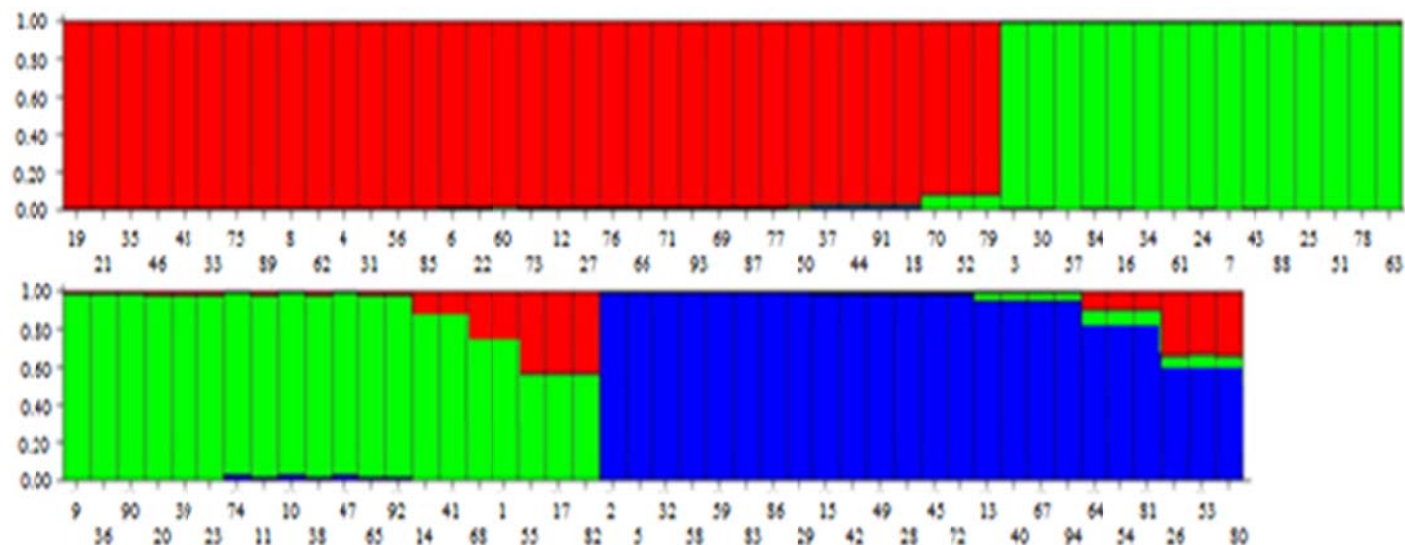
Figure 1b. A model based clustering of 94 genotypes of *Salix sps* based on RAPD profiling and using STRUCTURE without prior knowledge about the populations and under an admixed model. (A) The relationship between K and ΔK., that is, ΔK is reaches its maximum when K = 3, suggesting that all genotypes fall into one of the 3 clusters. (B) Grouping of genotypes when K = 3. The genotypes were more likely clustered with respect to one of the 3 clusters. Genotypes from different clusters are represented with different colours.

Table 3a. Summary of genetic variation statistics RAPD profiling among the genotypes of *Salix sps* grouped according to sex.

Sex	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Male	10	2.00 (0.00)	1.698 (0.234)	0.398 (0.094)	0.583 (0.108)	0.398 (0.009)	61	100
Half sibs	80	2.00 (0.00)	1.720 (0.178)	0.412 (0.065)	0.601 (0.071)	0.412 (0.004)	61	100
Female	4	1.917 (0.279)	1.697 (0.284)	0.390 (0.132)	0.563 (0.182)	0.390 (0.017)	60	91.67

Table 3b. Summary of genetic variation statistics SSR profiling among the genotypes of *Salix sps* grouped according to sex.

Sex	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Male	10	2.00 (0.00)	1.561 (0.174)	0.351 (0.074)	0.533 (0.086)	0.351 (0.005)	59	100
Half sib	80	2.00 (0.00)	1.389 (0.070)	0.278 (0.036)	0.450 (0.045)	0.278 (0.001)	59	100
Female	4	1.983 (0.129)	1.677 (0.188)	0.396 (0.073)	0.581 (0.094)	0.396 (0.005)	58	98.33

10 males and 80 half sibs plants.

A relatively high genetic variation was detected among the genotypes categorized into 3 different groups. Genetic diversity analysis in terms of Na, Ne, H, I, Ht, Hs, and PPL reveals higher value for the group with 4 female, 10 male, and 80 half sib plants. This disparity may be because of more number of genotypes included in the group with 4 female, 10 male and 80 half sibs (Table 3). Overall genetic variability across all the 94 genotypes in terms of Na, Ne, H, I, Ht, Hs, Gst, NPL, PPL and Gene flow (Nm) were also included in Table 4.

The Nei's genetic diversity index was 0.296 and Shannon information index was 0.471 demonstrating high rate of genetic variability. AMOVA for among groups (0.88%) and among genotypes (99.16%) indicated that there are more variations across the genotypes and not among the groups (Table 5). The estimated gene flow was 32.83.

Table 4. Overall genetic variability across all the 94 genotypes of *Salix sps* based on RAPD only, SSR only and combination of both RAPD and SSR markers.

Marker	Sample size	Na	Ne	H	I	Ht	Hs	NPL	PPL	Gst	Nm
RAPD	94	2.00 (0.00)	1.721 (0.179)	0.412 (0.065)	0.601 (0.071)	0.412 (0.004)	0.410 (0.004)	61	100	0.042	70.93
SSR	94	2.00 (0.00)	1.422 (0.063)	0.296 (0.031)	0.471 (0.038)	0.296 (0.001)	0.291(0.001)	59	100	0.025	32.83
RAPD + SSR	94	2.00 (0.00)	1.572 (0.201)	0.354 (0.078)	0.536 (0.086)	0.354 (0.006)	0.350 (0.006)	120	100	0.037	49.5

Gst, Genetic differentiation; Nm, gene flow.

Table 5. Summary of analysis of molecular variance (AMOVA) based on (a) RAPD only (b) SSR only and (c) combination of both RAPD and SSR markers among the genotypes of *Salix sp.* Levels of significance are based on 1000 iteration steps.

Source of variation	Degree of freedom	Variance component	Percentage of variation	P-value
Based on RAPD profiling				
Among groups	2.0	0.042	0.496	-
Among genotypes	91.0	8.953	99.53	< 0.001
Based on SSR profiling				
Among groups	2.0	0.063	0.880	-
Among genotypes	91.0	7.094	99.16	< 0.001
Based on combination of both RAPD and SSR profiling				
Among groups	2.0	0.069	0.944	-
Among genotypes	91.0	7.242	99.06	< 0.001

RAPD and SSR combined data for cluster analysis

Based on combined data set of RAPD and SSR markers, the dendrogram obtained gave similar clustering pattern like RAPD and SSR (Figure 3a). This result is corroborate with STRUCTURE analysis; the estimated likelihood of distribution for all the 94 genotypes was highest for example, ΔK was maximum with K = 3 (Figure 3b [A]), reveals that all the genotypes were clustered better (with high likelihood probability) with 3 clusters (Figure 3b [B]). Other genetic variation studies were also performed on RAPD and SSR combined data

which are represented in different tables (Tables 3, 4 and 5). The differences found among the dendrograms generated by RAPDs and SSRs could be partially explained by the different number of PCR products analyzed reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships as observed by Loarce et al. (1996) in barley. Another explanation could be the low reproducibility of RAPDs (Karp et al., 1997). The genetic similarity of these genotypes is probably associated with their similarity in the genomic and amplified region.

Comparative analysis of RAPD with SSR markers

RAPD markers were found more efficient with respect to number of polymorphism detection (based on average NPL value), as they detected 61 polymorphism loci as compared to 59 polymorphism loci for SSR markers. This is in contrast to the results obtained for several other plant species like wheat (Nagaoka and Ogihara, 1997) and *Vigna* (Ajibade et al., 2000). More polymorphism in case of RAPD than SSR markers might be due to the fact that 10 SSR primers used in the study only amplified 732 numbers of fragments
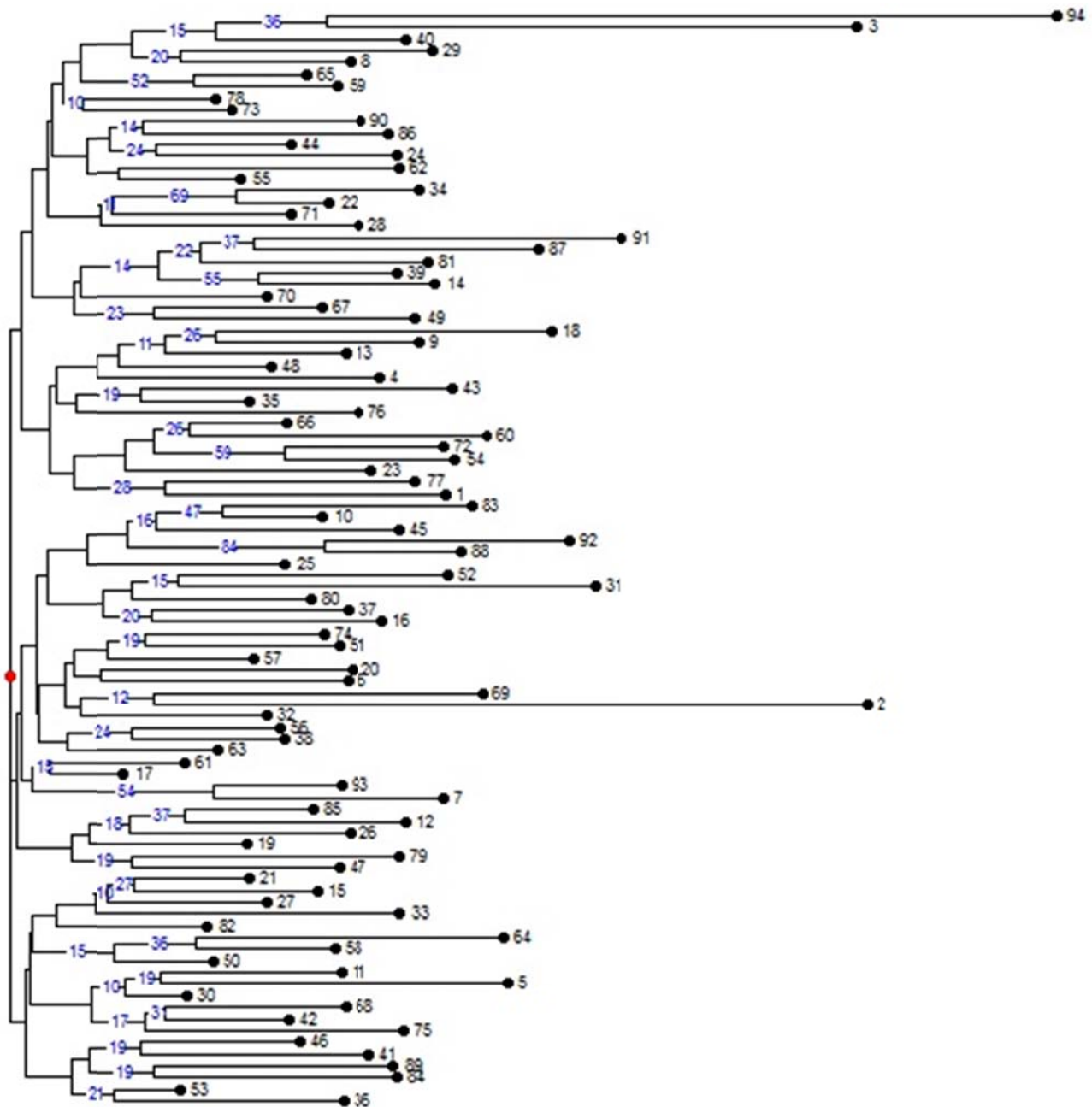
Figure 2a. Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 94 Salix genotypes based on SSR profiling. Number indicates bootstrap support values.

(Table 2). While in case of RAPD, all the 15 primers which were used in the investigation amplified 1795 number of fragments (Table 1). Similar polymorphism pattern was also observed in case of *Jatropha* (Gupta et al., 2008) and *Podophyllum* (Alam et al., 2009). This shows that RAPD data is more close to RAPD+SSR combined data. A possible explanation for the difference in resolution of RAPDs and SSRs is that the two-marker techniques target different portions of the genome. The

mean effective multiplex ratio is more for RAPD (6.246) than that for SSR (4.588) and similarly marker index is more for RAPD (0.876) than that for SSR (0.751) markers.

Conclusion

In this study, we may conclude that molecular analyses of
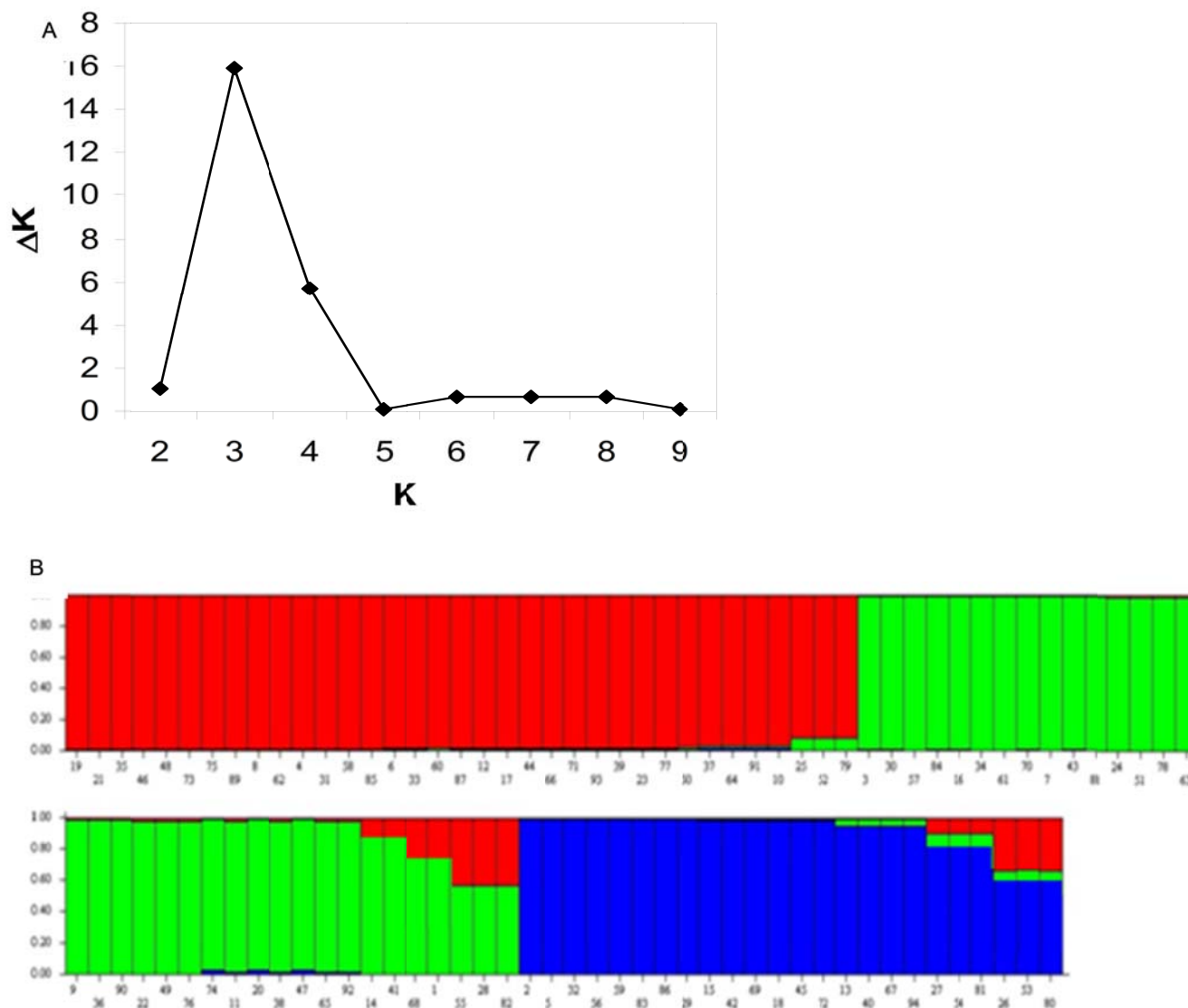
Figure 2b. A model based clustering of 94 genotypes of *Salix sps* based on SSR profiling and using STRUCTURE without prior knowledge about the populations and under an admixed model. (A) The relationship between K and ΔK., i.e. ΔK is reaches its maximum when K = 3, suggesting that all genotypes fall into one of the 3 clusters. (B) Grouping of genotypes when K = 3. The genotypes were more likely clustered with respect to one of the 3 clusters. Genotypes from different clusters are represented with different colours.

both RAPD and SSR markers were extremely useful for studying the genetic relationships of *Salix* genotypes. The results indicates the presence of high genetic variability, which should be exploited for the future conservation and breeding of willow sp. Since no single, or even a few plants, will represent the whole genetic variability in willow, it is essential to maintain sufficiently large popu-lations in natural habitats to conserve genetic diversity in willow to avoid genetic erosion. Based on polymorphic feature, genetic diversity, genetic similarity, and gene flow among the populations of *Salix* based on molecular markers study, we recommend that any future conser-vation plans for this species should be specifically designed to include representative populations with the highest genetic variation for both in situ conservation and germplasm collection expeditions.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Figure 3a. Dendrogram generated by neighbor joining (NJ) clustering technique showing relationships between 94 genotypes of *Salix* based on combination of both RAPD and SSR profiling. Number indicates bootstrap support values.
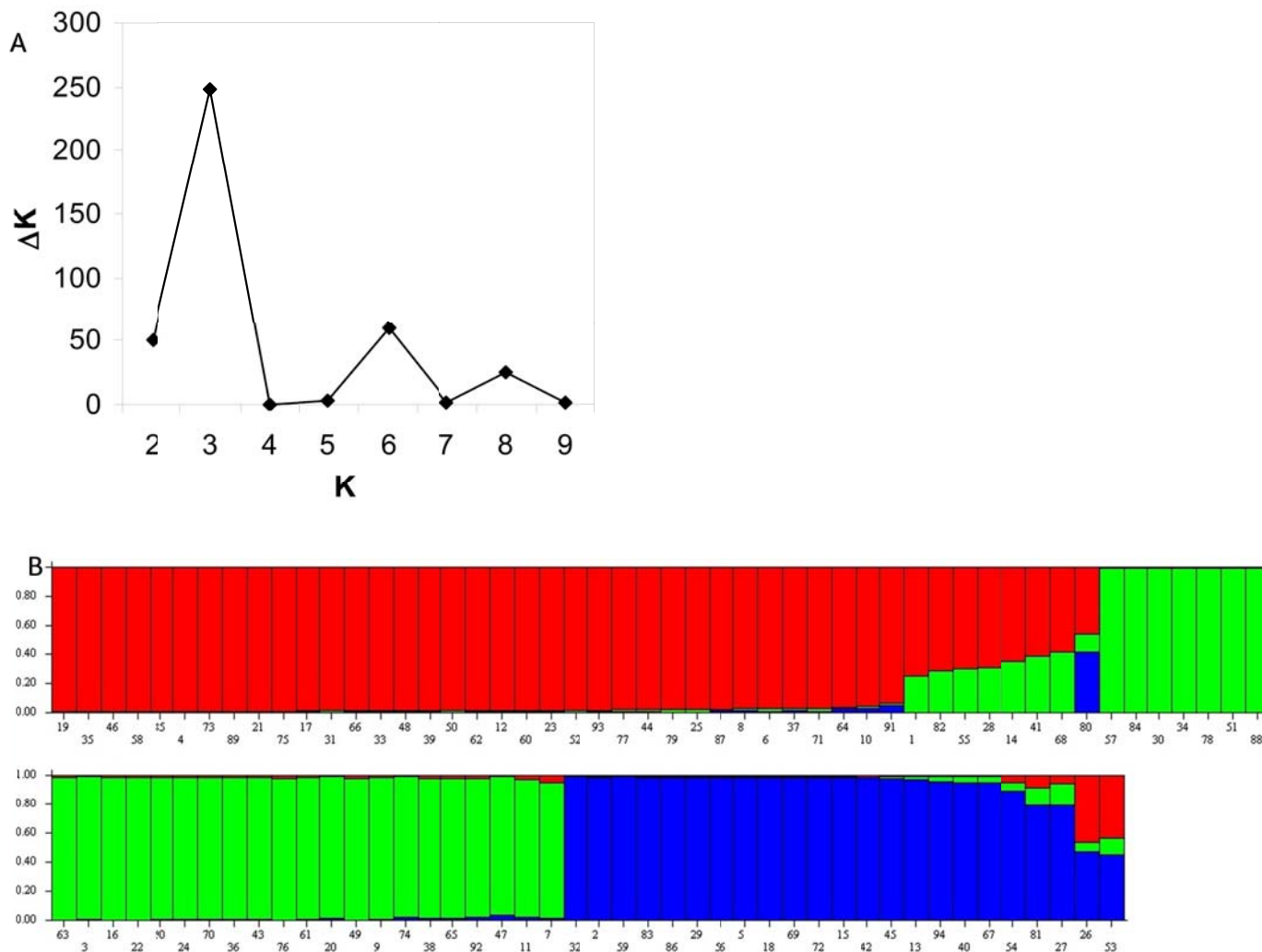
Figure 3b. A model based clustering of 94 genotypes of *Salix sps* based on combination of RAPD and SSR profiling and using STRUCTURE without prior knowledge about the populations and under an admixed model. (A) The relationship between K and ΔK., that is, ΔK is reaches its maximum when K = 3, suggesting that all genotypes fall into one of the 3 clusters. (B) Grouping of genotypes when K = 3. The genotypes were more likely clustered with respect to one of the 3 clusters. Genotypes from different clusters are represented with different colours.

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Full Length Research Paper

Growth, physiology and flowering of chrysanthemum var. Punch as affected by daminozide and maleic hydrazide

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Growth retardants have been proven to prevent excessive stem elongation and reduce internode length in plants by inhibiting the effect of cell division and enlargement of cell in plants. This study was aimed to evaluate the effect of concentrations of daminozide and maleic hydrazide on growth, physiology and flowering of chrysanthemum cultivar Punch under protected condition. The treatments composed of two growth retardants: daminozide at four different concentrations (1000, 1500, 2000 and 2500 ppm) with three frequencies (7, 14 and 21 days after darkening) and maleic hydrazide at three different concentrations (500, 750 and 1000 ppm) with two frequencies (7 and 21 days after darkening) along with control (no spray given). Hence based on the results obtained, it can be concluded that single application of daminozide at 2500 ppm at seven days after darkening reduced the plant height at considerable height (34.33, 83.33 and 103.58 cm) at critical stages and improved total leaf area (1694.55 and 1745.27 cm^2) at bud appearance and peak flowering stage and showed earliness in flowering and days to harvest (44.72 and 81.33 day), increased pedicel length (5.87 cm), cut stem girth (3.24 cm), stem fresh weight (68.00 g/stem) and chlorophyll (1.93, 2.53 and 2.80 mg/g) and soluble protein content (65.66, 72.33 and 80.66 mg/g) at critical stages. Increased cut stem yield (77.34 stems/m^2) and improved vase life (12.50 day) were also recorded. This may be recommended to improve growth and flower quality of 'Punch' chrysanthemum under greenhouse conditions.

Key words: Chrysanthemum, daminozide, maleic hydrazide, growth, flowering.

INTRODUCTION

Chrysanthemum (Family: Asteraceae) is one of the most beautiful leading commercial flower crop grown as cut flower for interior decoration and as well as pot plant in the world. It ranks second in the international cut flower trade. Plants are able to modify their growth, development and physiology according to the variable

environment. In tropical country like India, during summer months when the day length exceeds 12 h it resulted in excess stem elongation due to translocation of stored energy to the apical growing tip which causes ultimate reduction of flower stalk girth and flower quality. These problems associated and affected in meeting out the standard of cut chrysanthemum in the export market. So as to overcome this, in recent years scientists have given due attention to the idea of regulating the plant growth as the most important factor in improving the growth, yield and flower quality with the application of plant growth regulators in various ways. Growth retardants have been proven to prevent excessive stem elongation and reduce internode length in plants (Kuehny et al., 2001). Height control in floriculture can be succeeded by using growth retardants, since it has an important role in reducing "long neck" in chrysanthemum resulting in lower and stronger plants with less lodging (Karlovic et al., 2004). Retardants such as daminozide commercially known as B-Nine or Alar and Maleic Hydrazide are successfully applied to control lodging of plants, height, branching and obtain higher yield in chrysanthemum, like many other plant species (Karlovic et al., 2004). Kahar (2008) found that single application of B-9 at 2500 ppm in Chrysanthemum cv. Reagan Sunny delayed the time of flowering and improved the synchrony of flowering and double the vase life. In *Chrysanthemum morifolium*, foliar spraying of B-9 at 1000 ppm significantly decreased the plant height, the branch number and leaf number but the content of chlorophyll and soluble sugar in leaves was significantly increased was reported by Zhi-kai (2008).

Various growth regulators, especially growth retardants are nowadays being used for controlling growth with a view to have compact plants and to stretch out or retard the plant growth (Navalae et al., 2010). Daminozide inhibited gibberellin biosynthesis or action and induced flowering. Kazaz et al. (2010) reported that different day lengths and daminozide application at 3000 ppm had significant effect on days to flower by 42 days, increased chlorophyll a (11.50 µg/mg), chlorophyll b (5.93 µg/mg), compact and high quality flowers with desired stem length as compared to short day conditions. Piotr and Anita (2012) found that foliar spraying of daminozide 2500 ppm two times found no retardation of flowering in chrysanthemum cv. Leticia Time Yellow. Minimum plant height (142.25 cm) and internodal length (9.85 cm) was recorded in the plants treated with Alar at 1500 ppm reported by Vijai Ananth and Ramesh Kumar (2012) In this context, the objective of this study was aimed to evaluate the effect of concentrations of daminozide and maleic hydrazide on growth, physiology and flowering of spray chrysanthemum var. Punch grown under greenhouse conditions. The research findings are based on the key parameters necessary for regulating the growth of chrysanthemum under protected conditions and hoped to be valuable information for flower growers and researchers.

MATERIALS AND METHODS

The study was conducted in a naturally ventilated greenhouse at a private farm of M/s Salem Green Plants Limited, Yercaud (Latitude 11° 04" to 11° 05" N, Longitude 78° 05" to 78° 23" E and Altitude 1500 m MSL), Salem, Tamil Nadu during 2011 - 2013 to evaluate the effect of concentrations of daminozide and maleic hydrazide on growth, physiology and flowering of spray chrysanthemum var. Punch. A naturally ventilated poly house of 50 × 56 m^2 area in East-West orientation was erected with 800 gauge UV stabilized poly ethylene sheet. The average temperature (Maximum: 22°C, Minimum: 16°C), relative humid (75%) and photoperiod (long day: 13 h during early vegetative stage and short day - 14 h during bud appearance stage) was maintained throughout the growing period. The experiment was laid out in a Randomized Block design (RBD) with 19 treatments and three replications. The treatment comprises two growth retardants: daminozide at four different concentrations (1000 ppm, 1500, 2000 and 2500 ppm) with three frequencies (7, 14 and 21 days after darkening) and Maleic Hydrazide at three different concentrations (500 ppm, 750 ppm and 1000 ppm) with two frequencies (7 and 21 days after darkening) along with control (no spray given) (Table 1).

Rooted cuttings of spray chrysanthemum var. Punch obtained from the mother stock were planted in raised beds (height - 45 cm) containing growing media consortia [Coco peat (3 kg/ m^2), Vermicompost (500 g/ m^2), Perlite (500 g/ m^2) and microbial consortia (50 g/m^2)] with a spacing of 12.5 x 10 cm accommodating 80 plants per m^2. The characteristics of greenhouse soil was analysed (soil type: sandy clay loam, pH: 5.5, EC (ds/m): 0.3, N - 526 kg/ha, P - 61.7 kg/ha and K - 332 kg/ha). Daminozide was sprayed to the experimental plots at 7, 14 and 21 days after darkening and Maleic Hydrazide at 7 and 21 days after darkening. Five plants were randomly tagged in each of the plot (treatment and replication wise) and observed for growth, physiology and flowering parameters at critical stages of the crop growth. All other agronomic operations and plant protection measures were done as per the schedule.

Plant height

The height was measured from the base of the plant to the terminal tip at the critical stages namely: peak vegetative, bud appearance and peak flowering stage and the mean values were expressed in centimeters (cm).

Total Leaf area per plant

The total leaf area per plant was measured using the leaf area meter (Make: Leaf Area Meter, Li- 3100) at the critical stages and the mean was worked out and expressed in square centimeter (cm^2).

Determination of chlorophyll content

Fresh leaves were collected and the total chlorophyll contents in the leaves were determined by following the method of Yoshida et al. (1971) and expressed in mg/g.

Determination of soluble protein content

Soluble protein content was estimated with tricarboxylic acid extract of leaf sample following the method of Lowery et al. (1957) and

Table 1. Treatment comprising two growth retardants.

T	Treatment details	T	Treatment details
T_1	Daminozide 1000 ppm spray at 7th day after darkening	T_{11}	Daminozide 2500 ppm spray at 7th and 14th days after darkening
T_2	Daminozide 1000 ppm spray at 7th and 14th day after darkening	T_{12}	Daminozide 2500 ppm spray at 7, 14 and 21st days after darkening
T_3	Daminozide 1000 ppm spray at 7th, 14 and 21st day after darkening	T_{13}	MH 500 ppm spray at 7th day after darkening
T_4	Daminozide 1500 ppm spray at 7th day after darkening	T_{14}	MH 500 ppm spray at 7th and 21st day after darkening
T_5	Daminozide 1500 ppm spray at 7th and 14th days after darkening	T_{15}	MH 750 ppm spray at 7th day after darkening
T_6	Daminozide 1500 ppm spray at 7, 14 and 21st days after darkening	T_{16}	MH 750 ppm spray at 7th and 21st day after darkening
T_7	Daminozide 2000 ppm spray at 7th day after darkening	T_{17}	MH 1000 ppm spray at 7th day after darkening
T_8	Daminozide 2000 ppm spray at 7th and 14th days after darkening	T_{18}	MH 1000 ppm spray at 7th and 21st day after darkening
T_9	Daminozide 2000 ppm spray at 7, 14 and 21st days after darkening	T_{19}	Control (no spray)
T_{10}	Daminozide 2500 ppm spray at 7th day after darkening		

T = Treatment.

expressedin milligrams per gram (mg/g) of fresh weight.

$$\text{Amount of soluble protein} = \frac{X}{1} \times \frac{25 \times 1000}{250}$$

Where, X = Corresponding concentration in the standard graph (µg)

Days to first flower bud appearance

The number of days taken from the date of planting to visible flower bud appearance was recorded and the mean was expressed in days.

Days to first harvest

The number of days taken from the date of planting to the first harvest was counted and expressed in days.

Flower stalk length (cm)

The length of flower stalk was measured from the fourth node to the base of flower and expressed in centimeters (cm).

Pedicel length

The pedicel length was measured from the point of pedicel attachment to the stalk to its attachment to the base of the flower and it expressed in centimeter (cm).

Stem girth

The girth of the stem at peak flowering stage was measured using thread and expressed in centimeters (cm).

Stem fresh weight

The cut stem along with flowers was cut at the peak flowering stage and weighed using weighing balance and the mean was expressed in gram (g).

Cut stem yield/m^2

The number of stems harvested per square meter area was counted and expressed in numbers for yield/m^2.

Vase life

The flower stalks harvested from experimental plot were kept in distilled water at room temperature and the vase life was evaluated daily by counting the number of days taken for the symptom of shriveling and wilting (Halevy and Mayak, 1979).

Statistical analysis

Data were analyzed statistically for analysis of variance per the method suggested by Panse and Sukhatme (1985). The critical differences were worked out for 5 per cent (0.05) probability. The mean differences were compared using the LSD test (p<0.05).

RESULTS AND DISCUSSION

Plant height recorded in the experiment were significantly (p<0.05) affected by growth retardant applications at varied frequencies and concentration (Table 2). The mean data presented in the table shows a difference in significant height reduction (14.66 and 7.48 cm) in chrysanthemum with single spray of daminozide 2000 ppm at 7th day after darkening (T_7) when compared to

Table 2. Influence of daminozide and maleic hydrazide on plant height (cm) and total leaf area per plant (cm^2) of spray chrysanthemum var. Punch.

Treatment	Plant height (cm)			Total leaf area per plant (cm^2)		
	Peak vegetative stage	Bud appearance stage	Peak flowering stage	Peak vegetative stage	Bud appearance stage	Peak flowering stage
T$_1$	31.20[gh]	79.87[efgh]	108.53[gh]	657.17[hi]	799.52[j]	892.97[hi]
T$_2$	33.00[hi]	82.89[fgh]	115.27[hij]	1547.69[a]	1694.55[a]	1745.27[a]
T$_3$	27.03[d]	81.87[fgh]	117.06[ij]	984.00[cd]	1095.22[def]	1128.49[de]
T$_4$	28.87[def]	82.13[efgh]	117.90[j]	1142.71[b]	1183.24[c]	1235.04[c]
T$_5$	22.13[ab]	78.87[efgh]	106.33[fg]	732.36[gh]	819.44[j]	879.81[hi]
T$_6$	27.00[d]	80.60[fgh]	107.60[gh]	838.24[defg]	921.24[i]	984.04[fg]
T$_7$	21.67[a]	77.27[efgh]	118.20[j]	1048.14[bc]	1167.63[cd]	1211.03[c]
T$_8$	24.80[c]	80.60[efgh]	109.96[ghi]	956.40[cde]	1102.46[de]	1187.06[cd]
T$_9$	29.63[efg]	81.73[fgh]	108.55[gh]	830.69[efg]	953.62[ghi]	1054.08[ef]
T$_{10}$	34.33[ij]	83.33[gh]	103.58[efg]	1473.03[a]	1576.95[b]	1634.92[b]
T$_{11}$	30.13[fg]	82.40[fgh]	114.80[hij]	908.23[cde]	1026.52[fg]	1066.80[e]
T$_{12}$	29.50[efg]	79.50[efgh]	106.97[g]	939.58[cd]e	1071.66[ef]	1093.32[e]
T$_{13}$	27.67[de]	67.37[ab]	99.01[def]	662.31[hi]	915.37[i]	944.70[gh]
T$_{14}$	23.97[bc]	69.36[bc]	91.60[cd]	767.45[fg]h	829.67[j]	885.00[hi]
T$_{15}$	26.93[d]	74.93[cde]	97.33[de]	862.25[defg]	940.69[hi]	984.03[fg]
T$_{16}$	23.50[abc]	75.47[bde]	87.80[bc]	672.49[hi]	805.54[j]	817.64[ij]
T$_{17}$	23.17[abc]	71.30[acd]	83.37[b]	872.95[def]	997.58[gh]	1058.08[ef]
T$_{18}$	23.67[abc]	63.37[a]	72.07[a]	562.64[ij]	723.14[k]	768.66[j]
T$_{19}$ (control)	36.33[j]	84.75[h]	120.77[j]	450.75[j]	686.84[k]	826.20[ij]
S.Ed	1.029	2.806	3.836	74.682	36.238	38.092
CD (p=0.05)	2.087	5.690	7.780	151.479	73.503	77.263

Means followed by a common letter are not significantly different at the 5% level by LSD.

control (T$_{19}$) at peak vegetative and bud appearance stage. At the peak flowering stage, a single spray of daminozide 2500 ppm (T$_{10}$) recorded a considerable reduction in height difference (17.19 cm) without affecting the total stem length which is the most important criteria to be considered in the export market. Height reduction in the daminozide treatments might be due to reduction in number of cells in stem and complete suppression of apical dominance which inhibits the cell division. The result shows that single application of daminozide to 'Punch' chrysanthemum was sufficient because the inhibitory effect was long enough. This is in agreement with the findings of Mahalle et al. (2001), Khobragade et al. (2002), Karlovic et al. (2004), Zhi-kai (2008) and Piotr and Schroeter (2011) in chrysanthemum. Instead, maleic hydrazide treatments recorded drastic height control over daminozide treatments which resulted in severe reduction of stem length in spray chrysanthemum but poor flower quality was observed in terms of flower size and flowering synchrony.

Leaves serve as the index of measurement of vegetative growth and in determining the yield potential. Improvement in the total leaf area was observed in all the treatments receiving daminozide irrespective of concentrations. Foliar spray of daminozide 2500 ppm at 7th day after darkening (T$_{10}$) significantly increased the total leaf area per plant at peak vegetative (1473.03 cm^2) and T$_2$ (daminozide 1000 ppm at 7 and 14th day after darkening) at bud appearance (1694.55 cm^2) and flowering stage (1745.27 cm^2) over control. This might be due to the supplementary action of daminozide in cell division and the ability of the compound to be translocated to the meristematic tissue. This probably accounts for its effect on axillary leaf growth which resulted in increased number of leaves and ultimately reflected on the leaf area and leaf area index. This result is not in agreement with the findings of El-Mokadem and Hadia (2008) who reported that daminozide reduce the leaf number and area. On the other side, leaf area registered significantly a decreasing trend (T$_{18}$) with the increase in concentration of maleic hydrazide.

The leaf chlorophyll content is an important physiological factor as it directly influences the photosynthesis and it occurs in chloroplast as green pigments in all photosynthetic plant tissues. It also acts as an index of metabolic efficiency of plant to utilize the absorbed light radiation for dry matter production. Growth retardants affected the total chlorophyll contents at all the critical stages of the crop growth (Figure 1). Levels of total
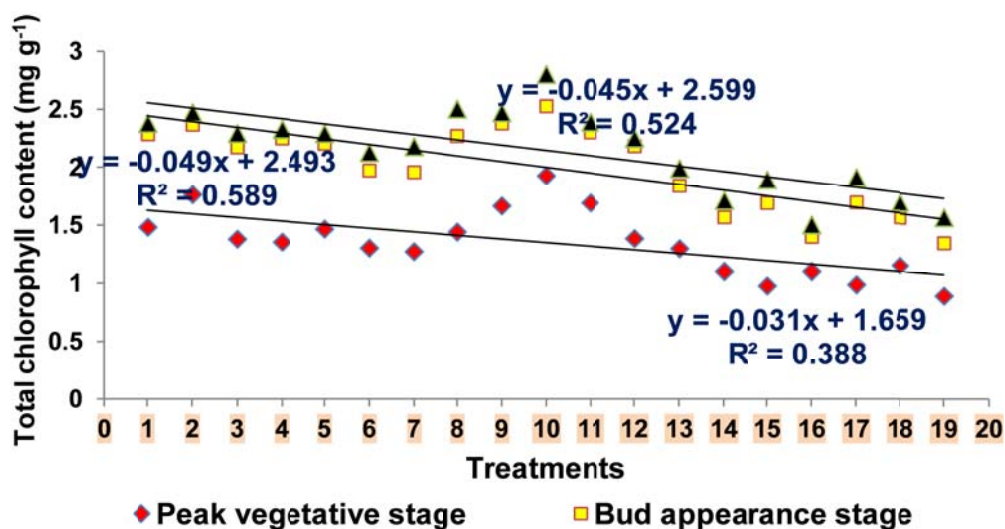
Figure 1. Total chlorophyll content in chrysanthemum 'Punch' with the application of different concentrations of daminozide and maleic hydrazide.

chlorophyll at peak vegetative, bud appearance and peak flowering stage was found to be higher (1.62, 1.81 and 2.05 mg/g) in T_{10} (daminozide 2500 ppm at 7 days after darkening) which was followed by T_2 (1.52 mg/g, 1.71 mg/g and 1.75 mg/g) and T_9 (1.46 mg/g, 1.70 mg/g and 1.78 mg/g) respectively. Daminozide significantly increased the content of chlorophyll in leaves, the leaf colour became deeper and photosynthetic efficiency was increased (Kazaz et al., 2010). Control (T_{19}) recorded the lowest total chlorophyll content at all the stages (0.85, 1.25 and 1.33 mg/g) of growth.

Soluble protein is a key factor in determining the photosynthetic efficiency of the crop plants. Analysis of soluble protein revealed that spraying of daminozide at 2500 ppm at 7th day alone (T_{10}) increased the protein content (65.66, 72.33 and 80.66 mg/g) at the critical stages. The results indicated that more soluble protein in the plant system leads to high RuBisCO activity, which ultimately induce high carbon fixation, improve photosyn-thetic efficiency and result in high yield (Table 3). The least soluble protein content was registered in the plots that not received any of the concentration of growth retardants at any frequency (control).

Days taken for first flowering were significantly affected by daminozide and maleic hydrazide application at different concentrations and intervals (Table 4). The earlier flowering (44.72 day) was recorded in the plants receiving daminozide at 2500 ppm spray at 7th day after darkening (T_{10}). However, the treatments receiving daminozide spray irrespective of frequencies showed positive influence on earliness in flowering and days to harvest. The earliness in flowering might be due to the effect of chemicals on height control, the stored food reserves translocated and diverted to the sink. This is not in

agreement with the findings of Kahar (2008), Zhi-kai (2008), (Kazaz et al., 2010) and Piotr and Schroeter (2011) who have reported that daminozide delayed flowering in chrysanthemum. The delayed flowering in MH treatments was observed more than control. This might be due to the result of growth inhibition as it is necessary for increasing / decreasing the flower formation and their development. However there was improvement in the flowering synchrony with daminozide applications.

In general, daminozide application regulated the stalk length in 'Punch' chrysanthemum. Foliar application of daminozide at 2500 ppm at 7th day after darkening (T_{10}) recorded marketable reduction in flower stalk length than control. The results of the present study are in agreement with the findings of Karlovic et al. (2004) and El-Sheibany et al. (2007). On the other hand, maleic hydrazide treatments viz., T_{13}, T_{14} T_{15} T_{16} T_{17} and T_{18} recorded poorest stem length (93.61, 86.10, 92.43, 82.70, 79.07 and 67.07 cm) respectively. In the present studies, the effectiveness of daminozide and maleic hydrazide were compared. The effects of these substances depend on their concentration and frequencies. It was also observed that increasing concentrations and frequencies showed maximum reduction in plant height and also total stalk length in chrysanthemum var. Punch.

Significant differences were observed among the growth retardants for pedicel length. The mean data presented in the Table 5 reveals that single spray at 7 days after darkening with daminozide 2500 ppm (T_{10}) recorded the maximum pedicel length (5.87 cm) which is followed by T_8 (5.73 cm) and T_{15} (5.38 cm). This might be due to the action of daminozide which removes apical dominance and increase the pedicel length. The increase

Table 3. Effect of daminozide and maleic hydrazide on soluble protein content (mg/g) at critical stages of spray chrysanthemum var. Punch.

Treatment	Soluble protein content (mg/g)		
	Vegetative stage	Bud appearance stage	Flowering stage
T_1	59.93^{cd}	64.56^d	68.31^c
T_2	63.15^b	69.28^b	75.69^b
T_3	61.17^{bc}	64.99^d	61.06^{fgh}
T_4	52.95^g	58.37^e	63.52^{ef}
T_5	54.32^g	60.56^e	60.35^{ghi}
T_6	58.67^{de}	66.10^c	64.93^{de}
T_7	53.71^g	55.51^f	59.14^{hi}
T_8	56.73^{ef}	55.26^f	62.86^{efg}
T_9	60.02^{cd}	65.21^d	74.93^b
T_{10}	65.66^a	72.33^a	80.66^a
T_{11}	62.92^b	67.83^{bc}	67.06^{cd}
T_{12}	55.15^{fg}	48.95^g	66.30^{cd}
T_{13}	57.72^{de}	44.25^h	57.70^{ij}
T_{14}	54.64^{fg}	40.86^{jk}	50.27^{kl}
T_{15}	43.76^h	50.22^g	46.02^m
T_{16}	36.74^j	42.93^j	48.15l^m
T_{17}	32.75^k	38.75^{kl}	52.38^k
T_{18}	40.03^i	46.23^h	55.31^j
T_{19} (control)	30.50^k	36.41^l	45.74^m
SEd	1.162	1.222	1.345
CD (p=0.05)	2.358	2.478	2.728

Means followed by a common letter are not significantly different at the 5% level by LSD

Table 4. Effect of daminozide and maleic hydrazide on days to first flower bud appearance, days to harvest and flower stalk length (cm) of chrysanthemum.

Treatment	Days to first flower bud appearance (day)	Days to harvest (day)	Flower stalk length (cm)
T_1	50.95^{cde}	87.40^{abc}	104.53^{gh}
T_2	46.54^{ab}	84.67^{ab}	109.17^{hi}
T_3	54.03^{ef}	88.27^{bcd}	112.09^{ij}
T_4	53.60^{def}	90.37^{cde}	114.40^{ij}
T_5	49.56^{bcd}	95.78^{efg}	101.29^{fg}
T_6	52.64^{de}	89.33^{bcd}	102.10^{fg}
T_7	51.60^{cde}	92.56^{def}	113.60^{ij}
T_8	57.80^{fg}	86.93^{abc}	104.66^{gh}
T_9	47.31^{abc}	85.87^{abc}	104.25^{fgh}
T_{10}	44.72^a	81.83^a	98.58^{ef}
T_{11}	53.54^{def}	93.67^{ef}	109.30^{hi}
T_{12}	52.47^{de}	87.60^{bcd}	102.37^{fg}
T_{13}	66.50^{ij}	101.63^h	93.61^{de}
T_{14}	60.81^{gh}	95.97^{fg}	86.10^c
T_{15}	64.37^{hi}	99.37^{gh}	92.43^d
T_{16}	69.88^j	102.33^h	82.70^{bc}
T_{17}	69.60^j	104.73^h	79.07^b
T_{18}	74.81^k	110.50^i	67.07^a
T_{19} (control)	54.95^{ef}	90.07^{bcd}	115.37^j

Table 4. Contd,

SEd	2.138	2.769	2.905
CD (p=0.05)	4.337	5.616	5.891

Means followed by a common letter are not significantly different at the 5% level by LSD.

Table 5. Effect of daminozide and maleic hydrazide on pedicel length (cm), cut stem yield/m^2 and vase life (d) of spray chrysanthemum var. Punch.

Treatment	Pedicel length (cm)	Cut stem yield / m^2	Vase life (day)
T$_1$	4.36^{ij}	74.65^{abc}	10.00^{fg}
T$_2$	5.30^{cd}	76.21^{ab}	11.76^b
T$_3$	4.56^{ghi}	73.65^{bcd}	10.04^{fg}
T$_4$	4.51^{hi}j	71.33^{ef}	9.76^g
T$_5$	5.02^{def}	73.67^{bcd}	10.02^{fg}
T$_6$	4.71^{gh}	70.97^{efg}	8.97^h
T$_7$	4.73^{fgh}	73.15^{ce}f	10.75^{cd}
T$_8$	5.73^{ab}	72.66^{def}	9.68^g
T$_9$	5.47^{bc}	75.68^{abc}	10.95^c
T$_{10}$	5.87^a	77.34^a	12.50^a
T$_{11}$	5.26^{cde}	70.21^{fg}	10.20^{ef}
T$_{12}$	5.00^{ef}	68.34^g	10.47^{de}
T$_{13}$	4.25^j	64.84^h	8.44^{ij}
T$_{14}$	4.27^{ij}	60.21^i	8.21^{jk}
T$_{15}$	5.38^c	55.29^j	7.67^l
T$_{16}$	4.39^{ij}	52.40^j	8.64^{hi}
T$_{17}$	4.32^{ij}	47.31^k	6.95^m
T$_{18}$	4.81^{fg}	45.25^k	7.86^{kl}
T$_{19}$ (control)	3.89^k	74.35^{bcd}	5.27^m
SEd	0.142	1.457	0.208
CD (p=0.05)	0.287	2.956	0.426

Means followed by a common letter are not significantly different at the 5% level by LSD.

in pedicel length due to daminozide was also observed by Zhang (2011) in *Chrysanthemum morifolium* Ramat. Cv. Youxiang. Maleic hydrazide treated plants recorded lesser pedicel length when compared to daminozide treated plots.

Stem girth is an important criterion for determining stem strength as it directly relates ability of the stem to bear numerous flowers and also in improving post harvest life. Growth retardant applications significantly increase the cut stem girth (Figure 2). The stem girth was measured as 5.87 cm in T$_{10}$ (daminozide 2500 ppm at 7 days after darkening) which is on par with the treatment (T$_8$) which received daminozide 2000 ppm at 7 and 14 days after darkening (5.73 cm). In this study daminozide application irrespective of concentration increased the stem girth. Similar results were reported by Mahalle et al. (2001); Khobragade et al. (2002) and Kim et al. (2010), While T$_{19}$ recorded the lowest stem girth of 3.89 cm.

The effect of daminozide and maleic hydrazide on stem

fresh weight was found statistically significant (Figure 3). A higher stem fresh weight (68.00 g/stem) was recorded in T$_{10}$ (daminozide 2500 ppm at 7 days after darkening) followed by T$_2$ (65.79 g/stem) and T$_9$ (61.98 g/stem) and lowest was recorded in control (39.50 g/stem). Spray chrysanthemums are graded by stem length, stem fresh weight and maturity at the Dutch Flower Auction Association (VBN). In this grading, stem fresh weight ranges from 25 to 105 g in spray chrysanthemum depending on the grading codes. The data on fresh weight of stems obtained in the study are in agreement with the above mentioned standards.

In general, the flower yield of any crop is determined by various yield components. Yield attributing characters *viz.,* total stalk length, pedicel length, stem girth and stem fresh weight have definite role in successful commercial cultivation of greenhouse chrysanthemums. Important yield components namely: marketable stem yield per sq.m was favourably influenced in the treatments receiving damino-
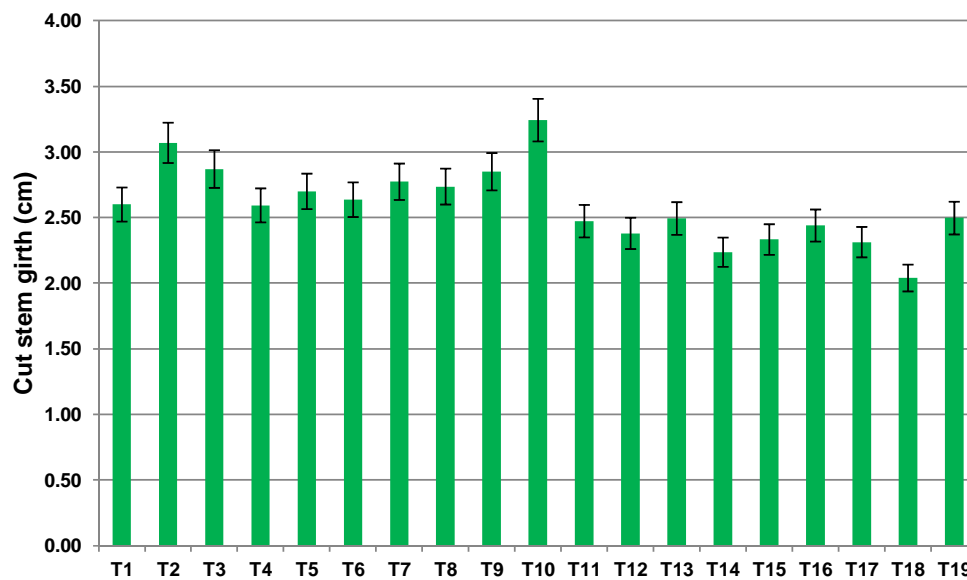
Figure 2. Cut stem girth of flowers in chrysanthemum 'Punch' with the application of different concentrations of daminozide and maleic hydrazide.
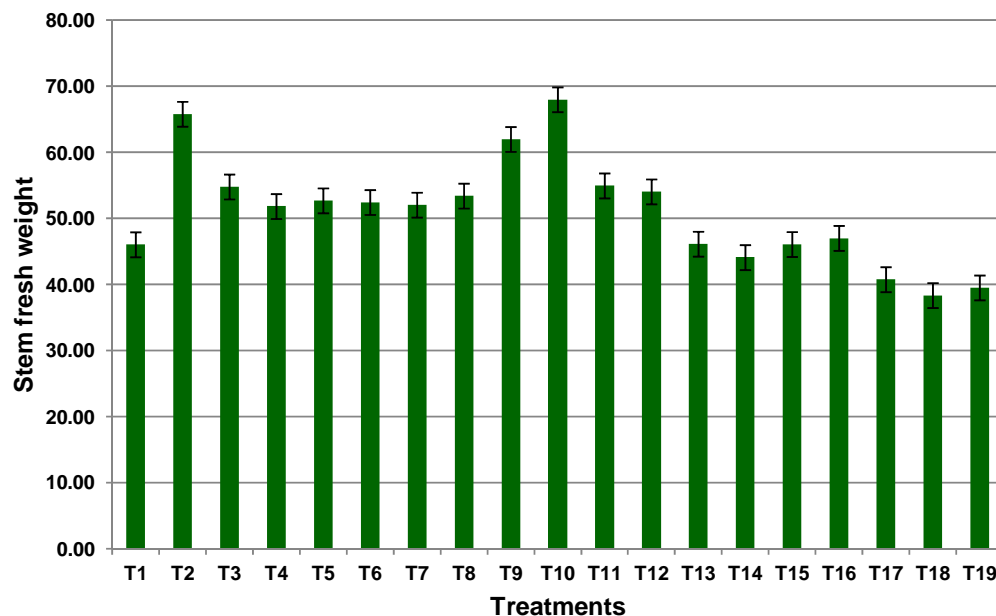
Figure 3. Fresh weight of cut stem chrysanthemum 'Punch' with the application of different concentrations of daminozide and maleic hydrazide.

zide, irrespective of frequencies (Table 5). This illustrates the ability of daminozide to enhance the number of flowers per spray, stem girth and stem fresh weight at proper concentration and in reliable frequency.

Longest vase life (12.50 days) was achieved in the treatment receiving daminozide at 2500 ppm at 7 days after darkening than the control (5.27 days). Internal physiolo-

gical status of the cut stems might have helped in delaying early onset of senescence. Positive effect of daminozide on inhibiting suberin formation, improvement in water relations, hydraulic conductance, antirespiratory properties, maintaining carbohydrates and antimicrobial effect prevented vascular blockage and increased vase life of cut chrysanthemum var. Punch. This is not in

agreement with the findings of Navale et al. (2010) who reported that growth retardants decreased the vase life of cut flowers.

Conclusions

The effects of daminozide and maleic hydrazide at varied concentrations and frequencies on growth, flowering and chlorophyll content in spray chrysanthemum var. Punch were tested in the study. Daminozide had comprehensive effects on improving plant type of *D. grandiflora* and increasing its ornamental value and physiological quality. Daminozide also showed a longer inhibitory action than maleic hydrazide. Hence based on the results obtained, it can be concluded that single application of daminozide at 2500 ppm at seven days after darkening may be recommended for reducing the height of spray chrysanthemum var. 'Punch'.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Storage of 'Laetitia' plums (*Prunus salicina*) under controlled atmosphere conditions

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The optimum condition for storage of 'Laetitia' plums (*Prunus salicina*) under controlled atmosphere (CA) is not currently known. This study was aimed at assessing the effects of controlled atmospheric (CA) conditions on the postharvest quality preservation of 'Laetitia' plums. Besides regular atmosphere (RA; 21kPa O_2 + 0.03 kPa CO_2) as condition/treatment control, the following CA conditions (kPa O_2 + kPa CO_2) were assessed: 1+3; 1+5; 2+5; 2+10; and 11+10. In all cases, the fruit were stored for 60 days at 0.5°C±0.1°C and 96±2% of relative humidity (RH). Upon removal from the cold storage chamber and after four days in ambient conditions (20±2°C/60±5% RH), the fruit were assessed in terms of: respiration and ethylene production rates; flesh firmness; texture; titrable acidity; red color index and hue angle (h^o) of the skin; incidence of fruit cracking and internal breakdown (flesh browning). The fruit stored under the different CA conditions presented lower respiration and ethylene production rates, higher values of flesh firmness, texture and titrable acidity, lower development of skin red color, and lower incidence of skin cracking compared to the fruit under RA. CA conditions of 2+5, 1+5, and 1+3 resulted in a more substantial delay of ripening. CA conditions of 2+5 and 1+3 resulted in lower incidence of internal breakdown.

Key words: Ripening, physiological disorder, postharvest, *Prunus salicina*.

INTRODUCTION

The maturation of plums (*Prunus salicina*) is extremely fast, and its harvesting period usually does not last more than 20 days, providing a large amount of fruit in a short period of time. Nevertheless, the storage conditions can extend the supplying period. Storage under controlled atmosphere (CA) is the storage system that allows the maintenance of quality by reducing the fruit metabolism.

Storage of plums under CA is still little explored, and in the case of the 'Laetitia' cultivar, studies evaluating the CA conditions for maintaining quality are scarce. The studies were not able to define the ideal CA condition for storage. Nevertheless, better quality during storage under this system has been observed in plum, due to the reduction of chilling injury and the preservation of the

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physicochemical attributes (Streif, 1995; Maré et al., 2005; Manganaris et al., 2008).

Van de Geijn (1993) recommends the temperature of $0^{\circ}C$ and CA condition of 3 kPa O_2 and 7 kPa CO_2 for European plums. Manganaris et al. (2008) observed higher flesh firmness in plums stored under CA at 1 to 2 kPa O_2 and 3-5 kPa CO_2, than in regular atmosphere (RA). According to Streif (1995), the best CA conditions for plums are partial pressures of 1 to 3 kPa O_2 and 8 to 12 kPa CO_2. The author also states that the use of partial pressures of 10 kPa CO_2 or higher inhibits the development of pathogens that cause decay. However, the benefits of CA condition in plums are cultivar-dependent (Manganaris et al., 2008).

Alves et al. (2010), in a previous study assessing the effect of CA on the quality of 'Laetitia' plums in Brazil, observed a delay in fruit ripening, where the condition which presented the best results was 2 kPa O_2 + 5 kPa CO_2. Nevertheless, the authors observed an increased incidence of internal breakdown and suggested that storage under CA conditions with lower O_2 and CO_2 levels may allow for storage without the occurrence of this disorder. CA reduces internal breakdown in plums, but fruits are very sensitive to CO_2; higher CO_2 concentration is associated with more severe anaerobic respiratory and flesh browning (Hui and Bo-Xun, 2007).

This study was aimed at assessing the effect of CA conditions on the maintenance of the physicochemical quality of 'Laetitia' plums.

MATERIAL AND METHODS

The "Laetitia" plums were harvested from a commercial orchard located in Lages, SC, southern Brazil, in 01/15/2010, and immediately transported to the laboratory, where the fruit were selected. The fruit with lesions, defects, wounds and/or mechanical damages were eliminated by visual inspection, and afterwards, the experimental units were homogenized.

The experiment followed a completely randomized design, with four repetitions of 30 fruits. Besides regular atmosphere (RA; 21 kPa O_2 + 0.03 kPa CO_2) as condition/treatment control, the following CA storage conditions were assessed (kPa O_2 + kPa CO_2): 1+3; 1+5; 2+5; 2+10; and 11+10. In all conditions, the fruit were stored during 60 days at 0.5°C±0.1°C and 96±2% RH in experimental minichambers (180 L), and the variations tolerated were of 0.1 kPa for O_2 and CO_2. The partial pressures of gases in the treatments with low O_2 and high CO_2 were obtained by means of diluting O_2 in the environment with injection of N_2 from a nitrogen generator which operates by the "Pressure Swing Adsorption" – (PSA) system, and afterwards, by injection of CO_2 from high pressure cylinders up to the level set for the treatment. The maintenance of the partial pressures determined for the gases under the different storage conditions (that varied according to the respiration of the fruit) was carried out daily. This monitoring was carried out by means of Agri-datalog electronic analyzers for CO_2 and O_2, with correction until the preset levels were reached. The O_2 consumed by respiration was replaced by means of the injection of atmospheric air in the minichambers, and the excess of CO_2 was absorbed by a solution of potassium hydroxide (40%) though which the ambient storage air was passed.

Before storage, an initial analysis of two samples of 15 fruits was

carried out to determine the initial quality of plums. Fruit had flesh firmness of 42 N, soluble solids content of 9.3 ºBrix, and titratable acidity of 31.17 meq 100 mL^{-1}.

Fruit quality was assessed after 60 days of storage. Four samples of 15 fruit were analyzed after removal from the chamber, and another sample of 15 fruit were analyzed after four days of shelf life (20±2°C/60±5% RH). The variables analyzed were respiration and ethylene production rates, titratable acidity (TA), texture attributes, red color index and hue angle (h^{o}) of the skin and internal breakdown incidence, as described by Alves et al. (2010). Fruit were also assessed visually for the incidence (%) of skin cracks and decay (fruit affected with pathogen lesions higher than 5 mm in diameter)

Data were analyzed with SAS version 8.02 (SAS Institute, Cary, NC, USA) using analysis of variance (ANOVA) and Tukey's test to determine mean separation between treatments. The data expressed in percentage were transformed by arcsine $[(x+0.5)/100]^{1/2}$ before being submitted to ANOVA.

RESULTS AND DISCUSSION

Flesh firmness was higher in fruit stored under CA conditions than RA, after removal from the chamber as well as after four days of shelf life (Table 1). Similar results were obtained in other studies with 'Sapphire', 'Songold', 'Laetitia' and 'Stanley' plums (Maré et al., 2005; Golias et al., 2010). Among the CA conditions evaluated, the higher values obtained for flesh firmness after removal from the chamber were 2 kPa O_2 + 5 kPa CO_2, 1 kPa O_2 + 5 kPa CO_2 and 1 kPa O_2 + 3 kPa CO_2. CA conditions 11+10 and 1+10 had intermediate values of flesh firmness, but better than control treatment (Table 1). This result is partly in accordance with Streif (1995) and Manganaris et al. (2008), citing O_2 partial pressures between1 and 3 kPa as more appropriate for the storage of plums under CA. Nevertheless, Streif (1995) considers partial pressures of CO_2 between 8 and 12 kPa as ideal for storing plums under CA.

According to Golias et al. (2010), fruit softening, biochemically conditioned by spontaneous pectolysis of the contained pectin compounds, is noticeably slowed down in the atmospheres with lowered O_2 content; a higher content of CO_2 in the atmosphere (CA treatment) does not significantly slow down softening when compared to the low O_2. The authors observed that 'Stanley' plums stored for up to 55 days under CA had higher flesh firmness values in 0.5-0.6 kPa O_2 + 0.1-0.2 kPa CO_2 than in 1.2-1.4 kPa O_2 + 0.2-0.4 kPa CO_2 or 1.9-2.2 kPa O_2 + 8.7-8.9 kPa CO_2. These points out those different cultivars of plums behave differently with regard to storage conditions, and it might explain the difference between the results obtained in this study and the results obtained by other authors regarding the best partial pressures of O_2 and CO_2.

Fruit stored under CA conditions had higher values of peel break, flesh penetration and fruit compression forces than in RA upon removal from the chamber and after four days of shelf life (Table 1). The effect of CA on the texture attributes may be related to its role in reducing ethylene biosynthesis and action, therefore reducing the

Table 1. Flesh firmness, titratable acidity and texture attributes in 'Laetitia' plums stored under different atmospheres for 60 days, upon removal from storage and after four days of shelf life ($20\pm2°C/60\pm5\%$ RH).

Atmosphere O_2+CO_2 (kPa)	Titratable acidity (meq 100 mL^{-1})	Texture attributes			
		Flesh firmness (N)	Peel break force (N)	Flesh penetration force (N)	Fruit compression force (N)
Removal from chamber					
21 + 0.03	12.4[d]	16.9[d]	4.42[b]	1.11[b]	42.8[d]
1 + 3	19.4[a]	35.6[abc]	9.61[a]	2.56[a]	78.3[abc]
1 + 5	18.0[ab]	36.9[ab]	9.41[a]	2.47[a]	86.3[ab]
2 + 5	20.2[a]	40.5[a]	9.31[a]	2.49[a]	101.[2a]
2 + 10	15.9[bc]	30.2[c]	9.95[a]	2.23[a]	77.3[bc]
11 + 10	14.8[c]	33.7[bc]	9.15[a]	2.02[a]	62.5[bcd]
CV (%)	7.8	8.7	6.2	12.4	16.4
After four days of shelf life					
21 + 0.03	10.4[d]	12.2[b]	3.8[b]	0.87[b]	32.0[b]
1 + 3	19.6[a]	33.8[a]	10.1[a]	2.04[a]	74.3[a]
1 + 5	18.3[ab]	37.3[a]	10.6[a]	2.28[a]	74.6[a]
2 + 5	19.5[a]	35.8[a]	9.9[a]	2.17[a]	80.4[a]
2 + 10	16.4[bc]	32.4[a]	10.5[a]	2.00[a]	74.5[a]
11 + 10	13.5[c]	34.4[a]	9.8[a]	2.25[a]	67.1[a]
CV (%)	6.2	8.7	4.3	12.0	11.5

*Averages followed by the same letters in the columns are not different by the Tukey test ($p<0.05$). CV, coefficient of variation.

Table 2. Respiration and ethylene production rates in 'Laetitia' plums stored under different atmospheres for 60 days, upon removal from storage and four days of shelf life ($20\pm2°C/60\pm5\%$ RH).

Atmosphere O_2+CO_2 (kPa)	Respiration rate (nmol CO_2 kg^{-1} s^{-1})		Ethylene production rate (pmol C_2H_4 kg^{-1} s^{-1})	
	Removal from chamber	After four days of shelf life	Removal from chamber	After four days of shelf life
21 + 0.03	775.6[a]	498.4[a]	5.53[a]	15.6[a]
1 + 3	527.3[b]	352.9[a]	0.72[cd]	5.1[b]
1 + 5	114.1[d]	419.4[a]	1.16[c]	7.0[b]
2 + 5	529.6[b]	362.1[a]	0.57[cd]	3.4[b]
2 + 10	209.1[d]	350.3[a]	0.37[d]	7.0[b]
11 + 10	450.1[c]	362.7[a]	2.90[b]	6.1[b]
CV (%)	15.6	15.1	11.7	29.2

Averages followed by the same letters in the columns are not different by the Tukey test ($p<0.05$). CV, coefficient of variation.

activity of the hydrolytic enzymes responsible for degrading the cell wall components.

The TA was higher in the fruit stored under CA conditions than in RA upon removal from the chamber as well as after four days of shelf life (Table 1). This result is in accordance with the data presented by Hui and Bo-Xun (2007).. Among the CA conditions, 1 kPa O_2 + 3 kPa CO_2 and 2 kPa O_2 + 5 kPa CO_2 resulted in the highest TA values. However, these CA conditions did not differ from 1 kPa O_2 + 5 kPa CO_2 condition (Table 1). The higher values of TA under CA might reflect the lower respiration rates under these storage conditions (Table 2). The low partial pressure of O_2 and/or high partial pressure of CO_2

reduce the consumption of organic acids as a source of energy for the respiration process (Steffens et al., 2007). After four days of shelf life, no difference was observed between treatments in terms of respiration rate (Table 2). The reduction of the respiration activity under low O_2 is due to the decrease in the activity of several oxidases, such as cytochrome oxidase, polyphenol oxidase, ascorbic acid oxidase and glycolic acid oxidase (Kader, 1986). The high CO_2 might reduce respiration by inhibiting enzymes in the glycolytic pathway (phosphofructo kinase), and tricarboxylic acids cycle (succinate oxidase and isocitrate dehydrogenase), as well as by reducing the activation by ethylene of enzymes involved in the respiration

Table 3. Red color index (RCI) and hue angle (h^o) of the skin, decay and internal breakdown in 'Laetitia' plums stored under different atmospheres for 60 days, upon removal from storage and after four days of shelf life ($20\pm2°C/60\pm5\%$ RH).

Atmosphere O_2+CO_2 (kPa)	RCI* (1-4)	Hue angle (h^o)		Decay (%)	Internal breakdown (%)
		Fruit side with more intense red color	Fruit side with less intense red color		
Removal from chamber					
21 + 0.03	3.51a	29.0b	66.1b	19.1a	69.7c
1 + 3	2.84b	35.6a	91.2a	4.1b	53.9c
1 + 5	2.51b	39.7a	94.1a	13.3ab	79.0bc
2 + 5	2.71b	38.1a	87.7a	3.3b	56.6c
2 + 10	2.57b	39.4a	91.6a	5.8b	95.1ab
11 + 10	2.45b	38.3a	82.8a	6.7b	98.3a
CV (%)	6.5	7.2	7.4	15.1	15.7
Four days of shelf life					
21 + 0.03	3.91a	23.1b	35.3b	12.1a	84.7a
1 + 3	3.39a	30.6a	61.4a	12.8a	48.8c
1 + 5	3.36a	30.3a	67.3a	20.3a	63.1ab
2 + 5	3.49a	32.1a	62.3a	7.26a	52.6bc
2 + 10	3.49a	33.0a	68.4a	7.74a	87.4a
11 + 10	3.40a	33.1a	66.7a	10.4a	93.3a
CV (%)	16.4	7.7	9.5	57.4	26.8

Averages followed by the same letters in the columns are not different by the Tukey test ($p<0.05$). CV, coefficient of variation. *, Scores 1, 2, 3, and 4: 0 to 25%, 26 to 50%, 51 to 75%, and >74% of fruit red color surface, respectively.

process (Fonseca et al., 2002).

Fruit stored under CA conditions had lower ethylene production rates than in RA, after removal from the chamber as well as after four days of shelf life (Table 2). The reduced ethylene production under CA is caused by low O_2 and/or high CO_2, as reported by other authorss (Fonseca et al., 2002; Steffens et al., 2007). The suppressed ethylene production rate under CA results of reduced oxidation of 1-carboxilic-1-aminocyclopropane (ACC) by low O_2 and/or inhibition of the ethylene action in inducing autocatalysis by high CO_2 (Kader, 1986). As for the contents of soluble solids, no differences were observed among treatments (data not shown).

The fruit stored under CA conditions had lower skin red color percentage (lower values of red color index) upon removal from the chamber, as well as a less intense red color (higher h^o values in the both fruit sides, with more intense and less intense red color) upon removal from the chamber and after four days of shelf life than fruit in RA (Table 3). As observed for flesh firmness and texture, the less intense skin red color of the fruit must be related to the reduced biosynthesis and the action of ethylene in storage under CA (Alves et al., 2010).

The incidence of decay, upon removal from the chamber, was higher in the fruit stored under RA and, in general, CA conditions delayed the incidence of decay (Table 3). After four days under ambient conditions, decay was not different between treatments. The effects of the CA on decay reduction may be attributed to the low levels of

O_2 combined with high levels of CO_2, which presented a fungi static effect, inhibiting spore germination and fungal growth during the storage period (Sitton and Patterson, 1997; Wszelaki and Mitcham, 2000; Vieira et al., 2006).

The incidence of internal breakdown upon removal from the chamber was higher in the CA conditions with 10 kPa CO_2 (Table 3). According to Hui and Bo-Xun (2007), plums are very sensitive to CO_2, and the increase of CO_2 partial pressure causes an increase in anaerobic respiratory and internal breakdown. Conditions with very low O_2 partial pressure or very high CO_2 partial pressure might cause damages to the integrity of the tissue (Jayas and Jeyamkondan, 2002). The CO_2 works by reducing the speed of the tricarboxylic acid cycle, and CO_2 at very high levels might leave to the accumulation of succinic acid due to inhibition of the succinate dehydrogenase enzyme, which causes physiological disorders (Monning, 1983; Kader, 1986; Matthooko, 1996; Galvis et al., 2005). The occurrence of this physiological disorder might also be the result of reduced energetic metabolism and reduced content of phospholipids, with consequent cell decompartmentalization (Saquet et al., 2003).

After four days of shelf life, CA conditions with 10 kPa CO_2 had the highest incidence of internal breakdown (Table 3). Even fruit in RA had an increase of internal breakdown after shelf life, compared to fruit assessed upon removal from storage. After shelf life, the incidence of internal breakdown was not different between fruit in RA and fruit under CA condition with 1 kPa O_2 + 5 kPa CO_2.

'Laetitia' plum develops internal breakdown, especially when stored for over 30 days in RA (Alves et al., 2010). Although the the atmospheres with 1 kPa O_2 + 3 kPa CO_2 and 2 kPa O_2 + 5 kPa CO_2 had the best results in controlling the internal breakdown, its incidence was high even under these CA conditions. Therefore the results show that 'Laetitia' plums should not be stored for more than 60 days, even under the most suitable CA conditions.

Only fruit stored in RA had skin cracking, corresponding to 20% upon removal from the chamber. No cracking was observed under CA conditions (data not shown). In 'Royal Gala' and 'Galaxy' apples, low O_2 and high CO_2 under CA condition reduced the incidence of skin cracking compared to RA due to the delay in fruit ripening (Brackmann et al., 2008).

Conclusions

The CA conditions with 2 kPa O_2 + 5 kPa CO_2 and 1 kPa O_2 + 5 kPa CO_2 provided the best results in delaying fruit ripening, while the atmospheres with 2 kPa O_2 + 5 kPa CO_2 and 1 kPa O_2 + 3 kPa CO_2 had fruit with the lowest incidence of internal breakdown. Nevertheless, the results indicate that for 'Laetitia' plums, the storage period of 60 days is excessively long, even under these CA conditions, leading to high occurrence of internal breakdown.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Influence of plant growth regulators on development and polysaccharide production of cell cultures of *Pelargonium sidoides*

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Pelargonium sidoides* is a traditional medicinal plant from South Africa. An aqueous-ethanolic formulation of the roots and tubers is approved for the treatment of acute bronchitis. Therefore propagation of the plant material by cell cultures and the extraction of potential pharmaceutical active compounds are of great interest. Calli were established on different media from roots and shoots of seedlings and softness and colour of the tissue were compared. Optimum growth of callus cultures was achieved in MS-medium containing 1 mg/L 2,4-D and 0.2 mg/L kinetin or 2.2 mg/L TDZ supplemented with 50 mg/L ascorbic acid and 50 mg/L citric acid. Accumulation of phenolic deposits, responsible for inhibition of growth was avoided by addition of ascorbic and citric acid and a short period of sub-culture. Furthermore, the influence of different phytohormones [2,4-D, kinetin, 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), Thidiazuron (TDZ)] on the polysaccharide composition of the liquid media of suspension cultures was investigated. For the first time, arabinogalactan-proteins (AGPs) as bioactive components were isolated from cell cultures of *P. sidoides.

Key words: *Pelargonium sidoides*, cell culture, arabinogalactan-protein, 2,4-dichlorophenoxyacetic acid, kinetin, *β*-glucosyl Yariv reagent.

INTRODUCTION

Plant cell culture is an important system to produce secondary metabolites and to investigate biosynthetic pathways besides propagation of plants. Furthermore, the isolation of primary and secondary metabolism

products from plant cell cultures has a certain industrial relevance in medicine, pharmacy and food technology. Successful examples for production of pharmaceuticals in plant suspension cultures are secondary metabolites like

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **A,** ascorbic acid; **AG,** arabinogalactan; **AGP,** arabinogalactan-protein; **BAP,** 6-benzylaminopurine; **C,** citric acid; **IBA,** indole-3-butyric acid; **K,** kinetin; **MS,** Murashige and Skoog medium (1962); **NAA,** *α* naphthaleneacetic acid; **PA6,** polyamide 6; **TDZ,** Thidiazuron; **Yariv,** *β*-glucosyl Yariv reagent.

ginseng saponins, diosgenin and shikonin (Weathers et al., 2010), the biotransformation of methyldigitoxin to methyldigoxin in cell cultures of *Digitalis lanata*, as well as the production of the anti-cancer agents podo-phyllotoxin and paclitaxel (Malik et al., 2011; Nosov, 2012). Initially, paclitaxel was isolated from the bark of *Taxus brevifolia*, leading to die back of the slow growing trees. Since 2002, the active agent is mainly isolated from suspension cultures of *Taxus*. On one hand, this secures the long-ranging requirement of paclitaxel with high quality; while on the other hand, it ensures conservation of *T. brevifolia*. Considering the importance of plants like *Pelargonium sidoides* it is essential to look for conservation strategies and guarantee availability of high quality pharmaceutical products (Moyo and van Staden, 2014).

Pelargonium sidoides DC. (Geraniaceae) belongs to the pool of medicinal plants of South Africa, and an aqueous-ethanolic extract from the roots of this plant is approved in Germany and other countries for the treatment of acute bronchitis. This extract is charac-terized by different pharmacological effects (Moyo and van Staden, 2014). Diverse antibacterial and antiviral activities of the *Pelargonium* root extract have been proven to date and gallic acid and other phenolic substances seem to be responsible for these effects (Helfer et al., 2014; Kolodziej, 2011). It has been postulated that the also known immunomodulatory activities are probably a result of synergistic effects of polyphenols and coumarins (Brendler and van Wyk, 2008; Kolodziej, 2011). On the other hand, it is known, that *Pelargonium* root extracts contain high amounts of carbohydrates (Schoetz et al., 2008), which have not been characterized accurately up to now. Recently, special glycoproteins, the so-called arabinogalactan-proteins (AGPs) have been detected in *P. sidoides* (Duchow, 2012). These hydroxyprolin-rich cell wall glycoproteins are characterized by heterogeneity of carbohydrate chains, complexity of the protein backbone as well as the different possibilities in linking both parts (Nothnagel, 1997; Seifert and Roberts, 2007). Besides involvement in growth and developmental processes in plants (Goellner et al., 2013), AGPs play a commercial role especially as Gum Arabic, which is used in food and pharmaceutical industry due to its emulsifying, adhesive and water-binding attributes (Showalter, 2001). AGPs from other species, especially from *Echinacea purpurea*, are considered as stimulator of the human immune system and show immunomodulating activities in vitro, e.g. binding to human leucocytes (Thude et al., 2006). This implements the consideration that AGPs from *Pelargonium* could also be involved in the immuno-modulatory effects of the root extract. For suspension cultures of different plants, accumulation of high amounts of AGPs in the suspension media has been shown (Classen, 2007).

The aim of the present study was the establishment of callus and suspension cultures of the medicinal plant *P. sidoides*. Different media including different phyto-hormones have been tested to produce cell cultures of highest quality. Furthermore, the polysaccharide fractions of the different suspension media were compared with regard to their monosaccharide composition and used for isolation of AGPs. The possible influence of different phytohormones on the polysaccharide and AGP composition is a special focus of this work. Suspension cultures of *P. sidoides* offer a reproducible source of AGPs as putative immunomodulatory active compounds with high yields and independent of varying plant origin.

MATERIALS AND METHODS

Plant materials

P. sidoides DC. (Geraniaceae) has been grown in the Garden of the Pharmaceutical Institute of the University of Kiel, Germany. Plant material has been identified by Birgit Classen, the Department of Pharmaceutical Biology, University of Kiel, where voucher specimens are deposited in the herbarium (identification number Ps 2012).

Establishment of cell cultures

Calli from shoots of germinating seeds of *P. sidoides* DC. were induced as described by Classen (2007) for *Echinacea*. Seeds were disinfected by using absolute ethanol (8 min) and 10% Domestos®-solution (30 min, Unilever, Hamburg, Germany). After rinsing with distilled water (3 × 5 min) seeds were kept in sterile petri dishes having autoclaved wet filter paper for germination. Hypocotyls, 5 mm pieces, were transferred to solid MS-medium (Murashige and Skoog, 1962) containing 0.4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and stored at light intensity of 94 µmol m^{-2} s^{-1} (Osram L 58W/77 Fluora) and 25°C for callus induction.

For further callus proliferation, the following phytohormones: A) 0.4 mg/L 2,4-D, B) 1 mg/L 2,4-D + 0.2 mg/L kinetin (K), C) 5 mg/l 6-benzylaminopurine (BAP) + 1 mg/L 1-*α*-naphthaleneacetic acid (NAA) and D) 2.2 mg/L Thidiazuron (TDZ) were used. Calli were subcultured every 4-5 weeks.

In experiments to avoid accumulation of polyphenolic compounds during callus proliferation, medium was supplemented with ascorbic- and citric acid (A+C, 50 mg/L each) or polyamide 6 (PA6). In addition, fast subculturing (every second week) was also attempted to reduce the impact of phenolic compounds on callus growth.

Calli ($\varnothing$ 2 cm) were transferred into 100 ml liquid MS-medium supplemented with: A) 0.4 mg/l 2,4-D; B) 1 mg/L 2,4-D + 0.2 mg/L K; C) 5 mg/L BAP + 1 mg/L NAA and D) 2.2 mg/L TDZ and ascorbic- and citric acid (50 mg/L each) or PA6 (Roth, Germany) and stored in the dark at 25°C on a shaker (120 rpm, Edmund Bühler GmbH, Hechingen, Germany). Suspension cultures were subcultured every 3 weeks. A small amount of the cultures (16.5 ml) has been utilized as inoculum for the next culture; while the rest has been used for isolation of polysaccharides and AGPs and represents a batch. Micrographs of suspension cultures have been taken with a Zeiss light microscope (number 4760059901, Zeiss, Germany).

Gel diffusion assay for detection of AGPs

A solution containing 1% agarose, 0.9% NaCl a nd 1 mM $CaCl_2$ in

10 mM Tris-HCl buffer (pH 7.3) was autoclaved and poured into sterile Petri dishes. Five holes (Ø 1.2 mm) were punched into the gel with four holes arranged around one central cavity at 1 cm distance. 20 µl of β-glucosyl Yariv reagent solution (1 mg/ml) was poured in the central cavity; the other four holes were filled with solutions of high molecular weight polysaccharide fraction (20 µl) at different concentrations (1, 10, 25 and 50 mg/ml). The plates were stored for 24 h at room temperature.

Isolation of polysaccharides and AGPs from suspension cultures

Suspension cultures (1-2 L) were centrifuged (3875 g, 15 min), cells deleted and the cell-free medium used for isolation of polysaccharides. After heating (95°C, 10 min) of small portions (100 ml) of the liquid medium, denatured proteins were removed by centrifugation (5000 rpm, 10 min) and the supernatant was precipitated with ethanol (80% V/V). After centrifugation (20000 g, 15 min), the precipitated material was freeze-dried to obtain the high-molecular weight polysaccharide fraction.

The polysaccharide fraction (100-300 mg) was dissolved in distilled water (putative AGP; 1 mg/ml) and precipitated by addition of an equal volume of an aqueous solution of 1 mg/ml β-glucosyl Yariv reagent (Yariv et al., 1962) containing NaCl (0.15 M) to get AGPs. After formation of the AGP-Yariv complex at 4°C overnight, it was centrifuged (20000 g, 10 min, 4°C), dissolved in distilled water and degraded by addition of $Na_2S_2O_4$ before heating to 50°C (Kreuger and van Holst, 1995). Final dialysis (molecular weight cut off: 12-14 kDa) against distilled water and freeze-drying resulted in pure AGPs.

Analysis of neutral monosaccharides

Neutral sugar analysis of the high molecular weight polysaccharide fraction and the Yariv reagent-precipitated AGPs was performed according to the method of Blakeney et al. (1983). After acid hydrolysis (1 M trifluoracetic acid, 1 h, 121°C) followed by reduction and acetylation of monosaccharides, the resulting alditol acetates were investigated by gas liquid chromatography with a flame ionization detector (HP 5890 series, Hewlett-Packard, Nürnberg, Germany) and a fused silica capillary column (Optima-OV-225, 0.25 µm, L = 25 m, i.d. = 0.25 mm, Machery & Nagel, Düren, Germany). Nitrogen was used as carrier gas with a flow rate of 1.2 ml/min and temperatures of 230°C for the oven (isothermal), 250°C for the injector and 240°C for the detector.

RESULTS AND DISCUSSION

Establishment of callus cultures on different media

Callus induction was achieved on different MS-media (Murashige and Skoog, 1962) varied by additions of phytohormones and other supplements. Both auxins (2,4-D and NAA) as well as cytokinins (K and BAP) and TDZ with cytokinin like activity in different concentrations led to growth of undifferentiated calli (Table 1).
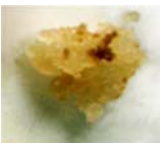
2,4-D (0.4 mg/L) supplementation led to viable, but slow growing callus tissue with many brown deposits. To improve the growth rate of the calli, 2,4-D concentration was increased to 1 mg/L and the medium was additionally supplemented with the cytokinin K. The combina-

tion of both phytohormons (2,4-D and K) resulted in optimum growth rates but calli were still partly brown. The parallel testing of another phytohormone combination with 5 mg/L BAP and 1 mg/L NAA led to comparable growth rates, however resulted in hard and dry callus tissue, interspersed with many brown deposits. These excretions probably consist of phenolic compounds, which led to reduced growth rates. Therefore, to decrease phenolic deposits, media were supplemented with ascorbic and citric acid (50 mg/l each) and the cultures were subcultured in frequent intervals (every two weeks). After three month, light coloured calli having one month sub culturing period were obtained. Although calli obtained from BAP and NAA supplemented MS media recorded faster growth per month with little brown deposits (yielding 3 cm dia-size from 0.5 cm), callus tissue was dry and crumbly. Calli cultured in 2,4-D and K or TDZ supplemented media containing ascorbic and citric acid resulted in soft tissue with high growth rates and represented the optimal basis for permanent culture. In addition to the application of ascorbic and citric acid for inhibition of phenolic excretions polyamide 6 was also tested and supplemented to the solid basic medium with 2,4-D and K in three different concentrations: 0.2, 0.5 and 1 mg/100 ml media. All three concentrations eliminated the occurrence of polyphenolic deposits and resulted in highly soft and lightish callus tissue with good growth rates.

Until now, only little is known about the micropropagation of *P. sidoides*: especially about the establishment of callus and suspension cultures. Investigations concerning shoot organogenesis and hairy root cultures have been done by Moyo et al. (2012, 2013, 2014) and Colling et al. (2010). Successful induction of callus tissue from leaves, stems and seeds of *P. sidoides* under the influence of auxins like 2,4-D and IBA was first achieved by Lewu et al. (2007). The use of media with kinetin in combination with NAA for callus induction from petioles only led to calli with crumbly consistence (Moyo et al., 2012). These first results underline the need for further research with the aim to optimize callus growth and quality. Data for other *Pelargonium* species show that a balanced combination of auxins and cytokinins results in optimal cell growth. The ratio of these phytohormones is most important for the induction of undifferentiated tissues (Brown and Charlwood, 1986): high amounts of auxins result in root development whereas an excess of cytokinins leads to shoot differentiation (El-Nil et al., 1976; Rao, 1994; Beck and Hartig, 2009; Moyo et al., 2012).

Comparable to this, our experiments also show that a balanced combination of auxins (1 mg/L 2,4-D) and cytokinins (0.2 mg/L K) has a favourable effect on the growth of *P. sidoides* callus cultures. The use of 0.4 mg/L 2,4-D as single phytohormone, which was applied for the establishment of soft and lightish callus tissues from *Echinacea purpurea* (Classen, 2007), only led to slow

Table 1. Growth and quality of callus cultures obtained from shoots of *Pelargonium sidoides* on MS-media with different supplements.

| Culture | MS-medium + | | Characteristics of callus tissue | | | |
	Phytohormones	Other constituents	Size (ø) after 4 weeks (cm)	Colour	Consistence	Growth rate
	0.4 mg/L 2,4-D		1	Brown deposits	Soft	+
	1 mg/L 2,4-D 0.2 mg/L K		2	Brown deposits	Soft	++
	5 mg/L BAP 1 mg/L NAA		2	Brown deposits	Dry Hard	++
	0.4 mg/L 2,4-D	50 mg/L A+C	1	No brown deposits	Soft	+
	1 mg/L 2,4-D 0.2 mg/L K	50 mg/L A+C	2	No brown deposits	Soft	++
	5 mg/L BAP 1 mg/L NAA	50 mg/L A+C	3	Little brown deposits	Dry Hard	+++
	2.2 mg/L TDZ	50 mg/L A+C	2	No brown deposits	Usually soft	++
	1 mg/L 2,4-D 0.2 mg/L K	0.2 g/100 ml PA6	2	No brown deposits	Soft	++
	1 mg/L 2,4-D 0.2 mg/L K	0.5 g/100 ml PA6	2	No brown deposits	Soft	++
	1 mg/L 2,4-D 0.2 mg/L K	1 g/100 ml PA6	2	No brown deposits	Soft	++

Growth rate: +, weak; +, weak; +++, strong.
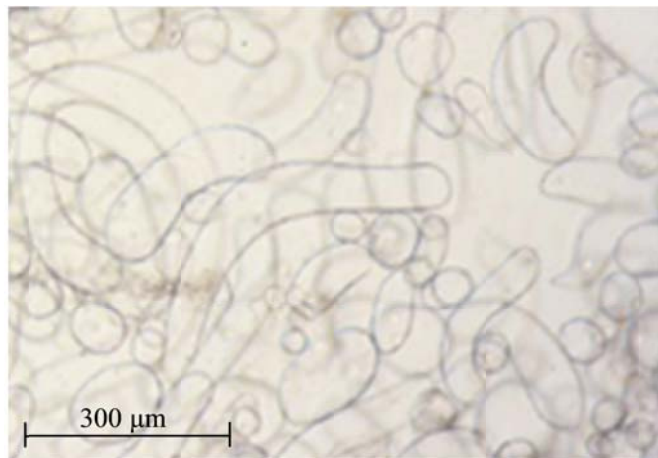
Figure 1. Suspension cultured cells of *P. sidoides* in a medium with 1 mg/L 2,4-D, 0.2 mg/L K and ascorbic and citric acid (50 mg/L each).

growing calli in case of *P. sidoides*. A critical point for all media was the occurrence of brown, fast spreading accumulations which inhibited callus growth, especially in the medium with BAP and NAA. These dark deposits of phenolic origin have often been observed in cell cultures from different plants with high amounts of tannins (Vatanpour-Azghandi et al., 2002; Zagoskina et al., 2003) and have been quantified in tissue cultures from *P. sidoides* by a Folin and Ciocalteu assay (Moyo et al., 2012). For callus cultures from other species it has been shown that supplementation of media with antioxidant compounds like ascorbic and citric acid (50 mg/L) results in suppression of the formation of polyphenolic compounds (Klein, 2004; Sathyanarayana and Varghese, 2007). For *P. sidoides*, addition of ascorbic and citric acid to the media in combination with a shortened subcultivation period (every two weeks) also led to inhibition of phenolic deposits. Ascorbic and citric acid probably act as inhibitors of polyphenoloxidases (Pizzocaro et al., 1993; Jang and Moon, 2011; Suttirak and Manurakchinakorn, 2010). The benefit of short cultivation intervals to reduce the occurrence of brown coloration has also been shown for teak cell cultures (Tiwari et al., 2002). Interestingly, a reduction of the amount of 2,4-D seems to minimise brown coloration in cell cultures of *Curcuma mangga* (Sundram et al., 2012). This could not be proven for *P. sidoides*: 0.4 mg/L 2,4-D as well as 1 mg/L 2,4-D produced comparable amounts of phenolic deposits.

Alternatively, suppression of polyphenols was achieved with polyamide 6 (PA6) which was added to the culture media. Fast growing, soft and bright calli impressively proved the adsorption of phenolic deposits on PA6.

In brief, our investigations have identified MS-media with 1 mg/L 2,4-D and 0.2 mg/L K or alternatively 2.2 mg/L TDZ, both enriched with 50 mg/L A+C each or 2 g/L

PA6 as optimal media for permanent callus culture of *P. sidoides*.

Establishment of suspension cultures

Comparable to callus cultures, quality of suspension cultures was investigated with regard to different phytohormones and further supplements. At first, calli from media with 0.4 mg/L 2,4-D were transferred to the liquid medium of the same composition. Although growth was acceptable, the colour of the suspension was rather dark, indicating production of phenolic compounds. Therefore calli from media with 0.4 mg/L 2,4-D, 1 mg/L 2,4-D and 0.2 mg/L K, 5 mg/L BAP and 1 mg/L NAA as well as 2.2 mg/L TDZ, all supplemented with ascorbic and citric acid, were transferred to liquid media and the suspension cultures investigated concerning their growth and colour. Suspension cell lines have been stable over a period of up to 16 subcultivations. Growth of all cultivations was good with comparable medium viscosity after three weeks. Suspensions only differed in their colour: media with BAP/NAA led to gloomy cultures, cells on 2,4-D/K were of bright colour and suspensions with TDZ were even brighter. Microscopic investigation of the cultured cells showed relatively homogeneous suspensions with undifferentiated, more or less round or sausage-like cells (Figure 1).

Isolation and analysis of secreted polysaccharides

The amount of water-extractable polysaccharides in suspension culture media varied between 1 and 4% related to the dry cell mass and was highest for the medium with TDZ (Table 2). This result shows that high amounts of polysaccharides can be isolated directly from suspension culture media with the advantage of a faciliated isolation process in comparison to isolation from native plant material.

Neutral monosaccharide composition of the polysaccharide fractions differed from medium to medium (Table 3). The polysaccharides from the medium with BAP and NAA are characterized by a special composition with glucose and galactose (about 35 and 30%) as the dominating monosaccharides, followed by nearly similar amounts of arabinose and xylose (nearly 15% each) and small amounts of mannose, rhamnose and fucose (2% each). In contrast to that polysaccharides which were isolated from media with 0.4 mg/l 2,4-D or 1 mg/L 2,4-D in combination with 0.2 mg/l K have another principal composition with galactose (about 43%) and arabinose (about 25%) accompanied by smaller amounts of glucose (about 11%), xylose (about 12%), mannose (about 4%) rhamnose (about 2%) and traces of fucose. Galactose (ca. 39%) and arabinose (about 33%) were also the main sugars in media supplemented with TDZ with a different

Table 2. Amounts of isolated polysaccharides and AGPs from different suspension cultures of *P. sidoides* (related to freeze-dried cell mass).

Cultivation with		Amount of polysaccharides (%)	Amount of AGPs (%)	Number of batches
0.4 mg/L 2,4-D		2.66 ± 0.97	0.39 ± 0.19	8
0.4 mg/L 2,4-D	A+C	1.54 ± 0.01	0.41 ± 0.17	2
1 mg/L 2,4-D + 0.2 mg/l K	A+C	1.75 ± 0.78	0.41 ± 0.24	19
5 mg/L BAP + 1 mg/l NAA	A+C	1.20 ± 0.59	0.24 ± 0.11	4
2.2 mg/L TDZ	A+C	4.06 ± 4.00	0.67 ± 0.51	4

Table 3. Neutral monosaccharide composition of polysaccharide fractions from suspension cultures of *Pelargonium sidoides* with different phytohormones.

Neutral monosaccharide (% w/w)	Polysaccharide fractions from suspension cultures with:				
	0.4 mg/L 2,4-D	0.4 mg/L 2,4-D + A/C[a]	1 mg/L 2,4-D + 0.2 mg/L K + A/C[a]	5 mg/l BAP + 1 mg/L NAA + A/C[a]	2.2 mg/L TDZ + A/C[a]
Number of batches	8	2	19	4	4
Gal	43.3 ± 3.8	45.6 ± 4.3	42.8 ± 4.4	29.7 ± 4.2	38.6 ± 10.3
Ara	26.3 ± 3.7	26.4 ± 3.6	24.5 ± 3.4	13.4 ± 1.4	32.5 ± 11.1
Glc	11.6 ± 4.0	7.6 ± 6.8	11.3 ± 5.2	35.5 ± 6.8	12.1 ± 10.9
Xyl	11.2 ± 1.9	12.3 ± 2.8	13.6 ± 3.0	14.7 ± 1.0	8.2 ± 4.4
Man	4.1 ± 0.7	4.3 ± 1.1	3.8 ± 0.8	2.5 ± 0.4	4.7 ± 1.0
Rha	2.4 ± 1.3	3.1 ± 0.8	2.4 ± 0.8	2.2 ± 0.6	2.0 ± 0.8
Fuc	1.1 ± 0.5	1.0 ± 0.4	1.7 ± 1.1	2.1 ± 0.3	1.8 ± 0.7
Ara:Gal ratio	1:1.7	1:1.8	1:1.8	1:2.3	1:1.3

[a]Concentration 50 mg/L.
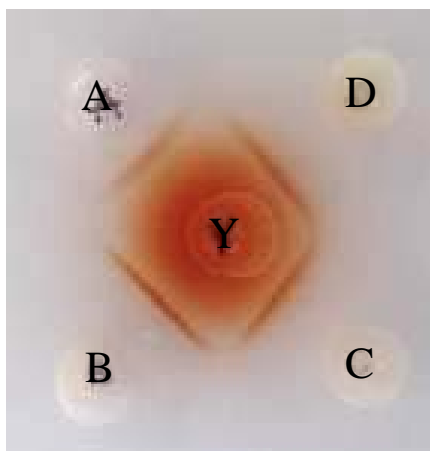
Figure 2. AGP-positive gel diffusion assay with Yariv reagent (Y) and four different concentrations of the polysaccharide fraction from medium supplemented with 0.4 mg/L 2,4-D (A=1 mg/ml, B=10 mg/ml, C=25 mg/ml, D= 50 mg/ml).

ratio of galactose to arabinose. All other monosaccharides occured only in small amounts (Glc 12%, Xyl 8%, Man 5%, Rha and Fuc 2%), indicating that

a high percentage of this polysaccharide fraction are possibly AGPs.

Detection of AGPs

Using a gel diffusion assay, the presence of AGPs could be proven to be part of the ethanol-precipitated polysaccharide fraction from cell culture media. AGPs can be detected by formation of a red precipitation line with Yariv reagent (Figure 2), which is a typical feature of many AGPs (Seifert and Roberts, 2007). For suspension cultures from different plants, secretion of high amounts of AGPs into the suspension media has already been shown (Immerzeel et al., 2004; Classen, 2007; Sánchez-Sampedro et al., 2008), but for *Pelargonium* species, the presence of AGPs has not been reported before, neither for the plant nor for suspension cultured cells.

Isolation and characterisation of AGPs

AGPs are predominantly secreted into the extracellular matrix in plants, and this probably is the reason, why they can be found in the liquid media of suspension cultured cells. Precipitation with Yariv reagent yielded purified AGPs which accounted for 0.24-0.67% of dry weight of

Table 4. Neutral monosaccharide composition of AGPs from suspension cultures of *Pelargonium sidoides* with different phytohormones.

Neutral monosaccharide (% w/w)	AGP fractions from suspension cultures with:				
	0.4 mg/L 2,4-D	0.4 mg/L 2,4-D + A/C[a]	1 mg/L 2,4-D + 0.2 mg/L K + A/C[a]	5 mg/L BAP + 1mg/L NAA + A/C[a]	2.2 mg/L TDZ + A/C[a]
Number of batches	8	2	19	4	4
Gal	61.7±0.3	62.2±3.8	62.6±2.3	65.6±2.6	58.6±7.0
Ara	32.5±2.1	30.2±2.2	31.2±1.9	27.2±2.2	31.5±1.6
Glc	0.9±0.4	1.1±1.6	1.5±1.5	1.9±1	1.9±1.8
Xyl	1.1±0.1	1.7±1.6	0.8±0.4	1.4±1	2.9±3.2
Man	2.1±0.4	2.6±1.3	2.4±0.8	2.3±1.8	2.4±2.3
Rha	1.3±0.3	1.6±1.3	1.1±0.3	1.5±0.2	2.0±0.5
Fuc	0.4±0	0.6±0.4	0.5±0.4	0.4±0.2	0.7±0.5
Ara:Gal ratio	1:1.9	1:2.1	1:2.0	1:2.5	1:1.9

[a]Concentration 50 mg/L.

the cell material (Table 2), which is in good agreement with yields of AGPs from liquid media of *Echinacea* suspensions (Classen, 2007). The amount of AGPs was highest for the medium with TDZ and lowest for that with BAP and NAA. Compared to isolation from plant material (Classen et al., 2006), yields of AGPs from suspension cultures are tenfold higher, pointing out that suspension cultures are an excellent source of AGPs.

After isolation of AGPs, the influence of the phytohormones on the neutral sugar composition of AGPs was investigated. In contrast to the composition of high molecular weight polysaccharide fractions which differed with regard to the used phytohormone(s), monosaccharide composition of AGPs was rather stable. AGPs typically consisted of about 60% galactose and 30% arabinose accompanied by low amounts of glucose, xylose, mannose, rhamnose and traces of fucose (Table 4).

Compared to literature, neutral sugar composition of *P. sidoides* AGP reflects the typical qualitative and quantitative distribution of characteristic monosaccharides within an AGP such as high amounts of galactose and arabinose in a ratio of about 2:1 and minor amounts of accompanying monosaccharides like rhamnose and glucose (Showalter, 2001; Ellis et al., 2010). AGPs isolated from media with BAP and NAA might be a little different with a higher Gal:Ara ratio of 2.5:1, but statistical relevance of this should be verified by investigations of further batches.

Conclusion

With this work optimal conditions for growth of callus and suspension cultures of *P. sidoides* could be established. Future work should clarify whether suspension cultures might be a good source for *Pelargonium* coumarins which seem to be responsible for antibacterial, antiviral and immunomodulating effects of the *Pelargonium* root

extract together with polyphenols (Brendler and van Wyk, 2008; Kolodziej, 2011; Moyo and van Staden, 2014).

From suspension cultures of *P. sidoides* high amounts of potentially biological active AGPs were isolated. Prospective investigations of the *Pelargonium*-AGP will help to characterize the carbohydrate moiety more precisely; especially the linkage types between the single monosaccharide units as well as the mode of linkage between the sugar and the protein part. Together with the testing of biological activities with focus on immunomodulating effects, structure-activity-relationships are of high interest.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

The effect of ethylene on transgenic melon ripening and fruit quality

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Melons are good models used for explaining the physiological and biochemical changes in postharvest ripening. In this study, transgenic melons expressing apple ACC oxidase gene when treated with ethylene (AS3) were evaluated. Cell wall expression (MPG1; MPG2), ethylene synthesis ACC oxidase (ACCO1), flavour synthesis AAT (alcohol acyltransferase) and physiochemical parameters such as pulp firmness, titratable acidity (TA), soluble solid content (SSC), volatile esters, ethylene productions, antioxidant assay capacity and ascorbic acid content were evaluated. In cell wall expression analysis, MPG1 increased when fruits of transgenic melons were exposed to ethylene; showing they are ethylene-dependent. MPG2 decreased gradually when fruits were subjected to ethylene application. Fruit firmness was modified in transgenic fruits when ethylene was applied. There was a great reduction similar to that of non- transgenic fruits. However, TA in transgenic fruits remained lower than in non-transgenic fruit. The ethylene applied in transgenic fruit made the titratable acid to increase during 48 h and after it, a reduction was observed. In relation to soluble solid contents, transgenic fruits treated with or without ethylene did not reduce gradually compared to the wild type melons in all the periods. Ethylene productions in transgenic fruits were reestablished when ethylene was applied, exhibiting the same behavior as transgenic fruits. Antioxidant assay levels were more active in transgenic fruits when ethylene was applied than in control fruits, and it was only in transgenic fruits without ethylene. Ascorbic acid was kept in transgenic fruits with or without the application of ethylene. Results obtained show that the application of ethylene in transgenic ACC oxidase melons is able to change the metabolism of the cell wall, flavors and antioxidant capacity levels in fruit during the ripening process.

Key words: Esters, antioxidant, solid soluble content, ascorbic acid.

INTRODUCTION

Melon (*Cucumis melo* L.) var. *cantalupensis* Naudin is a climacteric fruit characterized by its peak of respiration and an autocatalytic ethylene production during ripening (Périn et al., 2002; Kays and Paul, 2004). The genetic

transformation of melons with antisense ACC oxidase gene reduces ethylene production and increases marketable postharvest preservation (Ayub et al., 1996; Silva et al., 2004; Nuñez-Palenius et al., 2007). In similar studies, Ayub et al. (1995) used antisense ACC oxidase gene from melons, that were isolated and characterized by Balagué et al. (1993); other authors (Silva et al., 2004) used a clone antisense ACC oxidase pAP4 from 'Royal Gala' apple constitutively expressed in climacteric ripening index.

The ethylene-suppressed ACC oxidase gene in melons allows the studying of ethylene-dependent and independent ripening pathways. Skin coloration and sugar accumulation are ethylene-independent, whereas yellowing of the rind, flesh softening, peduncle development abscission zone, volatile flavour compounds and climacteric respiration are totally or partially ethylene-dependent (Guis et al., 1997; Bauchot et al., 1998; Bower et al., 2002). Climacteric and non-climacteric regulation coexists during climacteric fruit ripening (Pech et al., 2008). Similar observations were made in *Charentais cantaloupensis* melons transformed with an antisense ACCO from apple (Silva et al., 2004). These authors showed prolonged fruit ripening cycle in an average of 10 days later, which supports the highest accumulation of sugars, in an average of 2.5°Brix higher than untransformed melons. Moreover, important phetotipics changes were observed; for example, vegetative cycle prolongation, increased fruits size, increased extensive root growth and minor leaves senescence. These characteristics were not described in earlier studies (Ayub et al., 1996; Bauchot et al., 1998).

Climacteric melons such as *cantaloupensis* are aromatic, but the ethylene suppressed to extend shelf-life can affect sensory qualities, especially aroma responsible for sensitive flavor (Pech et al., 2008). The synthesis of volatile compounds was significantly reduced in transgenic melons of Ayub et al. (1996), Bauchot et al. (1998) and Silva et al. (2004).

Bauchot et al. (1998), studying the behavior of transgenic melons by applying ethylene, verified that flavor intensity was restored by increase in the production of volatile compounds and induction of the peduncle abscission zone. In comple-mentary studies, Flores et al. (2002) and Yahyaoui et al. (2002) verified that the reestablishment of the overall production of volatile compounds and esters, in particular, was the consequence of alcohol acyltrans-ferases synthesis (AAT) induction and enzyme-key in the biosynthesis pathway of these compounds.

As a result, four clones of AAT (Cm-AAT1, Cm-AAT2, Cm-AAT3 and Cm-AAT4) were isolated and partially characterized in melons. Cm-AAT1 and cm-AAT4 are stronger and they are expressed during the ripening and under ethylene action (Yahyaoui et al., 2002; El-Sharkawy et al., 2005; Lucchetta et al., 2007).

However, in preliminary assays with transgenic melons (AS3 clone) (Silva et al., 2004), the responses to ethylene treatment were different from those observed by Bauchot et al. (1998), Flores et al. (2002) and Yahyaoui et al. (2002). The ripening was not completely re-established, where aroma intensity restoration was partially complete; although the treatment conditions with ethylene were similar to the ones described by other authors.

Some authors like Buttery and Ling (1993) and Goff and Klee (2006) state that, the improvement of plants to obtain a cultivar that is more productive, resistant to diseases and/or with extended shelf life can generate physiological changes and make the product to lose some important qualitative attributes. Silva et al. (2004) found that, *cantaloupensis* melons transformed (AS3) showed extended shelf life in postharvest. However, there was a significant reduction of its aroma intensity and low succulence of the fruits compared to control fruits. Goff and Klee (2006) co-related the volatile compounds production with the nutritional and functional quality of fruits. Also, they cited that the emission of volatile compounds results in the functional quality potential of fruits. Buttery and Ling (1993) observed that, the tomatoes selected for prolongation of shelf life have lesser nutritional quality and volatile compounds production than wild type tomatoes. In addition, it was verified that aroma, besides being the determinant of consumers' preference, can be associated with the best nutritional quality, essentials fatty acids, vitamins, carotenoids, licopens, folates, and other molecules with antioxidants properties (Goff and Klee, 2006).

This study explains the hypothesis which states that, the transformation with antisense ACC oxidase gene promotes other physiological modifications in melons. The practical non-existence of similar studies on fruits suggests that studies on the possible inter-relations between changes in ethylene production reduced (greater than 99%) as well as the postharvest behavior of melons.

In this work, the authors studied the effect of ethylene reduction and the exogenous treatment of this hormone on physiochemical characteristics, volatile compounds and expression of some genes with ethylene characteristics regulated during fruit ripening such as ACC oxidase (ACO), alcohol aciltransferase (AAT) expression and polygalacturonase genes (MPG1 and MPG2). Exogenous ethylene treatment was performed in AS3 fruits in order to verify if it was possible to restore the condition of ripening similar to that of WT.

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Table 1. Specific primers used for RT-PCR analysis of target genes.

Gen	Primers (5' - 3')	Author
CM-ACOO1	(F) AAG GAT CCG CAC AAA CCA AAT CTT GTA C (R) AAG GAT CCT AAG CTG AAA GTG AAT TTA AAT TA	Lassère et al., 1996
CM-AAT	(F) GTGATGGTGTGAGTCACACTGTTC (R) CGACCAGCAAGGTCCAAAC	John 1997
MPG1	(F) CTCTCATGCGCTGCAGTCTG (R) GCTTGGGCAATTTGATCCTT	
MPG2	(F) CCGCATGGAAGCAGGCTTGT (R) CCATGTCAACAGTAGAGCCT	Hadfield et al., 1998
ACTIN	(F) GAT GAC GCA GAT AAT GTT TGA GAC (R) AAG GTC ACG ACC AGC AAG GTC C	Bouquin et al., 1997

MATERIALS AND METHODS

Plant material

Non- transformed fruit (WT) and ACC oxidase antisense (AS3) Cantaloupe melons (*Cucumis melo* var. Cantalupensis, Naud cv.Vedrantais) were used (Silva et al., 2004). They were grown in a greenhouse under standard cultural practices for fertilization and pesticide treatments. Hermadrofite flowers were tagged on the day and self-hand pollinated. After this step, non- transgenic fruit plants were monitored during the period just to get to the actual 32 days after day pollination (DAP). During delayed ripening, AS3 fruits were harvested, 42 DAP and immediately exposed to 100 μL.L^{-1} ethylene for 24, 48 and 120 h in vessels of 7.2L. Vegetative root tissues were picked up from control and AS3 plants immediately after the harvesting of 2nd fruit per plant. After treating the fruits with ethylene, pulp firmness, total soluble solids, titratable acid and samples were frozen in liquid N and stored at - 80°C prior to analysis.

Soluble solid content (SSC), titratable acidity (TA) and pulp firmness

Fresh pulp firmness was determined using an 11-mm Effegi tester penetrometer and the results were expressed in Newton (N). Soluble solid content was measured by a digital refractometer (ATAGO PR-101, Tokyo, Japan), using filtered juice; the results were expressed by percentage (m/m). Titratable acidity was perfomed by titulometric method, using NaOH (0, 1 N) with pH 8.1. The results were expressed in mg citric acid g FW-^{1}.

Measurements of ethylene production

The ethylene content was determined by gas chromatography (Varian$^{®}$ 3300). The treatments were replicated three times, and values represented the mean ± SE. The results were expressed in nL of ethylene.g^{-1}.h^{-1}.

RT-PCR of ACC oxidase and CmAAT e polygalacturonase (MPG1 e MPG2)

Total RNA was extracted from 50 mg of frozen melon pulp with TRIZOL$^{®}$ Reagent (I*nvitrogen*) buffer according to the manufacturer's instruction. First strand cDNA was synthesized from 1 μg of total RNA (DNAse treated) using a poly (T) 15 as a primer and Kit SuperScript^{TM} First-Strand System for RT-PCR (Invitrogen). The reaction was stopped by heating at 70°C for 10 min, and treated with RNAse H. Forward (F) and reverse (R) primers (50 nM) used for RT-PCR amplification of the target genes in each RNA sample are described in Table 1.

The RT-PCR conditions were: 35 cycles at 95°C for 30 s (2 min for the first cycle), 47°C for Cm-AAT at 1 min and 53°C for β-Actin and CM-ACO1, and 72°C for 1 min (5 min for the last cycle). MPG1 and MPG2 cycle performance was: 35 cycles at 95°C for 1min (2 min for the first cycle), 47°C for 1:30 min and 72°C for 2 min (5 min for the last cycle). Actin gene was used as constitutive promoter.

Volatile compounds (esters)

All analyses were performed as described by Bauchot et al. (1998), with minor changes. SPME carboxen-PDMS (0.75 m × 1 cm, Supelco, USA) was used as the adsorbent matrix. All analyses were performed on a Varian 3800 gas chromatograph interfaced with a Shimadzu QP-50000 mass spectrometer. Volatiles were identified by comparing each mass spectrum with spectra from authentic compounds analyzed with spectra in reference collections (NIST/ EPA/NIH Mass Spectral database).

Antioxidant assay activity

Antioxidant activities were determined as a free radical according to Brand-Willams et al. (1995), by using 2, 2- Diphenyl-1-picrilhidrazil (DPPH) D-9132, Sigma-Aldrich, Dorset, UK. The samples analyzed were obtained from 100 g of fruits pulp dissolved in 250 ml of ultrapure water and centrifuged at 14000 x g for 15 min. The measurement of reduction absorbance was processed with 3.9 ml of free radical DPPH (100 µM) dissolved in 80% methanol. Then 0.1 ml of sample or standard was added to homogenize the mixture carefully. It was left in the dark for 30 min at a wavelength of 517 nm. The DPPH concentration in reaction was calculated by a linear regression obtained from calibrated curve. The results were expressed in TEAC activity equivalent to Trolox (acid 6-hidroxi-2, 5, 7, 8-tetramethylcrome-2-acid carboxilic, 97%; µM g of fresh weight^{-1}). The antioxidant synthetic Trolox was used based on calibrated curve.
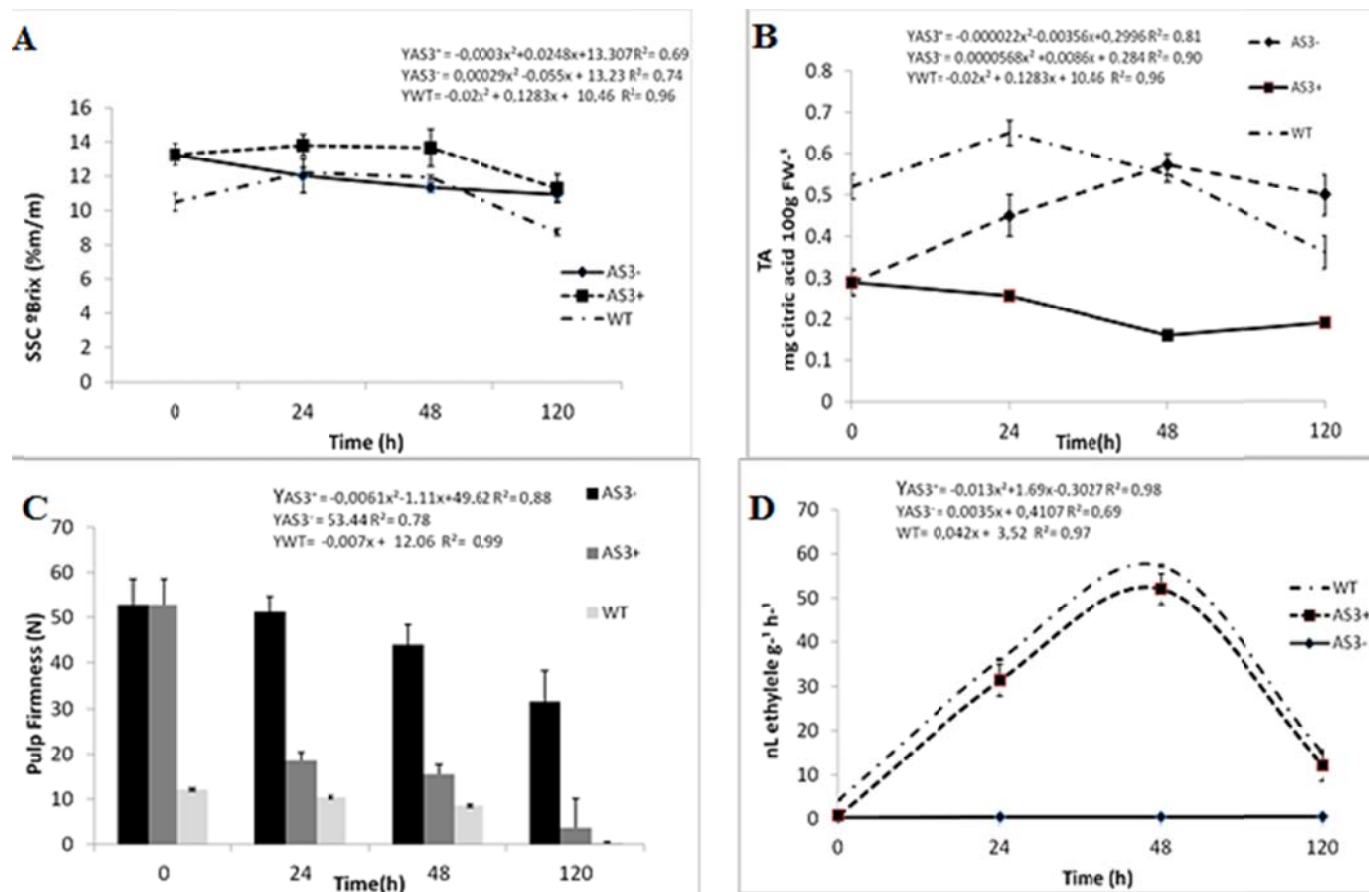
Figure 1. Characterization for Soluble solids content (A), titratable acidity (B), Pulp firmness (C) and Ethylene production (D) in melons in wild-type (WT) (C. melo, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h.

Ascorbic acid content

Ascorbic acid or vitamin C content in the melon pulp was measured using a high-performance liquid chromatography (HPLC) system. A Shimadzu liquid chromatography equipped with an auto sampler and a detector of 254 nm was used. A reversed-phase RP-18 column (5 mm particle size, 4.6 mm diameter, 150 mm length) with octadecyl stationary phase, operating at 25°C with a flow of 0.8 ml^{-1} min^{-1} was used.

Along with RP-18 guard column, it was used to separate the vitamin C using methanol (100%) and acidified water as a mobile phase. The ultra-pure water was acidified with acetic acid (0.1%, v/v). The mobile phase was filtered using a 0.45 im membrane filter and degassed using helium gas before passing through the column at 25°C with a flow of 0.8 ml^{-1} min^{-1}. A standard calibrated curve was obtained using L-ascorbic acid (Sigma Chemical, 99.97% of purity) in the following concentrations: 10, 25, 50, 75 and 100 mg 100 ml^{-1}. The method was adapted from Vinci et al. (1995) and Ayhan et al. (2001).

A portion of 10 g of melon pulp was cut into small pieces and diluted to 30 ml of phosphoric acid solution (4.5%). This sample with phosphoric acid solution was filtered and the volume was completed to 50 ml with ultrapure water. An aliquot of 1.5 ml of this mixture was centrifuged at 10 000 rpm for 10 min (T = 20°C). The volume of the supernatant after centrifugation was always accurately measured. A fraction of 10 µL of the supernatant was

injected into the HPLC chromatograph.

Statistical analysis

Experimental setup was performed in a completely randomized blocks with 3 mode of treatments [Control fruit, transformed without ethylene (AS3$^-$) and transformed with ethylene (AS3$^+$)]; it was evaluated four times when ethylene was applied. Each treatment is made up of three replicates. Four fruits were used for the evaluations. The data were subjected to variance analysis by Test-F $p < (0.05)$. For each treatment, regression analyses were performed to represent time index analysis.

RESULTS

Pulp firmness, soluble solid content (SSC), titratable acidity (TA) and ethylene production

The AS melon, Cantaloupe cv. Vedrantais had low production of ethylene, approximately 0.5 nL.h.g^{-1} (Figure 1A). This result is in accordance with that described by Silva et al. (2004) in previous studies, where it was verified that AS melon had 99.5% lower ethylene produc-
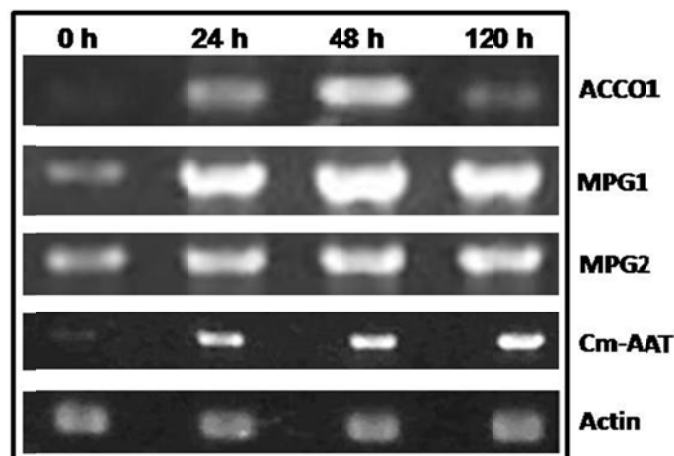
Figure 2. Expression of mRNA transcripts of ACC oxidase (ACCO1), poligalacturonase MPG1 and MPG2, alcohol acyl transferase (CmAAT) and Actin (Cm-Actin) in transgenic cantaloupe Vedrantais melon fruit (AS3) treated by ethylene exogenous for 0, 24, 48 and 120 h.

tion than control fruits. In other studies, Ayub et al. (1996) showed that transgenic melons expressing ACO anti-sense gene reduced ethylene production by 95%. AS melon treated with ethylene for 24 and 48 h and non-transformed fruits had similar ethylene production, but it declined in both materials at 120 h.

The wild-type melon quickly lost pulp firmness after harvest (Figure 1C). The main important characteristic of AS cantaloupes melon is that it maintains high pulp firmness in postharvest (Figure 1C). In AS fruits (AS3-), reduced firmness was lower than in control fruits during all the time of the analysis. The dates showed reduced pulp firmness in AS fruits of 52, 51, 48 and 32 N (harvest), respectively in 24, 48 and 120 h after harvest. This rise in the values obtained can be considered because 10 N is the minimum limit for the commercia-lization of this fruit. Otherwise, the presence of exoge-nous ethylene contributed to the reduction of pulp firmness significantly (Figure 1C). After 24 h of exoge-nous ethylene treatment, there was reduction in pulp firmness of 51 to 14 N. At the end of 120 h of exogenous ethylene treatment, pulp firmness was lower than 10 N reaching a value of 4 N. These results show that the strong correlation between pulp firmness and ethylene treatment was characterized by an ethylene-dependent event (Figure 1B).

In relation to SSC contents, AS3- increased during the first 24 h and kept on until the 48th hour; thereafter; there was a slight reduction of sugar contents. Slight reduction in AS3+ was observed until 120 h and was equal at the end. This is similar to AS3-. Also, in WT fruits, there was a slight increase in SS contents during 24 and 48 h and a strong reduction at the end of 120 h with value lower than 10°Brix (%m/%m).

Titratable acid (TA) in AS3- increased until the 48th hour.

It decreased after this period slightly until the 120th hour. On the contrary, when these fruits were exposed to exogenous ethylene treatment, there was a reduction in TA at the 48th hour and its establishment at the 120th hour. These results suggest that this reduction in TA content can be explained by higher respiration rate (data not shown), which must be in accordance with ethylene peak as shown in Figure 1D. Organic acids made part of the respiratory pathways. Already in non-transgenic fruit, there was a slight increase in acid within the first 24 h and a considerably decline at 120 h. Perhaps, in these fruits, sugars were transformed in organic acids and were used in the respiratory pathway.

ACC oxidase (ACCO1), polygalaturonase MPG1 and MPG2, and alcohol acyl transferase (CmAAT) gene expression

When evaluating the expressions of MPG1 and MPG2 genes in cantaloupensis melon under the action of ethylene, it was verified that both increased in the transcription genes; however, high levels of MPG1 gene were observed (Figure 2). In addition, the biggest difference in expression occurred within the first 24 h of ethylene treatment. These results suggest that MPG1 gene can be strongly involved with cell wall hydrolysis that leads to reduced pulp firmness (Figure 1C).

Considering that MPG1 and MPG2 correspond to an endo and exo-PG, respectively (Hadfield et al. 1998), it was expected that the increased expression of these genes would lead to reduction in pulp firmness. In fact, it happened so fast (Figure 2 and 1C). The ACC oxidase gene transcription in AS3 melons was lower than that in untransformed fruits (Figure 2). The ethylene applied in AS3 melons induced the transcription of ACC oxidase gene for 24 and 48 h (Figure 1A). The long exposure to ethylene (120 h) resulted in low amount of transcripts. Ethylene treatment induces the production of phytohor-mone like ACCO1 gene transcription (Figure 2). ACCO1 is a key enzyme in ethylene biosynthesis that shows the effect of induction treatment and recovery on the maturation of melons WT.

The mRNAs transcription of Cm-AAT1 was strongly expressed in control melons but weakly expressed in AS3 melons. This is because Cm-AAT1 gene is induced during ripening and under ethylene action. The ethylene applied in AS3 melons induced the transcription of Cm-AAT1, led to high expression of mRNAs and the attaining of similar levels with WT melons at 24 h. These elevated levels were maintained until 120 h (Figure 2).

Volatile compounds quantitation in AS melons

WT fruits had high production of esters. Ethylene produc-tion was reduced by transformed plants. It was observed as a significant reduction of volatile compounds synthesis
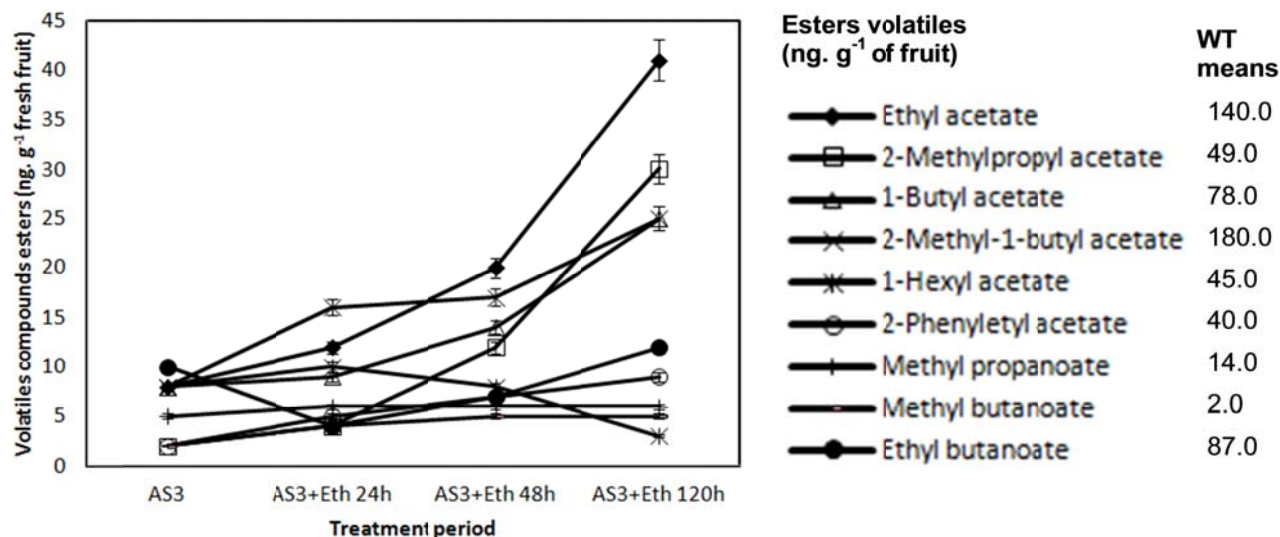
Figure 3. Contents of esters volatiles quantified (means) in melons wild-type (WT - right column) (C. melo, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h. Vertical bars represent standard error of the mean.

(Figure 3). Ethylene treatment restored the production of volatile compounds completely. This also happened in the study of Flores et al. (2002). It had partial effect, having recovered about 30% of volatile compounds; the fruits were maintained under ethylene action for 120 h (Figure 3).

Exogenous ethylene induced Cm-AAT1 transcription (Figure 2), but there was not a total effect on volatile compounds production (Figure 3). Within the compounds analyzed, 2-methylpropyl acetate, 1-butyl acetate, 2-Methyl-1-butyl acetate, 1-hexyl acetate, Methyl propanoate, ethyl propanoate, methyl butanoate and ethyl butanoate were more expressive than those treated with ethylene. By studying the possible cause of this behavior, ethylene was applied to enhance ACC oxidase expression in the proper phytohormone biosynthesis pathway, Cm-AAT1 and esters. The mRNAs expression of these genes was stimulated (Figure 2), which is in line with that of Yahyaoui et al. (2002).

Antioxidant total activity (TEAC) and ascorbic acid content

Also, in this study, some components of melon fruits that have nutritional and/or functional importance were evaluated. AS3 fruits that were harvested 42 DAA showed potential antioxidants levels higher than WT fruits (Figure 4A). The exposure of the fruits to exogenous ethylene for 24 h to 120 h enhanced antioxidants activity of AS3 melons. Moreover, to extend exposure of fruits to ethylene treatment, AS3 fruit exhibits values significantly higher than antioxidants activity compared to WT fruits.

Another component with antioxidant activity is ascorbic

acid. In transgenic plants, ascorbic acid is kept almost constant for 120 h. These results make one to believe that ethylene is able to regulate other genes related to secondary metabolism such as phenolic compounds. The ascorbic acid content (Vitamin C) was strongly influenced by genetic transformation (AS), resulting in drastic reduction in the fruits transformed (Figure 4B). The AS fruits with low ethylene production had significantly lower vitamin C than WT fruits during the harvest and after treating with exogenous ethylene.

DISCUSSION

The reduction of ethylene production in transgenic plants reduced ACC oxidase gene expression (Hamilton et al., 1991; Ayub et al., 1996; Silva et al., 2004) and led to improved long shelf life in postharvest. But with these changes in ethylene metabolism, the changes that occur by ethylene action were also modified. For example, majority of these studies verified lower chlorophyll degradation but not induced abscission zone, delayed leaves senescence, less pulp softening and reduced volatile compounds synthesis (Ayub et al., 1996; Bauchot et al., Silva et al., 2004).

Flores et al. (2002) demonstrated that when the transformed plants with antisense ACC oxidase gene from melon were exposed, ethylene was seen; this reestablished the ripening of the process. This also occurred in AS3 melons obtained by Silva et al. (2004).

The fruits treated with ethylene for 120 h had low production of ethylene. This is probably due to the induction of the ACCO1 gene expression (Figure 2); and production of ethylene was strong during the first 48 h.
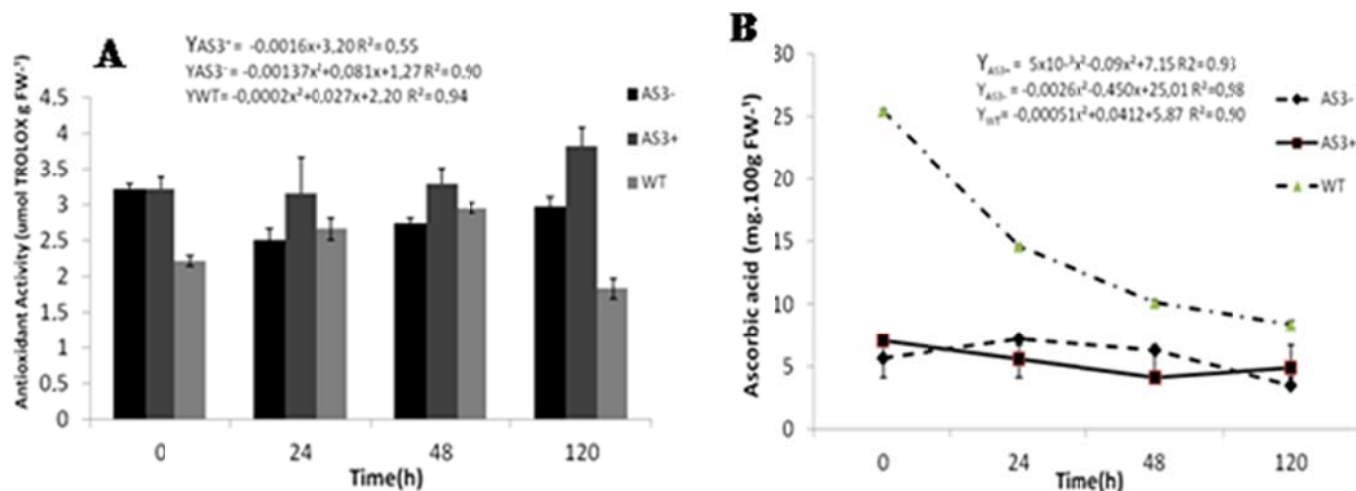
Figure 4. Characterization of antioxidant activity (A) and ascorbic acid (B) in melons in wild-type (WT) (*C. melo*, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h. Vertical bars represent standard error of the mean.

For 120 h, there were mature stadium and deterioration symptoms in this phase. They were characterized by low pulp firmness (Figure 1C). The effect of ethylene on the transcription induction of this gene was demonstrated by Lassere et al. (1996). However, these authors had studied the expression of this and other genes in vegetal models without genetic modification.

Ayub et al. (1996) and Bauchot et al. (1998), studying ACC oxidase melons antisense, related that the flesh softening in transformed fruits were practically inhibited. After 10 days of storage at 25°C, transgenic fruit remained fully firm, with a green rind and unaltered shape; whereas, wild-type fruit displayed senescence profile with a shriveled yellow rind, fungal infection, soft flesh and squashed shape. The continuous treatment of detached transgenic fruit with 100 ppm ethylene reversed the antisense phenotype result, which led to the activation of abscission zone melon, declined firmness and increased yellowing, like in control fruits.

The perception of autocatalytic ethylene is given by a set of receptor which transduces signal through a cascade of factors such as ctr, ein2, ein3/EIL and finally ethylene response factors (ERFs) (Bapat et al., 2010). In general, reducing the production and/or the action of ethylene, such as cold storage (CS), controlled atmosphere (CA) and the use of control systems (Zhou et al., 2000b; Dong et al., 2001) result in more mainte-nance of pulp firmness. Chaves et al. (1997) had verified, however, that the benefits of genetic modification in pulp firmness manifest just in fruits harvested in the stadium "breaker". When the fruits were matured, this did not present differences between fruits with low and high ethylene production. In AS3 melons, this did not take place. The reduction of ethylene production led to the maintenance of pulp firmness and exogenous phytohor-mone treatment softens the fruits (Figure 1C). Nishiyama et al. (2007) also demonstrated that in transgenic melon with ACO suppressed expression gene, there was a complete inhibition of softening of pulp, but was restored by exogenous ethylene treatments. In control fruits, when 1-MCP was applied, there was a significant reduction in the loss of firmness, which suggests an ethylene-dependent event.

In the harvesting of AS3 melons (42 DAA), the fruits did not show the peak of climateric maturity, which occurs in the maximum accumulation of reserves. Melons transformed with ACO1 antisense demonstrated greater accumulation of SS, which was also observed in previous experiments (Ayub et al., 1996; Silva et al., 2004; Grumet et al., 2007). The AS3 fruit was treated with exogenous ethylene, and rates of SS and TA were changed because there was an acceleration of maturation process. Probably, the solubilization of pectin that occurs in this period can explain the increase of SS and TA (Figure 1A and B). The solubilized carbohydrates, although less in melons, are in the order of 0.5 to 1.2%. Temporarily, during the post-harvest, this can result in the accumulation of SS content and increased acidity. In the reduction, when the fruit was treated with ethylene, between 48 and 120 h, there was consumption of sugars through the maintenance of aerobic respiration and senescence process.

The variations of pulp firmness are related with hydrolytic enzymes, where transcription can be ethylene-dependent (α-L-arabinosidase and β-D-galactosidase, endo-poligalactuonase) (Guis et al. 1997, 1999; Pech et al., 2008), ethylene-independent (pectil-metil-esterase) (Guis et al., 1997, 1999 and exo-poligalacturonase (Lelièvre et al., 1997). In melons, the main hydrolytic enzymes of cell wall are pectilmehyl esterase, endo and

exo-poligalacturonase, β-galactosidases/β-galactanases, expansins, endo-1, 4-b-glucanases, and xyloglucan endotransglycosylases (Rose et al., 1998; Hadfield et al., 1998).

Earlier, Gonçalves et al. (2013) showed that PG1 responds to ethylene treatment in pMEL1AS and pAP4AS cloned fruits. The regulation of gene expression during maturation and senescence related as wall cellular enzymes has generated a lot of discussion. Sitrit and Bennett (1998), studying polygalacturonase behavior gene in tomatoes expressing an ACC synthase gene with low ethylene production, verified that polygalacturonase mRNA gene was suppressed but when ethylene was applied, there was increase in mRNA transcripts levels of PG. However, in both cases, there was fruit softening. Regarding the gene MPG2, cited by Hadfield et al. (1998) as a possible exo-PG, the accumulation of mRNAs was gradual under the action of ethylene.

In mango, Sane et al. (2005) describe an expansion gene which correlates with the other genes of the cell wall metabolism during maturation induced by ethylene treatment. In advanced stages of ripening, endo- β-1, 4-glucanase enzymes correlate with increased activity of EGase (Chourasia et al., 2008). This study overlapping expression of cell wall enzymes shows synergistic action which explains why the change of part of the plant cell wall metabolism is directly influenced by ethylene, while another part depends on physiological factors correlated. Quesada et al. (2009) demonstrated that PG plays an important role in the ripening of strawberry and is negatively regulated by auxin. In strawberry fruits with PG transformed anti sense, the behavior is similar to that of melons, which maintain pulp firmness and increase the content of soluble solids during ripening.

MPG1 and MPG2 correspond respectively to an endo and exo-PG. Hadfield et al. (1998) state that both are involved in the reduction of pulp firmness, but MPG1 gene has stronger effect. This explains the quick and severe loss of firmness during the first 24 to 48 h of exposure to ethylene. It should be noted, however, that the interpretations of this study, which entail evaluating the mRNAs of genes and not enzymatic activity, give the assumption that the period between the transcription, translation, and post modification co-translational is long. This statement is made because in some cases, there is no relationship between the rate of transcription and enzymatic activity respectively. Like in the case of tomatoes, where the gene transcription PG is far above the máximum enzymatic activity (Sitrit and Bennett, 1998). However, Hadfield et al. (1998) and Rose et al. (1998) observed that these events are simultaneous and co-ordinated in melon.

The mRNAs transcription of Cm-AAT1 was strongly expressed in control melons than in AS3 melons. This behavior is based on the fact that, Cm-AAT1 gene is induced during the ripening and under ethylene action (Flores et al., 2002; Katzir et al., 2008); this is with pMEL

clone of ACC oxidase gene (Lassere et al., 1996). The WT fruits had high aroma production. When ethylene production was reduced, volatile compounds synthesis was reduced too. This behavior has been explained earlier by other authors (Bauchot et al., 1998; Yahyaoui et al., 2002; Silva et al., 2004). The ethylene treatment did not restor the volatile compounds production completely, as seen in the study of Flores et al. (2002). The effect was partial. This behavior is not due to ethylene treatment imperfections, but because it has the same conditions described by other authors (Bauchot et al., 1998; Yahyaoui et al., 2002; Flores et al., 2002). The ethylene treatment induced Cm-AAT1 transcription (Figure 2), but there was no total response to volatile compounds production, which is contrary to the results obtained by Flores et al. (2002).

By studying the possible causes of this behavior ethylene was applied to enhance ACC oxidase expression in the proper phytohormone biosynthesis pathway, Cm-AAT1 and esters. The mRNAs expression of these genes was stimulated (Figure 2), as described by Yahyaoui et al. (2002). This behavior could have occurred by controlling the processes of post-transcriptions phases and/or other metabolism pathways that reduced levels of subtrates such as Acyl CoA, organics acids, aldehyds, alcohols from fatty acids and amino acids degradation (Song and Bangerth, 2003; Fellman et al., 2000). In general, the metabolism is lower when ethylene production is low; so it suggests that the reserved degradation that gives substrates physiological events in secondary metabolism is affected (Baldwin et al., 2000; Bauchot et al., 1998). Moreover, the effect of ethylene in the processes of CoA-SH recycled; reaction product in the esters pathway by action of AATs (Lucchetta et al., 2007) is unknown. Hypothesis is not tested in this study. In climacteric fruit, the esters volatile compounds are prevalent in strawberries (Severo et al., 2011), apple (Villatoro et al., 2008) and melon (Obando-Ulloa et al., 2008).

The volatile compound was quantified in these fruits. This has already been done by Bauchot et al. (1998). WT fruits have high esters production. When it was reduced, the ethylene produced by transformed plants was observed as a significant factor responsible for the low volatile compounds synthesis (Figure 3). This behavior has been explained earlier by other authors (Yahyaoui et al., 2002; Silva et al., 2004; Pech et al., 2008). The ethylene treatment did not restore the volatile compounds production completely, as seen in the study of Flores et al. (2002). It was partial, having recovered around 30% of volatile compounds; although this maintained the fruits under 120 h ethylene actions (Figure 3). This behavior is not due to ethylene treatments imperfections, but due to its concentrations and times of exposure (data not shown). The compounds analyzed, 2-Methylpropyl acetate, 1-butyl acetate, 2-methyl-1-butyl acetate, 1-hexyl acetate, methyl propanoate, ethyl propanoate, methyl

butanoate and Ethyl butanoate were more expressive than the one treated with ethylene.

The aroma profile of the melon decreases with maturity and senescence. In the same species, the profile of volatile compounds is different between climacteric and non-climacteric fruits (Obando-Ulloa et al., 2008). Villatoro et al. (2008) demonstrated that during the ripening of apples, there was increased esters production primarily by the accumulation of substrate for the action of the enzyme alcohol acyltransferase. This is due to the action of other enzymes such as precursor lipoxygenase (LOX), hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) that give rise to the substrates.

Souleyre et al. (2005) showed that the substrate is not necessarily the profile of esters of fruit; this explains why there are no specific precursors of the esters. Severo et al. (2011) describe significant affinity between the transcription and physiological responses related to changes in sensory and nutritional strawberry, which highlights genes involved in cell wall metabolism, phenolic compounds biosynthesis, ascorbic acid and aroma (ADH and, AAT).

In earlier study (Shan et al., 2012), melons transformed with antisense AAT resulted in levels of mRNA transcripts and lower enzymatic activity than WT fruits. This caused a reduction in esters production. The reduction of esters contributed to a greater accumulation of aldehydes and alcohols that normally decrease with ripening. In previous studies, our group of collaborators (Yahayaoui et al., 2002; Ei-Sharkawy et al., 2005; Lucchetta et al., 2007) had already reported that these clones (Cm-AAT) after expression in yeast were active and showed different substrate preferences.

One of the molecules included in the antioxidant activity are phenolic compounds, although they are not evaluated in this study. These phenollic molecules can contribute to increase antioxidant activity. In studies with Kiwi fruits, Park et al. (2008) showed a strong correlation between applying ethylene with phenol compounds. The same authors showed that there are different phenolic compounds interacting in different moments at fruit ripening stage.

The evaluations also showed the functional/nutritional modifications in the melon pulp; therefore, the AS fruits had low accumulation of ascorbic acid (vitamin C), but they had a significant increased antioxidant potential. Ioannidi et al. (2009), studying the expression profile of ascorbic acid-related genes during tomato fruit development and ripening, showed that L-Galactose-1-phosphate phosphatase mRNA and transduction are dependent on ethylene. Perhaps, in our study, this gene can be changed by antisense ACC oxidase gene, showing a small loss of AA content compared to the wild type. The carotenoids contents were not altered by genetic modification and treatment with ethylene (data not shown).

The reduction of ascorbic acid contents in AS melons and genetic modification were on average five times the values found in fruits. After the harvest, in the fruits treated with exogenous ethylene, a stimulation of the maturation was verified. This led to a significant reduction of ascorbic acid contents, mainly in WT melons, that were more sensible to this phytohormone. This reduction is also observed in other fruits with mature stadium (Andrade et al., 2002) where, the oxidation of ascorbic acid (vitamin C) produces compounds with radical carbonyl that can react with amino groups and by polymerization produce dark pigments. The levels of ascorbic acid in AS fruits were practically unchanged.

An hypothesis is linked to cell wall metabolism. Di Matteo et al. (2010) verified that the up- regulation of a pectinesterase and two polygalacturonases suggests that AsA accumulation in tomato fruit is mainly achieved by increasing flux through the L-galactonic acid pathway, which is driven by pectin degradation and may be triggered by ethylene. Otherwise, in our AS3 melons, AA was kept due to low production of ethylene and consequently low expression of polygalacturonase as demonstrated in Figure 2.

The antioxidant activity of AS melons in the harvest was on average 100% more than that of WT fruits. The genetic modification resulted in reduction of ethylene production and in prolongation of maturation cycle. This led to high accumulation of compounds that result in antioxidant activity. The postharvest treatment with exogenous ethylene accelerated the process of maturation and the accumulation of compounds with antioxidant capacity. The effect of ethylene was more intense in WT fruits; however the indices of the antioxidant activity of AS continued to be significantly higher. The levels of potential antioxidant activity in cantaloupes melons have average good values in relation to some fruits commercialized; however they were lesser in red fruits (Kuskoski et al., 2005). The antioxidant capacity of fruits makes provision for some components, mainly phenols and the concentrations depend on environmental conditions, cultivar, species, etc. In this study, the genetic modification changed the composition and quantity of potential antioxidant. These differences in compounds can change the interaction for synergism or inhibitory effect (Rice-Evans et al., 1999; Robards et al., 1999).

To explain the behavior of WT melons and exogenous treatments, the possible interference of the ethylene production reduced can be related to cytokinins amounts and possible responses to the ethylene treatment (Zaicovski et al., 2008, Gonçalves et al., 2013). Zaicovski et al. (2008), evaluating different effects of depth irrigation on broccoli, showed that hydric stress was able to extend shelf life, gave high cytokinin levels and low ethylene production. Liu et al. (2013) transformed broccoli with isopentenyltransferase transformed (IPT), which encodes the key enzyme for cytokinin; and exoge-

nous treatment with N6-benzylaminopurine promoted postharvest conservation, establishing a system of protection.

The transgenic melons plants had significant phenoltypes alterations such as delaying leaves senescence, emission of more shoots and prolonging cycle of ripening. This indicates that other hormones interaction changes this phenotypes aspect, modifies ethylene sensibility, increases roots mass and more accumulation of the cytokinins levels in roots, pulp and rind of fruits (Gonçalves et al., 2013). The high accumulation of transcripts of genes involved in cytokinin synthesis shows that cytokines could be responsible for these different physiological behaviors of melon. Broccoli (Chen et al., 2001) and tomato (Martineau et al. 1995), induced to increase cytokinins, had significant effect on the ethylene responses. The irrigation management can stimulate the roots emission and increase the synthesis and translocation of cytokinins. This leads to reduction in the ethylene responses, leaves and flowers senescence (Zaicovski et al., 2008; Chang et al., 2003; Hedden and Philips, 2000; Martineau et al. 1995). In addition, cytokinins treatment in broccolis reduced the ethylene responses, which leads to the prevention of high green color degradation (Tian et al., 1995; Downs et al., 1997). In the case of melon, Gonçalves et al. (2013) applied exogenous cytokinin. But, it did not show any differences in ethylene production, firmness, soluble solids, titratable acidity, carotenoids, volatile ester compounds, or the contents of mRNA. Although the physiological mechanism has not been well described, the authors suggest the relation of cytokinins synthesis and accumulation increased with shelf life prolongation.

On the other hand, Yang et al. (2013) showed that in apple, the 1-MCP treatment induced changes in expression of genes involved in ethylene biosynthesis, perception and signal transduction. The 1-MCP blocked the system of perception and signal transduction of ethylene, resulting in decreased expression of genes involved in the ethylene response autocatalysis. In the case of AS3 melon, there were also changes in the perception and transduction system changed signal with low expression of related genes, causing a feeble response to ethylene treatment.

Conclusion

The exogenous ethylene treatments in transgenic fruits were reestablished and the metabolism was partially restored, changing fruit quality attributes. Transgenic melon expressing an antisense ACC oxidase under ethylene treatment was able to restore polygalacturonase genes (MPG1 and MPG2). Fruit firmness was greatly reduced similar to non- transgenic fruits. For CmAAT, the restoration of expression was similar to WT levels; however, there was no consistent amount of the esters productions, strengthening the hypothesis that, other

factors influence the aromatic compounds production. AS3 melons showed higher total antioxidant activity than WT maintained throughout the treatment with exogenous ethylene.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genetic variability and heritability of vegetative, fruit and seed yield traits in fluted pumpkin (*Telfairia occidentalis* Hook F)

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*Telfairia occidentalis (*fluted pumpkin*)* is a leafy vegetable crop popularly cultivated in the South-eastern part of Nigeria for its economic value and important role in nutrition and poverty alleviation. Nine genotypes of fluted pumpkin collected from different localities in Ogun, Lagos and Oyo States Nigeria were grown in the teaching and research farm of the School of Agriculture, Babcock University during the rainy season of 2011, in order to determine genetic variability and heritability of 15 characters comprising of seven vegetative, five fruit and three seed yield traits in fluted pumpkin. The experiment was laid out in a randomized complete block design with three replications. The results reveal huge genetic diversity among the genotypes studied. Number of branches per plant, vine length and number of fruits per plant were the most genetically divergent traits measured. Vine length (21.72, 97.38 and 44.16), number of branches per plant (23.86, 96.59, 48.31), seed weight per plant (18.43, 79.31 and 33.81) and leaf width (11.36, 80.73 and 21.03) showed high genotypic coefficient of variability (GCV), heritability and genetic advance (GA). These characters are most likely under additive gene control and can be effective in the prediction of vegetative, fruit and seed yield in *Telfairia.* Correlation analysis revealed that selection directed towards leaf length, leaf width, petiole length, vine length, vine width, number of fruit per plant, fruit length and fruit width will be efficient in improving vegetative and seed yield in *T. occidentalis.*

Key words: Fluted pumpkin, cucurbitaceace, divergent, vegetative, genetic advance.

INTRODUCTION

Telfairia occidentalis also known as fluted pumpkin is a leafy vegetable crop commonly cultivated in the South-eastern part of Nigeria (Odiaka et al., 2008); however, it is gradually gaining prominence in the western part of the country (Schippers, 2002). It belongs to the family Cucurbitaceae having over 90 genera (Akoroda, 1990). It

has a creeping growth habit that spreads across the ground to produce an efficient cover to the ground against erosion (Horsfall and Spiff, 2005) and produces large fruits with many large seeds. The seed is a rich source of oil and protein in the proportion of 30.1 and 47%, respectively (Akoroda, 1990; Ehiagbonare, 2008); while the leaf is a veritable source of protein, iron, saponin, tannins, phytic acid, vitamins and minerals (Akwaowo et al., 2000; Akanbi et al., 2007; Ehiagbonare, 2008). The leaf extract is commonly used in the treatment of anaemia, high blood pressure, convulsion and diabetes (Ehiagbonare, 2008; Fayeun et al., 2012); whereas the seed oil extract can be used in the production of margarine and pomade (Asiegbu, 1987). Beyond its nutritional and medicinal values, farmers prefer to grow fluted pumpkin because it generate appreciable cash to small family holdings thus alleviating poverty (Akoroda, 1990; Fayeun et al., 2012). It also portrays an ethnobotanical importance in the folklore of the Igbo's in the South-eastern part of Nigeria (Oyekunle and Oyelere, 2005)

The success of any crop breeding programme largely depends on the availability of huge genetic variability, genetic advance and character association, direct and indirect effects on yield and its attributes (Nwangburuka et al., 2012). Genetic diversity is important for selection of parents to recover transgressive segregants (Kiran Patro and Ravisankar, 2004). Genetic variability and heritability studies have been conducted on various vegetable crops in their breeding programme. For instance, *Solanum anguivi* (Denton and Nwangburuka, 2011), okra (Nwangburuka et al., 2012), *Corchorus olitorius* (Nwangburuka and Denton, 2012), kenaf (Mostofa et al., 2002), roselle (Ibrahim and Hussein, 2006), tomato (Foolad et al., 2006), cowpea (Aremu et al., 2007) or eggplant (Islam and Uddin, 2009). However, there is sparse information on the heritability studies in fluted pumpkin (Aremu and Adewale, 2012). The few information on heritability in fluted pumpkin has focused more on the vegetative yield characters (Oyekunle and Oyelere, 2005; Fayeun et al., 2012, Aremu and Adewale, 2012). Meanwhile, abundance and availability of improved seeds is a major factor in the continuous cultivation of fluted pumpkin for economic purpose and nutrition. Hence, there is need to evaluate the diversity in fluted pumpkin in both vegetative, seed and fruit characters and the heritability of these characters as a tool in the improvement of the overall yield of this crop.

Determination of heritability estimates, using different methods (Obilana and Fakorede, 1981; Wray and Visscher, 2008) will provide information on the proportion of phenotypic variance that is due to genetic factors for different traits but heritability estimate alone is not a sufficient measure of the level of possible genetic progress that might arise not even when the most outstanding individuals are selected in a breeding programme. The value of heritability estimates is enhanced when used together with the selection differential or genetic advance (Ibrahim and Hussein, 2006). Information on the amount and direction of association between yield and yield related characteristics is important for rapid progress in selection and genetic improvement of a crop (Asish et al., 2008). This will indicate the interrelationship between two or more plant characters and yield, providing suitable means for indirect selection for yield.

This study is aimed at evaluating the genetic variability in fluted pumpkin and further determining the traits associated with the seed, fruit and vegetative yield in fluted pumpkin. The heritability of those traits will be evaluated for indirect selection to improve fluted pumpkin fruit, seed and leaf yield.

MATERIALS AND METHODS

The nine accessions of fluted pumpkin used for this study were collections from different localities in Ogun, Lagos and Oyo State, where fluted pumpkin is intensively cultivated and deposited in the germplasm of the Department of Agriculture Babcock University, Ogun State, Nigeria.

This study was conducted in the Teaching and Research Farm of the Department of Agriculture Babcock University, Ilishan-Remo, Ogun State, Nigeria located on 6° 5'N, 6° 43'E during the rainy season of May 2011 to December 2011. The experiment was laid out in a randomized complete block design (RCBD) with three replications. Each replication consists of nine plots of double rows of *Telfairia* genotypes identified as follows: BUTEL 001, BUTEL002, BUTEL003, BUTEL004, BUTEL005, BUTEL006, BUTEL007, BUTEL009 and BUTEL010. Each row was 9 m long with intra-row spacing of 1 m and inter row spacing of 1 m whereas the distance between each plot was 2 m following the procedure provided in the Department of Agriculture Babcock University. The seeds lot were confirmed viable after germination test before planting. There were a total of nine plants per row, given a total of 18 plants per plot of genotype. The seeds were sown directly into the already prepared soil after allowing it under the sun for about 6 h as a pre-germination condition to enhance early germination.

Data collection

Data were collected on the following vegetative and seed yield characters as shown in Table 1.

Data analysis

The plot means for each character was subjected to analysis of variance using SAS (1999) employing the method prescribed by Steel and Torrie (1980). The yield and its component were used to determine the genotypic and phenotypic variances according to Prasad et al. (1981).

Genotypic variance $(\delta^2 g)$ = (MSG - MSE)/r

Phenotypic variance $(\delta^2 ph)$ = MSG/r

Error variance $(\delta^2 e)$ = MSE/r

Where, MSG = Genotype mean squares; MSE = error mean squares; R = number of replications

Table 1. Quantitative characters of *Telfeiria occidentalis* and the method of measurement.

Quantitative characters	Method/Unit of Measurement
Leaf length at 9 weeks	Measured from five middle row plants (cm)
Leaf width at 9 weeks	Same as in above using a ruler (cm)
Vine length at 9 weeks	Same as in above using a tape (cm)
Vine width	Same as in above using a vernier caliper (cm)
Number of branches at 9 weeks	Same as in above
Petiole Length at 9 weeks	Same as in above using a ruler (cm)
Number of leaflet at 9 weeks	Determined from 5 random leaves on the vine
Number of fruit per plant at harvest	Average Count of all fruits per plot/rep
Fruit weight per plant at harvest	Average weight of 10 fruits/plot (kg)
Fruit length at harvest	Same as in above using a ruler (cm)
Fruit width at harvest	Same as in above using a tape (cm)
Number of seeds per fruit at harvest	Mean from 5seeds/ 10 random fruit per row (g)
Seed length at harvest	Mean from 20 bulked seeds per row(cm)
Seed width at harvest	Same as in above using a ruler (cm)
Total seed weight per fruit harvest	Mean of seeds from 5 random fruits /row (g)

The variance components was used to compute the genotypic coefficient of variability (GCV), phenotypic coefficient of variability (PCV) as follows according to the methods of Burton (1952), Johnson et al. (1955) and Kumar et al. (1985).

Genotypic coefficient of variability = $100 \times [(\delta g)/x]$

Phenotypic coefficient of variability = $100 \times [(\delta ph)/x]$

Where, δg and δph are the genotypic and phenotypic standard deviations, respectively and X is the grand mean for the character under consideration.

The Broad-sense heritability and expected genetic advance, assuming a selection intensity of 5% were estimated according to the formula of Allard (1960) and Miller et al. (1958) as follows:

Heritability (Broad-sense) = $(\delta^2 g)/ [\delta^2 g + \delta^2 e]$

Where, $\delta^2 g$ is the estimate of genotypic variance and $\delta^2 e$ is the estimate of environmental variance.

Genetic advance = heritability x k x δph

Where, K (selection differential expressed in phenotypic standard deviations) = 2.06

Estimates of genotypic and phenotypic correlation coefficients among the characters were obtained using the formula of Miller et al. (1958):

$$r_{x,y} = \frac{CoV_{(x,y)}}{\sqrt{(\delta^2 x)(\delta^2 y)}}$$

Where, $r_{x,y}$ is either genotypic or phenotypic correlation between variables x and y $CoV_{(x,y)}$ is the genotypic or phenotypic covariance between two variables; $\delta^2 x$ is the genotypic or phenotypic variance of the variable x, $\delta^2 y$ is the genotypic or phenotypic variance of the variable yield y.

In order to determine the interrelationships amongst the character traits (yield characters) correlations were calculated. Experimental correlation coefficients were determined according to Falconer (1981).

RESULTS AND DISCUSSION

The result of analysis of variance indicated significant differences ($p>0.01$ and 0.05) in genotypes in all the characters studies except in number of fruits per plant at harvest, fruit weight per plant at harvest, fruit length at harvest, fruit width at harvest, seed length at harvest and seed width at harvest (Table 2). This suggests significant diversity among the genotypes studied. This finding is at variance with earlier observation by Ajayi et al. (2006), who reported a narrow genetic diversity in *T. occidentalis*. However, our finding agrees with the reports of Aremu and Adewale (2012) and Fayeun et al. (2012) who observed significant genetic variability in *Telfairia* genotypes in vegetative characters such as number of branches per plant, vine length and vine width, number of foliage and foliage width.

This observation further suggests that there is relatively low variability in fruit and seed traits compared to the vegetative traits in *T. occidentalis.* Table 3 shows the means, genotypic and phenotypic variances, genotypic coefficient of variability (PCV and GCV), estimates of broad-sense heritability and genetic advance in all the characters studied in the nine *Telfairia* genotypes. Generally, the genotypic variance is higher than the environmental variance, except in number of fruits per plant and fruit yield per plant. This may suggests that the genotype component contributed more to the expression of the vegetative and few of the seed and fruit characters compared to the contribution of the environment. This information will enhance selection towards vegetative yield as well as seed yield in *Telfairia*.

This observation is contrary to the report of Fayeun et al. *(*2012), who observed higher environmental variance in number of branches over genotypic variance. Meanwhile, procurement of sufficient seeds has been

Table 2. Mean Squares of leafy vegetable and seed yield related characters of Nine *Telfairia* accessions.

Source of Variation	df	Leaf length at 9 weeks	Leaf width at 9 weeks	Vine length at 9 weeks	Vine width at 9 weeks	Number of branches at 9 weeks	Petiole length at 9 weeks	Number of leaflets at 9 weeks	Number of fruits per plant at maturity	Fruit weight per plant at maturity	Fruit length at maturity	Fruit width at maturity	Number of seeds per fruit at maturity	Seed length at maturity	Seed width at maturity	Total seed weight per fruit at maturity
Block	2	27.97**	6.46**	144.05	0.007	0.02	0.21	0.02	61.18*	5.50*	8.44	14.78	278.86	0.12	0.003	0.06*
Genotype	8	6.02*	5.35**	3526.15**	0.01*	2.11**	3.63**	0.15**	26.62	1.76	62.82	20.75	291.13*	0.06	0.04	0.06**
Error	16	2.19	1.03	92.37	0.002	0.07	0.10	0.01	14.92	1.25	17.53	8.69	120.84	0.07	0.04	0.01
CV %		9.99	9.61	6.17	9.23	7.76	2.48	3.26	39.44	32.01	12.27	10.62	26.21	7.47	5.50	10.24

*Significant at 5% ($p > 0.05$) level of probability; **significant at 1% ($p > 0.01$) level of probability.

Table 3. Estimates of Phenotypic and Genotypic variance, Phenotypic and Genotypic coefficient of variability, Broad sense Heritability and Genetic Advance in 15 characters of *T. occidentalis*.

Character	Phen var	Gen var	Env var	PCV	GCV	Heritability	Gen adv%
leafL9wk	2.01	1.28	0.73	9.57	7.63	63.62	12.54
leafW9wk	1.78	1.44	0.34	12.64	11.36	80.73	21.03
Petlgt9wk	1.21	1.18	0.03	8.53	8.41	97.16	17.08
Vinelgt9wk	1175.39	1144.60	30.79	22.02	21.72	97.38	44.16
Vinewdth9wk	0.00	0.00	0.00	11.23	10.16	81.82	18.93
Nobranches9wk	0.70	0.68	0.02	24.28	23.86	96.59	48.31
Noleaflet	0.05	0.04	0.00	6.52	6.24	91.72	12.32
Nofruit	8.87	3.90	4.98	30.41	20.15	43.93	27.52
Fruityield	0.59	0.17	0.42	21.93	11.82	29.03	13.12
Noseed	97.04	56.76	40.28	23.49	17.96	58.49	28.30
Fruitlgt	20.81	14.96	5.84	13.37	11.34	71.92	19.81
Fruitwdt	6.92	4.02	2.90	9.47	7.22	58.10	11.33
Seedlgt	0.02	0.00	0.02	4.33	1.68	15.15	1.35
Seedwdt	0.01	0.00	0.01	3.46	1.38	15.91	1.13
Wgtseed	0.02	0.02	0.00	20.69	18.43	79.31	33.81

LeafL9wk = Leaf length at 9 weeks; leafW9wk = leaf width at 9 weeks; Petlgt9wk = petiole length at 9 weeks; Vinelgt 9wk = vine length at 9 weeks; Vinewdth9wk = vine width at 9 weeks; Nobranches9wk = number of branches at 9 weeks; Noleaflet = number of leaflets; Nofruit = number of fruits per plant; Fruityield = fruit yield per plant; Noseed = number of seeds per fruit; Fruitlgt = fruit length; Fruitwdt = fruit width; Seedlgt = seed length; Seedwdt = seed width; Wgtseed = weight of seed per plant; Phen var = phenotypic variance; Gen var = genotypic variance; Env var = environmental variance; PCV = phenotypic coefficient of variability; GCV = genotypic coefficient of variability; Gen adv% = genetic advance.

observed as a major challenge confronting *Telfairia* farmers (Oyekunle and Oyelere, 2005) and can be significantly addresses via selections towards vegetative and seed yield. The higher environmental variance observed in number of fruits per plant and fruit yield per plant may be due

to environmental interference encountered during the commonly practiced exercise of removing more of the male plants in order to allow for more of female plants to favour fruit production in *T. occidentalis*.

This practice can lead to abortion of young developing fruits. Hence, selection on the basis of number of fruits and fruit yield per plant for yield improvement is environment specific. There was higher genotypic variance over environmental variance in number of branches per plant, indicating a significant contribution of the genotypic component in the branching of the accessions. This further suggests adequate genetic gain when selection favours number of branches per plant. This observation is contrary to the report of Fayeun et al. (2012) who observed higher environmental variance against genotypic variance in branching per plant. Similarly, phenotypic variance was slightly higher than the genotypic variance in all the characters studied as expected, since the phenotype variance is the sum of both the genotypic and environmental variance. This report agrees with the observation of Fayeun et al. (2012), who observed higher phenotypic variance in all the character studied above the genotypic variance. High genotypic variance facilitates selection for improvement and widens the probability for heritability of traits from parents to offsprings (Ayanley et al., 2012).

The values of phenotypic coefficient of variability (PCV) and the genotypic coefficient of variability (GCV) are useful in comparing the relative amount of phenotypic and genotypic variation among different characters. The PCV values were slightly higher than the GCV values in all the characters studied, confirming slight environmental influence on the expression of all the characters studied. This corresponds to the report of Fayeun et al. (2012), who observed higher PCV in all the vegetative characters studied in *T. occidentalis*, except in vine width and leaf length.

Similarly, Nwangburuka and Denton (2012), also observed higher PCV above GCV in all the vegetative characters studied in *Corchorus olitorius*. Number of branches per plant, Vine length and Number of fruits per plant were the most genetically variable traits compared to the others with values (23.86, 21.72 and 20.15%) respectively and therefore may be considered in the distinguishing the genotypes. This also agrees with the report of Aremu and Adewale (2012) as well as Fayeun et al. (2012), who observed high GCV in number of branches per plant and vine length in *Telfairia*. Similarly, the relatively high PCV and GCV values recorded in the above mentioned three traits suggests huge prospects for selection based on these traits in the improvement of the crop (Denton and Nwangburuka, 2011; Ayanley et al., 2012).

Estimates of broad-sense heritability varied from 15.91 in seed width to 97.38 in vine length. Estimates of broad-sense heritability has been categorized (Dabholkar 1992) as low (5-10%), medium (11-30%) and high (>30%). All

the traits expressed heritability between medium and high, with the following characters exhibiting extremes of high heritability petiole length (97.16), vine length (97.38), number of branches per plant (96.59), number of leaflet (91.72), vine width (81.82) and leaf width (80.73). This agrees with the reports of Aremu and Adewale (2012), who observed high broad-sense heritability in foliage numbers, vine and branching traits in *Telfairia*. These high estimates of heritability observed in the traits mentioned above suggests that inheritance of these traits are under additive control, hence selection on the basis of these character will result in crop improvement and therefore reliable (Ullah et al., 2011). However, predictions of an individual's response to selection is more reliable when the estimate of broad-sense heritability is combined with GCV and genetic advance (GA) (Ibrahim and Hussein, 2006), instead of relying on GA values alone. Thus characters such as vine length and number branches per plant with high combination GCV, heritability and GA are most likely under additive gene control and will be effective in the prediction of yield (Bello et al., 2006). This agrees with the recent observation by Mohammed et al., (2012) on their report on wheat. Meanwhile high heritability and GA is an estimate of how much selection to improve a character in plants can be based on phenotypic observation (Johnson et al., 1955; Idahosa et al., 2010).

Estimates of genotypic and genotypic correlation of characters was also determined and expressed in Table 4. The result shows that there was strong significant positive phenotype and genotypic correlation between seed weight per pod and vine width (0.62. 0.81), number of fruits per plant (0.59, 0.86), fruit yield (0.77, 1.28), fruit length (0.73, 0.75) and fruit width (0.69, 0.70). This suggests that selections directed to any of the traits mentioned above may likely favour seed weight in *T. occidentalis*.

Similar reports have been presented by Nawngburuka et al. (2012) in okra. However, significant positive genotype correlation was observed between seed weight per pod and seed length (0.69), number of leaflet (0.40) and leaf width (0.40). This suggests that selection made on the basis of the genotypic expression of these characters will result in seed weight increase in *T. occidentalis*. Similarly, there was strong negative significant phenotypic and genotypic association between seed width and leaf length (-0.43, -1.70), vine width (-0.52, -1.47) and number of seeds per pod (-0.56, -1.03). This strongly suggests that a selection that is based on leaf length and vine width will not favour seed width. Seed width is one of the seed characters in *T. occidentalis* that determine seedling vigour and early crop performance. Meanwhile strong genotypic association exists between seed width and petiole length (0.71), vine length (0.40), number of branches per plant (0.79), number of leaflet (1.13), fruit width (0.75), but very strong negative genotypic association with seed length (-2.41).

Table 4. Phenotypic and Genotypic Correlation Coefficients of nine vegetative and seed yield characters in *Telfairia occidentalis.*

Character	leafW 9wk	Petlgt 9wk	Vinelgt 9wk	Vinewdth 9wk	Nobranches 9wk	Noleaflet	Nofruit	Fruityield	Noseed	Fruitlgt	Fruitwdt	Seedlgt	Seedwdt	Seedwgt
Leafl9wk														
P	0.08	0.47*	0.77**	0.07	0.06	0.41*	0.22	-0.23	0.02	0.25	0.22	-0.06	-0.43*	0.13
G	0.39*	0.67**	1.05**	-0.11	0.09	0.56**	0.14	-0.45*	0.18	0.47*	0.29	-0.32	-1.70**	0.13
Leafw9wk														
P		0.45*	0.58**	-0.29	0.46*	0.51**	0.31	0.40*	-0.58**	0.60**	0.72**	-0.21	0.62*	0.29
G		0.49*	0.62**	-0.23	0.53**	0.57**	0.66**	0.74**	-0.91**	0.82**	1.19**	-0.36	2.13	0.40*
Petlgt9wk														
P			0.66**	-0.19	0.20	0.51**	0.23	0.08	-0.12	0.37	0.35	0.33	0.24	-0.15
G			0.67**	-0.19	0.20	0.52**	0.37	0.18	-0.24	0.39*	0.40*	0.71**	0.71**	-0.19
Vinelgt9wk														
P				-0.08	0.01	0.67**	0.36	0.07	-0.45*	0.49**	0.51**	-0.04	0.10	0.11
G				-0.03	0.01	0.70**	0.52**	0.17	-0.65	0.55**	0.66**	-0.08	0.40*	0.11
Vinewdth9wk														
P					-0.65**	0.09	0.48*	0.56**	0.61**	0.49*	0.41*	0.26	-0.52**	0.62**
G					-0.71**	0.11	0.79**	1.15**	0.98**	0.66**	0.63**	0.97**	-1.47**	0.81**
Nobranches9wk														
P						-0.07	-0.12	-0.23	-0.12	-0.06	0.05	-0.36	0.29	-0.02
G						-0.08	-0.14	-0.37	-0.20	-0.11	0.00	-0.97**	0.79**	-0.03
Noleaflet														
P							0.32	0.43*	-0.31	0.45*	0.47**	0.46*	0.38	0.36
G							0.43*	0.71**	-0.57**	0.54**	0.61	1.19**	1.13**	0.40*
Nofruit														
P								0.65**	0.33	0.55**	0.54**	0.05	0.11	0.59**
G								1.35**	0.70**	1.08**	0.97**	-0.91**	-0.25	0.86**
Fruityield														
P									0.20	0.76**	0.69**	0.20	0.26	0.77**
G									0.19	1.37**	1.52**	-0.37	0.05	1.28**
Noseed														
P										0.04	-0.08	0.22	-0.56**	0.33
G										-0.24	-0.44*	0.63**	-1.03**	0.23
Fruitlgt														
P											0.95**	-0.07	-0.05	0.73**
G											1.03**	-0.47**	0.27	0.75**
Fruitwdt														
P												-0.10	0.07	0.69**
G												-1.02**	0.75**	0.70**
Seedlgt														
P													0.18	-0.08
G													-2.41**	-0.69**

Table 4. Contd

Seedwdt	
P	-0.14
G	-0.19

LeafL9wk = Leaf length at 9 weeks; leafW9wk = leaf width at 9 weeks; Petlgt9wk = petiole length at 9 weeks; Vinelgt 9wk = vine length at 9 weeks; Vinewdth9wk = vine width at 9 weeks; Nobranches9wk = number of branches at 9 weeks; Noleaflet = number of leaflets; Nofruit = number of fruits per plant; Fruityield = fruit yield per plant; Noseed = number of seeds per fruit; Fruitlgt = fruit length; Fruitwdt = fruit width; Seedlgt = seed length; Seedwdt = seed width; Wgtseed = weight of seed per plant; P = phenotypic correlation; G = genotypic correlation.

This suggests that selection base on seed length may yield seeds with small width, whereas those based on petiole length, number of branches number of leaflet, may affect seed width positively. Result further shows that there was significant phenotypic and genotypic correlation between seed length and number of leaflets (90.46, 1.19). Whereas seed length had negative genotypic correlation with number of branches (-0.97), number of fruits per plant (-0.91), fruit length (-0.470) and fruit width (-1.02), there was a positive significant genotypic association between seed length and petiole length (0.71), vine width (0.97) and number of seeds per pod (0.63). This suggests that selection based on number of branches, number of fruits per plant, fruit length and fruit width may depress seed length expression, while selection based on petiole length, vine length will favour seed length. Seed size is a function of seed length and seed width. However, large seed sizes have a reproductive advantage over small seed sizes under adverse shaded conditions and hence promotes seedling vigour. It is very important for the establishment of seedlings (Kenji and Kihachiro, 1999). The result for fruit width indicated strong positive phenotypic and genotypic correlation between leaf width (0.72, 1.19), vine length (0.51, 0.66), vine width (0.41, 0.63), number of fruits per plant (0.54, 0.97), fruit yield per plant 90.69, 1.52) and fruit length (0.95, 1.03). This result suggests that fruit width increase is dependent on the vegetative characters such as vine length and width, which serves as connecting tissues between the leaves and the fruits. However, fruit width is positively associated in genotypic term with petiole length (0.40) and negatively associated to fruit length (-0.47). This may imply that selections directed towards petiole length will favour fruit width while selection based on fruit length will not be meaningful. The correlation between fruit length and the other characters follow the same trend like that of fruit width and the other characters except that fruit length had a positive genotypic association with leaf length (0.47) and petiole length (0.39). Though selection based on petiole length may not favour fruit width, however it favours fruit length in *T. occidentalis*. The number of seeds per pod is a strong determinant of seed yield in *Telfairia* and has a strong positive phenotypic and genotypic correlation with vine width (0.61, 0.98), while it showed a negative correlation with leaf width. This could be because the leaf width contributes to accumulation of photosynthetic assimilate which will boost seed size rather than the seed number. Similarly there was a strong positive genotypic correlation between number of seeds and the number fruit per plant. The result of the correlation analysis further reveals a positive phenotypic and genotypic correlation between fruit yield and leaf length (0.40, 0.70), vine length (0.56, 1.15), number of leaflet (0.43, 0.71) and number of fruits (0.65, 1.33). This suggests that selection on the basis of the above mentioned characters will promote fruit yields. However, number of fruit per plant is positively associated at phenotypic and genotypic levels with vine width (0.48, 0.79), whereas, it only had a positive genotypic association with leaf width (0.66), vine length (0.52) and number of leaflets (0.43). This result portrays the strong relationship between vegetative traits and reproductive trait such as fruit and seed characters. Meanwhile, number of leaflet, a component of the vegetative yield in *Telfairia* had a strong significant phenotypic and genotypic correlation with other vegetative characters such as leaf length (0.41, 0.56), leaf width (0.51, 0.56), petiole length (0.51, 0.52) and vine length (0.67, 0.70). This also suggests that genetic improvement of *Telfairia* in vegetative yield will be effective when such characters highlighted above are considered. Number of branches per plant is negatively associated with vine width on the phenotypic and genotypic level (-0.65, -0.71) whereas it is positively associated with leaf width on phenotypic and genotypic levels (0.46, 0.53), respectively. This suggests that vine thickness in *Telfairia* may hinder the branching characteristic in *Telfairia*. Meanwhile branching characteristic in *Telfairia* is a vegetative yield component. However, a positive phenotypic and genotypic correlation exists between number of branches and leaf width, suggesting that selection directed toward leaf width will favour number of branches. This agrees with the report

of Fayeun et al. (2012), who reported positive correlation between number of branches per plant and leaf width. Vine length showed significant positive phenotypic and genotypic correlation with leaf length (0.77, 1.05), leaf width (0.58, 0.62) and petiole length (0.66, 0.67), while petiole length had a strong positive phenotypic and genotypic correlation with leaf length (0.47 and 0.67) and leaf width (0.45 and 0.49). This results suggest that vegetative yield in *Telfairia* is promoted by traits such as leaf length, width, petiole length and vine width. This agrees with the report of Fayeun et al. (2012). The higher genotypic correlation coefficient over phenotypic correlation coefficient observed in almost all the characters suggests very strong inherent association between various characters at genetic level. This is similar to the report of Ibrahim and Hussein (2006) on roselle (*Hibiscus sabdariffa*).

In conclusion, this study reveals that there is significantly large diversity in *T. occidentalis* genotypes studied which is sufficient enough for its genetic improvement. This diversity is contributed mostly by the vegetative traits. Vegetative traits such as number of branches per plant, vine length and number of fruits per plant can be used in distinguishing *T. occidentalis,* being the most genetically divergent traits in the genotypes. Characters such as vine length and number branches per plant, seed weight and leaf width with high GCV, heritability and GA were most likely under additive gene control and could be effective in the prediction of vegetative, fruit and seed yield in *Telfairia*. Hence, selection directed towards leaf length, leaf width, petiole length, vine length, will be efficient in improving vegetative yield in *T. occidentalis*, whereas selection on the basis of vine width, number of fruit per plant, fruit length, fruit width and leaf width will favour improvement in seed yield.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial activity of *Calotropis procera* and *Ficus sycomorus* extracts on some pathogenic microorganisms

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In vitro antibacterial potential of chloroform, absolute ethanol, methanol, ethanol (70%) and aqueous extracts of *Calotropis procera* and *Ficus sycomorus* leaves and latex were evaluated against five Gram-negative bacteria (*Neisseria lactamica* ATCC 23970, *Salmonella typhi* ATCC 19430, *Shigella flexenri* ATCC 12022, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922) and two Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* MRSA ATCC 43300, *S. aureus* CONS ATCC 29213). The antibacterial activities were expressed as zone of inhibition; minimum inhibitory concentrations (MIC) and also the survival curve was determined as kinetic studies. Interestingly, among all the tested extracts, aqueous and ethanol (either absolute or 70%) of *C. procera* and *F. sycomorus* leaves and latex were the best solvents for elute polar antibacterial substances and showed bacteriocidal effect against most Gram-positive and negative bacteria. Also, latex extracts were more pronounced than leaf extracts on human pathogenic bacteria. The most resistant bacterium was *E. feacalis* against both plant extracts. On the other hand, *S. areus* MRSA was the most sensitive bacteria especially with ethanol 70% extract of leaves and latex for both plants. The results of MIC for these extracts show more or less values higher than the chloramephnicol. Our conclusion confirms that, susceptibility of Gram-positive bacteria to the aqueous or ethanolic extracts of leaves for both plants was more than those of Gram-negative bacteria. The activities of 70% ethanol extracts recorded highest activity against Gram-negative bacteria than those of other extracts. The results therefore established a good support for the use of *C. procera* and/or *F. sycomorus* in traditional medicine against Gram-positive and negative pathogenic bacteria.

Key words: *Caltropis procera*, *Ficus sycomorus*, plant latex, leaf extract, pathogenic bacteria.

INTRODUCTION

Some bacterial infections cause high rate of mortality in human population and aquaculture organisms (Kandhasamy and Arunachalam, 2008). For example, *Enterococcus faecalis* is the causative agent of

inflammatory bowel disease (Balish and Warner, 2002). *Escherichia coli* and *Staphylococcus aureus* cause diseases like mastitis and abortion, while *Salmonella* sp. causes diarrhea and typhoid fever (Jawetz et al., 1995). *Shigella flexneri* strains are most frequently linked with endemic outbreaks of shigellosis in the developing world. *S. flexneri* invades the colonic and rectal epithelium of its host and causes severe tissue damage ranging from watery diarrhea to severe dysentery characterized by fever, abdominal cramping and bloody mucoid stool (Jennison and Verma, 2004).

On the other hand, *Neisseria lactamica* is not associated with infection in normally healthy people, and lives as a harmless commensal (Snyder and Saunders, 2006). Unlike *Neisseria meningitidis* and *Neisseria gonorrhoeae*, the rare cases of disease caused by *N. lactamica* are largely due to some compromise in the patient, and it is not normally pathogenic (Denning and Gill, 1991).

Control of infectious diseases is seriously threatened by the continuous increase in the number of microorganisms that are resistance to the chemical antimicrobial drugs; although new antibiotics are being steadily synthesized (Jazani et al., 2011). Due to the development of antibiotic resistance by various known and unknown reasons, in pathogenic and opportunistic microorganisms, the scientific community is constantly trying to develop new drugs and drug targets at present. In the last two decades, few new antibiotics, developed by the pharmaceutical companies, did not show the enhanced activity against the multidrug resistance bacteria (Sosa et al., 2003). Hence, the interest in plants, as a potential and promising source of pharmaceutical agents, has been dramatically increased (Kareem et al., 2008; Nenaah and Essam, 2011; Salem et al., 2011).

For example, dried latex and chloroform extract of *Calotropis procera* roots has been reported to possess anti-inflammatory activity (Kumar and Basu, 1994). Aqueous extract of *C. procera* latex has been found to exhibit antibacterial activity against carcinogenic bacteria (Kalpesh et al., 2012). The alcoholic extract from different parts of C. *procera* possess antimicrobial and spermicidal activity (Kamath and Rana, 2002). The anti-inflammatory property of the latex of *C. procera* was studied *in vivo*. A single dose of the aqueous suspension of the dried latex was effective to a significant level against an acute inflammatory response (Kumar and Basu, 1994). The decoction of the aerial part of C*. procera* is commonly used in Saudi Arabian traditional medicine for the treatment of various diseases including fever, joint pain, muscular spasm and constipation. The ethanol extract of the plant was tested on laboratory animals for its antipyretic, analgesic, anti-inflammatory, antibacterial, purgative and muscle relaxant activities (Mueen et al., 2004).

Ficus sycomorus, another medicinal plant belonging to the family Moraceae, was reported for its inhibitory effect

on bacterial growth (Ahmadu et al., 2007; Kubmarawa et al., 2007; Hassan et al., 2006a). The phytochemical analysis of *F. sycomorus* revealed the presence of tannins, anthraquinones, flavonoids, saponins, steroids and alkaloids (Adeshina et al., 2009). The presence of flavonoids in all the *Ficus sycomorus* extracts could probably be responsible for the observed antibacterial activity (Salem et al., 2013). Hence, the present study aimed to screen and evaluate the efficiency of different solvent extracts of *Caltropis procera* and *Ficus sycomorus* latex and leaves as antibacterial agents against some common pathogenic bacteria.

MATERIALS AND METHODS

Sample collection and preparation of plant extracts

Leaves and latex of *C. procera* and *F. sycomorus* were collected from South Valley University campus at Qena city, Egypt. Plant latex was collected by cutting the green stems and receiving the white milky latex in sterile bottles. Latex was centrifuged using a Biofuge™ Primo R, (Germany) cooling centrifuge at 1500 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was evaporated till dryness on a water bath at 100°C. Crude latex was stored at -20°C until being used (Singhal and Kumar, 2009). Healthy leaves of C*. procera* and *F. sycamorus* were collected, washed thoroughly with tap water followed by distilled water and air dried on a paper towel for 4 days. Dry leaves were ground in a tissue grinder (IKA® A10, Germany) to fine powder. Ten grams of each dried sample (leaves and latex of *C. procera* and *F. sycomorus*) were dissolved in 100 ml of five different solvents (water, methanol, absolute ethanol, 70% ethanol and chloroform) under stirring condition (150 rpm) for five days at room temperature. All solutions were filtered through Whatman #1 sterile filter paper. For aqueous extracts, the filtrates were stored at 4°C while for other extracts, the filtrates were dried at room temperature then the residues were dissolved in its original solvents to give 50 mg ml^{-1}. The extracts were stored at 4°C until being used against the tested bacteria (modified from Verastegui et al., 1996).

Bacterial strains and culturing conditions

The used bacterial strains were kindly provided by Luxor International Hospital, Luxor, Egypt. Bacterial cultures were maintained on Muller- Hinton (Muller and Hinton, 1941) agar slants and subcultured on Muller- Hinton broth then incubated at 37°C for 18 h before carrying out the test. Seven strains were used including five Gram negative bacteria including *N. lactamica* (ATCC23970), *S. typhi* (ATCC19430), *S. flexenri* (ATCC12022), *E. faecalis* (ATCC29212), *E. coli* (ATCC25922) and two Gram positive bacteria including methicillin- resistant *S. aureus* (MRSA, ATCC43300) and *S. aureus* (CONS, ATCC29213).

Antibacterial activities and minimum inhibitory concentrations (MICs)

Antibacterial activity was determined against the above bacterial strains using the disk diffusion method as described previously (modified from, Bauer et al., 1966). Whatman # 1 filter paper disks of 6 mm diameter were sterilized by autoclaving for 15 min at 121°C. The sterile disks were impregnated with different concentra-

tions of the used extracts (50, 40, 30 and 20 mg ml^{-1}) for MIC determination. Agar plates were inoculated with 0.1 ml of the tested microorganisms broth cultures using spread plate method. The bacterial inocula were adjusted to approximately 1.5 x 10^8 CFU ml^{-1}. The impregnated disks, in extracts and control solutions, were placed on the inoculated medium and the plates were incubated at 37°C for 24 h. Methanol, absolute ethanol, 70% ethanol and chloroform were used as negative controls while chloromophenicol (25 mg/ ml) was used as a positive control. Diameter of the growth inhibition halos (clearing zones) around each treated disk was measured in millimeters. Finally, the minimum inhibitory concentration (MIC) was defined as the lowest concentration of plant extracts that inhibit the growth of each strain, with the highest clearing zone value. All tests were carried out in triplicate.

Kinetic study of the extracts

Kinetic studies for the most active plant extracts on the tested microorganisms were carried out according to Kareem et al. (2008). An overnight broth culture of each strain (5 ml) was mixed with 25 ml fresh Muller- Hinton broth followed by the addition of 1 ml of the tested extract (10 mg ml^{-1}). The mixture was thoroughly shaken on a mechanical shaker. The optical density at 427 nm was determined at 30 min intervals for 5 h as described earlier by Kareem et al. (2008), using a "SPECTRONIC$^®$ GENESYS$^™$ 2PC" Spectrophotometer, Spectronic Instruments, USA.

RESULTS

Minimum inhibitory concentrations (MICs) and activity of the extracts

There were variations in diameters of inhibition zones caused by the tested plant extracts. In general, extracts of C. procera showed higher antibacterial activity compared to *F. sycomorus* extracts. The negative control, methanol, absolute ethanol, 70% ethanol and chloroform did not inhibit growth of the tested pathogens (data not shown). Among Gram +ve bacteria, *S. aureus* MRSA was the most sensitive to both leaves and latex extracts of *C. procera*; aqueous and 70% ethanol leaves extracts of *F. sycomorus*, and absolute ethanol extract of *F. sycomorus* latex (Tables 1 and 2). Compared to chloramephnicol (inhibition zone 14 mm), the higher antibacterial activity for the *S. aureus* MRSA was recorded for absolute ethanol extract of *F. sycomorus* latex and aqueous extract of leaves with 17 and 13.2 mm, respectively and an MIC of 50 mg ml^{-1} for both. For C. *procera*, methanol and 70% ethanol extracts of leaves and latex recorded the highest antibacterial activity (14.5 and 13 mm, respectively) as shown in Tables 1 and 2 with MIC value of 50 mg ml^{-1} for both.

S. aureus CONS was very susceptible to the most tested *C. procera* extracts with exception of methanol, absolute ethanol and chloroform extracts of latex. Among these extracts, that showed higher activity compared to chloramephnicol (inhibition zone of 13 mm), were the 70% ethanol extract of leaves and latex (17 and 15 mm, respectively with MIC of 20 mg ml^{-1}) and absolute ethanol

extract of leaves (15 mm with MIC of 50 mg ml^{-1}). Only ethanol 70% extract of *F. sycomorus* leaves recorded activity against the same organism (18 mm, MIC of 50 mg ml^{-1}).

Among all the pathogenic Gram -ve bacteria, only *E. faecalis* was resistant to both plant extracts (Table 1). On the other hand, *S. typhi*, *S. flexenri* and *E. coli* were sensitive to some of *C. procera* extracts such as absolute ethanol extract of leaves and ethanol 70% extract of latex and leaves on *S. typhi* (16; 15 and 13 mm with MIC of 20 and 50 mg ml^{-1}, respectively) and *E. coli* (11; 12 and 9 mm with MIC of 20; 50 and 40 mg ml^{-1}, respectively). Also, aqueous extract of *C. procera* leaves and chloroform extract of latex recorded antibacterial activity against *E. coli* (10 and 9 mm with MIC of 30 and 20 mg ml^{-1}, respectively). Only absolute ethanol extract of *C. procera* leaves recorded an inhibition zone higher than chloramephnicol on *S. flexenri* (24 mm with MIC of 50 mg ml^{-1}).

In general, the entire tested Gram -ve bacteria were resistant to *F. sycomorus* extracts. The exceptions were the aqueous extracts of latex on *N. lactamica* (zones of inhibition 13 mm and MIC of 20 mg ml^{-1}) as well as, absolute ethanol and 70% ethanol latex extract on *E. coli* (zone of inhibition 20 mm and MIC of 50 and 30 mg ml^{-1} respectively) (Tables 1 and 2). The commensal *N. lactamica* was very sensitive to *C. procera* extracts such as absolute ethanol and ethanol 70% (zones of inhibition 27 and 25 mm and MIC of 30 and 50 mg ml^{-1}, respectively).

Kinetic study of the extracts

The kinetic studies for both plant extracts were done only for the most active extracts against the most sensitive bacteria to confirm the activity of the extract as either bacteriostatic or bactericidal. The effect of 70% ethanol and aqueous extracts of *C. procera* latex and leaves on the growth dynamics of *E. faecalis*, compared to normal growth curve, showed that the extracts exhibited same characteristics. Although the inhibitory effect of *C. procera* aqueous extract of leaves was more pronounced than ethanol 70% extract of latex, compared to control, both extracts exhibited bacteriostatic effects (Figure 1A).

There were variations between the effect of *C. procera* leaves and latex extracts on the growth dynamics of *S. typhi*, compared to normal growth curve. The inhibitory effect of aqueous and 70% ethanol extracts of *C. procera* latex, absolute ethanol and 70% extract of leaves were more pronounced than water extract of leaves. The effect of the former extracts was bactericidal on *S. typhi* (Figure 1B). On the other hand, only 70% ethanol extract of *F. sycomorus* leaves had bactericidal effect on the same organism compared to control (Figure 2A). The bactericidal effect was obvious on *E. coli* for the extracts of 70% ethanol, chloroform (for *C. procera* latex), aqueous

Table 1. Inhibition halo diameter of leaves and latex extracts of *C. Procera* and *F. sycomorus* against some pathogenic bacteria.

Plant extract	Inhibition halo diameter (mm)*													
	S. aureus MRSA (ATCC 43300)		*S. aureus* Cons (ATCC 29213)		*S. typhi* (ATCC 19430)		*S. flexenri* (ATCC 12022)		*E. faecalis* (ATCC 29212)		*E. coli* (ATCC 25922)		*N. lactamica* (ATCC 23970)	
Chloramephenicol (25 mg/ml)	14 ± 0.3		13 ± 0.5		11 ± 0.4		17.3 ± 1		15 ± 0.1		13 ± 0.06		16± 0.1	
	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves
***C. procera* extracts**														
Aqueous	11±0.2	12.5±0.5$^{\neq}$	12±0.5	11±0.3	7±0.6$^{\neq}$	8±0.6	9±0.3	-ve	-ve	10±0.1	7±0.6	10±0.1$^{\neq}$	7±0.7	13±0.6
Methanol	9±0.3	14.5±0.5	-ve	11±0.1	-ve	-ve	7±0.5	-ve	-ve	10±0.3	-ve	-ve	-ve	-ve
Ethanol	11±0.0	11.2±0.3	-ve	15±0.3	-ve	16±0.4$^{\neq}$	-ve	24±0.1	-ve	-ve	7±0.1	11±0.3	-ve	27±0.1
Ethanol 70%	13±0.1	11±0.1	15±0.3	17±0.5	15±0.1$^{\neq}$	13±0.0$^{\neq}$	12±0.1	-ve	9±0.4	-ve	12±0.4$^{\neq}$	9±0.1$^{\neq}$	10±0.1	25±0.06
Cloroform	10±0.0$^{\neq}$	-ve	-ve	9±0.1	-ve	9.1±0.1	-ve	8±0.5	-ve	-ve	9±0.4$^{\neq}$	9±0.5	11±0.1	8±0.9
***F. sycomorus* extracts**														
Aqueous	-ve	13.2±1.3$^{\neq}$	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	13±0.1	-ve
Methanol	-ve	8.2±0.8	-ve	-ve	-ve	-ve	-ve	9±0.1	-ve	-ve	-ve	10±0.1	-ve	-ve
Ethanol	17±0	2.2±0.8	-ve	11±0.4	-ve	-ve	-ve	-ve	-ve	-ve	20.1±0.1	-ve	-ve	-ve
Ethanol 70%	-ve	10.2±0.8	-ve	18±0.06	-ve	9±0.1$^{\neq}$	-ve	7.5±0.1	-ve	-ve	20±0.1$^{\neq}$	-ve	-ve	-ve
Cloroform	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

*Mean ± SD, n=3; ≠ showed bacteriocidal effect; -ve = negative effect.

and 70% ethanol extracts of leaves (Figure 1C). While, for the same organism, 70% ethanol latex extract of *F. sycomorus* exhibited bacteriostatic effect (Figure 2B).

For *S. aureus* MRSA, only aqueous and chloroform latex extracts of *C. procera* exhibited bactericidal effect, while the other tested were bacteriostatic (Figure 1D). Furthermore, only *F. sycomorus* aqueous extract of leaves was bactericidal after 60 min compared to control (Figure 2C).

DISCUSSION

Drug resistance of human pathogenic bacteria has been reported all over the world and the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. Plants are important source of potentially useful structures for the development of novel chemotherapeutic agents and the first step towards this goal is the *in vitro* antibacterial assay (Valero and Salmerón, 2003). According to

the findings of the present study, the aqueous and ethanolic extracts (either absolute or 70%) and the latex of *C. procera* showed considerable antibacterial activities against most tested micro-organisms (Tables 1 and 2). In all cases, and regardless of the microorganism tested, the used solvent was a determinant factor for antimi-crobial agents extraction. Some findings was recorded by Nennah and Essam (2011) considering the antibacterial activities of aqueous and organic extracts of *C. procera* against human pathogenic

Table 2. MIC´s value of leaves and latex extracts of *C. Procera* and *F. sycomorus* against some pathogenic bacteria.

| Plant extract | Minimum Inhibitory concentrations MIC (mg ml^{-1})* | | | | | | | | | | | | | |
	S. aureus MRSA (ATCC 43300)		*S. aureus* Cons (ATCC 29213)		*S. typhi* (ATCC 19430)		*S. flexenri* (ATCC 12022)		*E. faecalis* (ATCC 29212)		*E. coli* (ATCC 25922)		*N. lactamica* (ATCC 23970)	
	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves	Latex	Leaves
***C. procera* extracts**														
Aqueous	20	20	50	40	20	20	50	-ve	-ve	50	40	30	20	50
Methanol	20	50	-ve	50	-ve	-ve	20	-ve	-ve	30	-ve	-ve	-ve	-ve
Ethanol	20	50	-ve	50	-ve	20	-ve	50	-ve	-ve	20	20	-ve	30
Ethanol 70%	50	30	20	20	50	50	50	-ve	50	-ve	50	40	30	50
Cloroform	20	-ve	-ve	40	-ve	40	-ve	20	-ve	-ve	20	20	50	50
***F. sycomorus* extracts**														
Aqueous	-ve	50	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	20	-ve
Methanol	-ve	20	-ve	-ve	-ve	-ve	-ve	50	-ve	-ve	-ve	20	-ve	-ve
Ethanol	50	20	-ve	20	-ve	-ve	-ve	-ve	-ve	-ve	50	-ve	-ve	-ve
Ethanol 70%	-ve	20	-ve	50	-ve	30	-ve	30	-ve	-ve	30	-ve	-ve	-ve
Cloroform	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve = negative effect.

bacteria. Also, in a study conducted by Kawo et al. (2009) a weak antibacterial properties of ethanolic extracts of *C. procera* leaves and latex against *E. coli*, *S. aureus*, *Salmonella sp.* and *Pseudomonas sp.* was recorded by using paper-disc diffusion and broth dilution techniques. The results obtained revealed that ethanol was the best extractive solvent for a fraction with antibacterial activity. Also, ethanol was reported for its efficiency for extracting the antimicrobial active substances from *Calotropis* compared to other solvents (Kareem et al., 2008). In our study, aqueous and ethanol (either absolute or 70%) extracts were the popular solvents that elute polar substances, and latex extracts are more active

than leaf extracts on human pathogenic bacteria. This may indicate that the used solvent is an important factor for the isolation of selective bioactive compounds (Salem et al., 2011). Some workers showed that methanol extraction yielded higher antimicrobial activity than hexane and ethyl acetate (Manilal et al., 2009; Rangaiah et al., 2010). In our results, aqueous and ethanol extracts of latex and leaves exhibited much more bioactivity than other extracts. The highest antibacterial activity (inhibition zone of 27 mm) was recorded for the ethanolic extract of *C. procera* leaves against *N. lactamica* (Table 1). On the contrary, methanol extracts of leaves and latex showed no bioactivity against all the tested

organisms (Tables 1 and 2). The greater resistance of Gram -ve bacteria, to latex extract, may be due to the differences in the cell wall structure between Gram +ve and -ve bacteria. The Gram -ve bacterial outer membrane acts as a barrier to many substances, including antibiotics (Burt, 2004). Chemically, the latex of *C. procera* is composed of various classes of phytochemical compounds. These were extensively proved in various studies which include proteolytic enzymes, cardenolides, alkaloids, cardioactive glycoside like calactin, calotropain, proceroside, syriogenine, calotoxin and uscharin, as well as tannins, flavonoids and procerain, a stable cysteine protease (Mossa et al., 1991; Dubey and
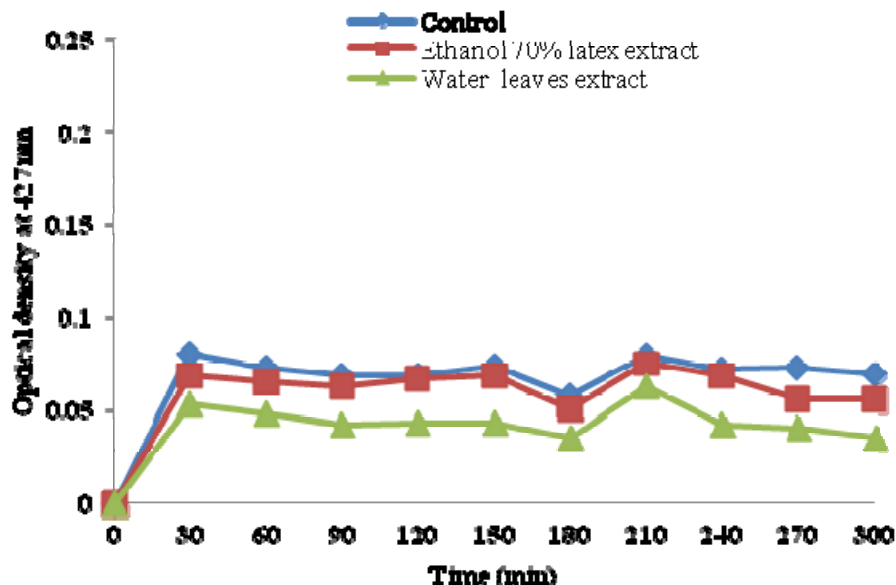
A. Enterococcus faecalis (ATCC 29212)
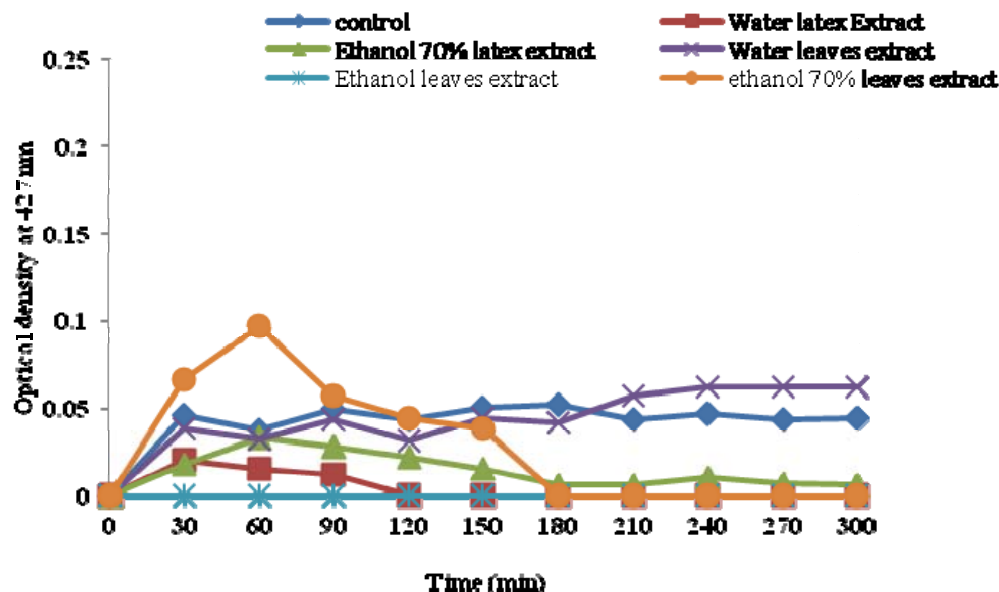
B. Salmonella typhi (ATCC 19430)

Figure 1. Time- kill kinetic analysis of *C. procera* extracts against *Enterococcus faecalis*, *Salmonella typhi*, *E. coli* and *Staphylococcus aureus* MRSA.

Jagannadham, 2003). One or more constituents of the latex, separately or in combination, may be responsible for the antibacterial activity of *C. procera* (Nennah and Essam, 2011; Nenaah, 2013). The action of extracts against clinical pathogenic organisms may be due to inhibition of cell wall (due to pore formation in the cell and leakage of cytoplasmic constituents) by the active com-ponents such as alkaloids, inhibition of electron transport

chain or sphingolipid biosynthesis (Dominguez and Martin, 1998; Hassan et al., 2007). Flavonoids have been reported to display strong antimicrobial activity against some pathogenic bacteria such as *Streptococcus mutans* (Koo et al. 2002; Özcelik et al., 2008; Salem et al., 2013). In our results, the antibacterial activity of aqueous and ethanolic (either absolute or 70%) extracts of *C. prodera* latex or leaves, against *S. typhi*, *E. coil* and *S. areus*
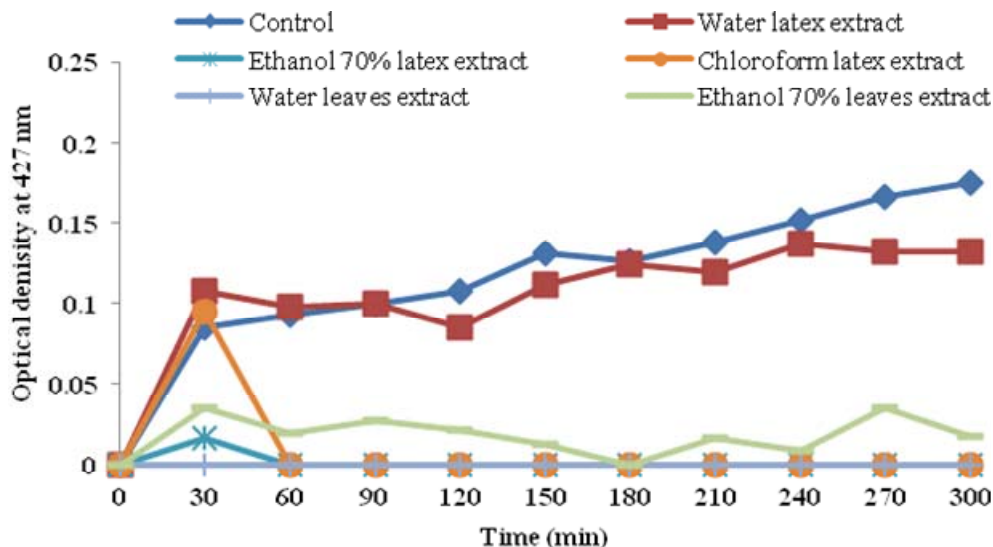
C. *E. coli* (ATCC 25922)
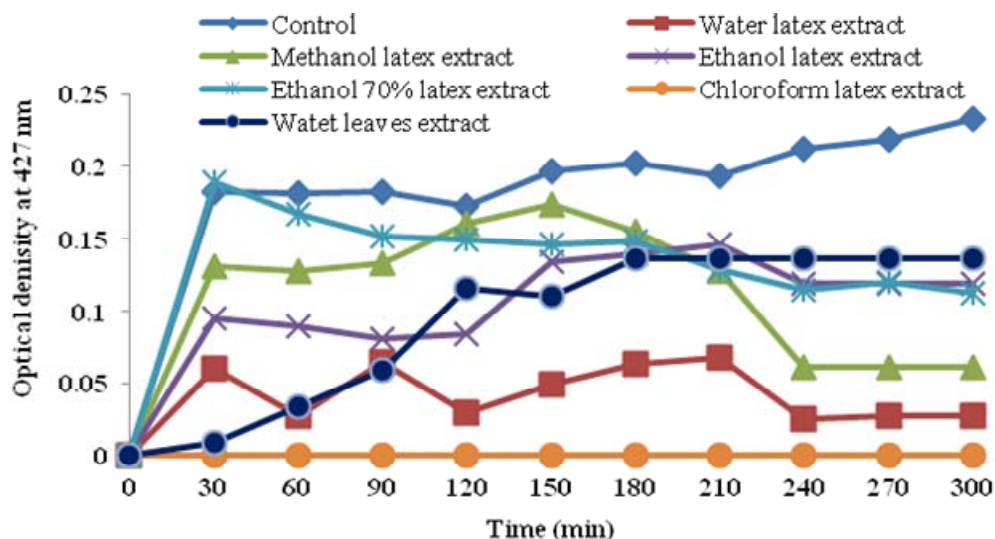
D. *Staphylococcus aureus* MRSA (ATCC 43300)

Figure 1. Contd.

MRSA was confirmed as bactericidal effect (Figure 1B, C and D). This may be indicative for the presence of broad spectrum antibiotics (Larhsini et al., 1999).

Aqueous extract of *F. sycomorus* was reported earlier for its inhibitory effect on bacterial growth (Shankar et al., 2004). It contains pharmacologically active substances including tannins, saponins, reducing sugars, alkaloids and flavones aglycones without any haematological, hepatic or renal toxicities (Kubmarawa et al. 2007).

In our results, the antibacterial activity of *F. sycomorus* extracts (absolute ethanol latex extract; aqueous and 70% ethanol leaves extracts) was reported against both

Gram +ve bacteria and only *S. typhi*, had a bactericidal effect (Tables 1 and 2; Figure 2A, C). These constituents could be responsible for the antibacterial activities of *F. sycomorus* against the two Gram +ve bacteria tested.

The low bioactivity observed for crude latex extracts, of both *F. sycomorus* and *C. procera*, against some Gram -ve bacteria (such as *S. flexenri and E. faecalis*) resulted from the previous effect, dilution of active constituents or from antagonism among extract constituents (Tables 1 and 2).

The same results were reported earlier for *Caltropis gigantea* latex against some pathogenic Gram negative
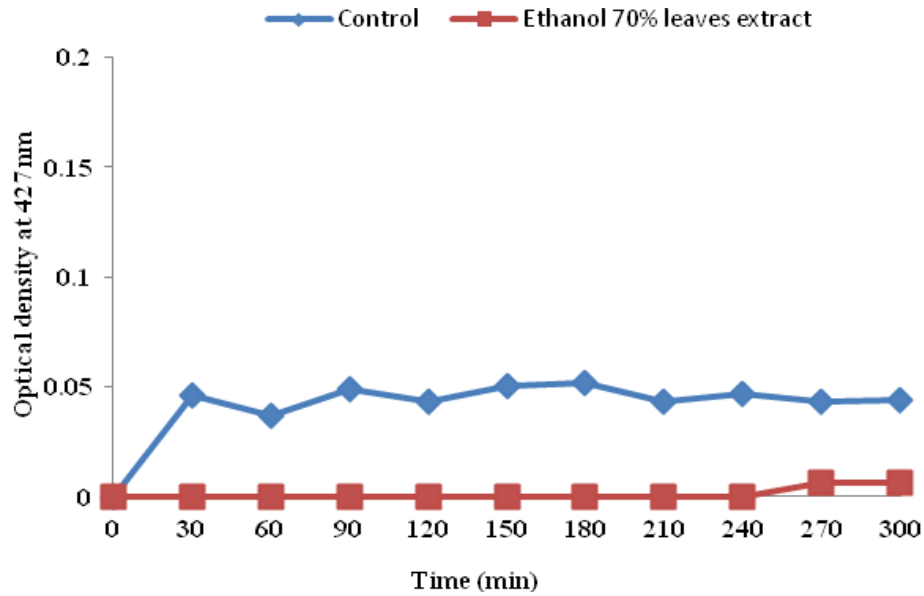
A. *Salmonella typhi* (ATCC 19430)
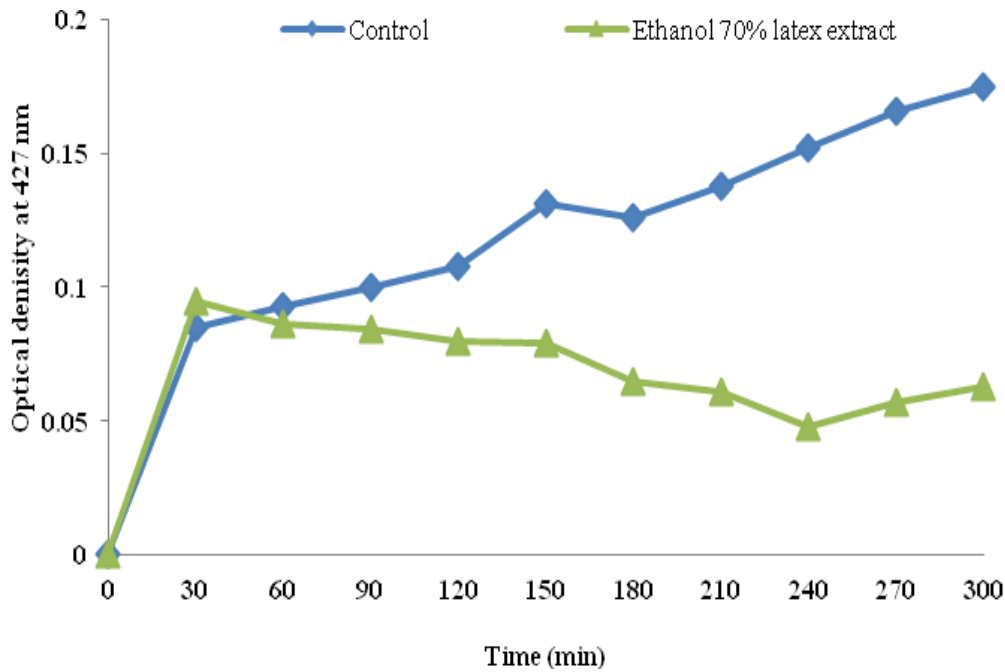
B. *E. coli* (ATCC 25922)

Figure 2. Time- kill kinetic analysis of *F. sycomorus* extracts against *Salmonella typhi*, *E. coli* and *Staphylococcus aureus* MRSA.

bacteria (Sorimuthu and Venkatesan, 2010).

MIC's are considered the "gold standards" for determining the susceptibility of microorganisms to antimi-crobials. MIC's are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods of
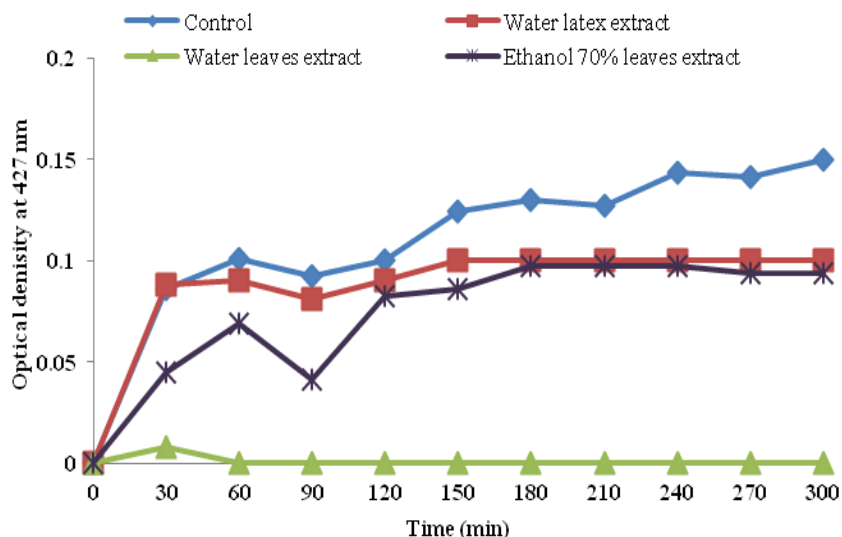
C. *Staphylococcus aureus* MRSA (ATCC 43300)

Figure 2. Contd.

testing, or when disc diffusion methods are not applicable. In general, the lowest MIC's (20 mg ml^{-1}) were recorded for latex extracts while the highest MIC's (50 mg ml^{-1}) recorded for leaves extracts for both plants (Table 2). According to Rangaiah et al. (2010) and Patra et al. (2008), this indicates the presence of active constituents in the plant extracts that can be used in pharmaceutical industry. This was also attributed to the presence of high concentrations of polysaccharides in some plants including *C. procera* and *F. sycomorus* that have antimicrobial properties (Akhtar et al., 1992; Yamashita et al., 2001; Doughari, 2006; Hassan et al., 2006b).

The results of our preliminary screening assays justify the use of 70% ethanol latex and leaves extracts of *C. procera* in the ethnomedicine field. However, it is important to note that the crude extract of *C. procera* latex need to be further purified through antibacterial activity- guided fractionation to isolate and identify the compounds responsible for this activity. In conclusion, the remarkable bactericidal effects of *C. procera* latex extract and *F. sycomorus* leaves extracts suggest that these extracts can be a useful source for the development of novel antibacterial formulations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Cloning and expression of an amylase gene from *Bacillus* sp. isolated from an agricultural field in West Bengal, India

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The use of fertilizer is pivotal to ensure soil fertility. Green fertilizers have many advantages and bio-molecules generated in a bacterial cell that can be used as green fertilizer. To produce bio-molecules from a bacterial cell, a bacterium was isolated that uses natural starch as sole carbon source. The organism was grown in presence of various natural starchy materials. The organism was partially identified as *Bacillus* sp. The optimum conditions for growth of the strain were: temperature of 37.0°C and pH 7.0. However, it grows well even above 55.0°C. The optimum conditions for degradation of various starchy substrates using extra-cellular amylase of the strain were studied. The optimum pH was 4.0 for soluble starch whereas it was pH 5.0 for other substrates and temperature was 17.0°C for soluble starch and 37.0°C for others. Prolongation of time of incubation, velocity of enzyme reaction, and optimum concentration of various metal ions for amylase activity was also studied using optimum assay conditions. Molecular cloning and expression of amy gene of the strain was done in *Escherichia coli* DH5α. The host cell harboring the recombinant plasmid was grown in the presence of potato starch and incubated for many days to obtained cellular lysate and the liquid biomass might be used as bio-fertilizer.

Key words: Amylolytic bacteria, amylase, bio-molecules, bio-fertilizer, single cell fertilizer.

INTRODUCTION

Enormous use of inorganic fertilizers (Prasad et al., 2004; Tiwari, 2007; Tandon, 2010), insecticides, and pesticides (Pimentel, 1995) is the modern technique of agriculture.

Inorganic fertilizers, used in agriculture, deposit in food materials and can also cause underground water pollution (Nolan et al., 1997; McIsaac, 2003). Bio-

fertilizers have multiple advantages over the inorganic fertilizers (Prasanna et al., 2008; Sheoran et al., 2010).They improve soil structure, texture, porosity, air holding and water holding capacities (Prasad, 1998). Bio-fertilizers are nitrogen fixing bacteria, blue green algae, ferns etc (Kennedy et al., 2004; Mishra and Pabbi, 2004; Baset Mia and Shamsuddin, 2010). As they are living cells they compete for food and nutrition with the crop plants, even though, the competition is incipient (Sen, 2007), and therefore *in vitro* production of various bio-molecules from a bacterium and using them as bio-fertilizer is the theme of the present study. Bacteria have less generation time; therefore huge amount of bio-mass can be generated using fermentation technology. Single cell fertilizer (SCF) is made of cellular molecules such as proteins, free amino acids, carbohydrates, free sugars, lipids, free fatty acids, nucleotides, macro and micro elements, and others (Sen et al., 2009). Various molecules that are present in SCF will be absorbed by crop plants as readymade food for their growth. The main object of this study is the production of SCF from a bacterium that can utilize low cost carbon source for its growth. The present study includes isolation of an amylolytic bacterium, partial characterization of the organism, partial characterization of its extra-cellular enzymes, molecular cloning and expression of the amy in *Escherichia coli* host and use of the recombinant strain as the source of SCF. Furthermore, it is the first report on production of SCF using a recombinant bacterium.

MATERIALS AND METHODS

Soil samples were collected from various agricultural fields of the district Burdwan, and other districts of West Bengal, India. These soil samples were collected from 5 to 10 cm depth during the month of March 2011. Temperature was around $30.0 \pm 2.0°C$. The following chemicals were purchased from the suppliers indicated: soluble starch, glucose, DNS, NaOH, KOH, KH_2PO_4, K_2HPO_4, NaCl etc. were from Merck, India; other chemicals such as phenol, chloroform, ethyl alcohol etc. were from Glaxo Smith co., Mumbai, India; restriction endonuclease, T-4 DNA ligase, and alkaline phosphatase were purchased from GeNei, Bangalore, India.

Isolation of an amylolytic bacterium

Starch medium was prepared by using 1.0 g of starch, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.01 g beef extract in 100 ml distilled water, at pH 7.0. Using dilution plate technique various mesophilic amylolytic bacteria were isolated. Among them, those that showed higher extra-cellular amylase activity were grown in the presence of different natural starchy materials. The organism which showed highest growth in presence of natural starch was selected for further studies.

Partial identification of the organism

The organism was grown on potato starch medium. The morpho-logical characterization was carried out by Gram staining and electron microscopic observation. Electron microscopy of potato starch granules that was used in the medium was also studied. Endospore staining was performed following standard method (Schaeffer and Fulton, 1933). Various biochemical tests were carried out following Bergey's Manual of Systematic Bacteriology (2009). Extracellular and intracellular amylase activities in presence of different carbon source were also determined.

Optimization of pH, temperature and potato starch concentration for growth of the bacterium

Standardization of pH, temperature and substrate concentration for optimum growth of the bacterium were carried out. 1.0% potato starch was used in the medium with other ingredients as described before. pH was adjusted separately to 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0, respectively. The medium was sterilized, inoculated and incubated at 37.0°C in shake condition for 20 h. The bacterial growth was measured at $\lambda=540$ nm using a colorimeter (Klett colorimeter, Clinical model: 800-3, 115 VAC). Determination of optimum temperature was also carried out using same medium. The incubation temperatures were 17.0, 27.0, 37.0, 47.0 and 57.0°C, respectively. The conditions of growth were determined. Different concentrations of potato starch (0.5, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0%) were used in the medium to determine the optimum substrate concentration. After incubation at 37.0°C for 20 h, O.D was taken.

Assay of amylase activity of the isolated strain

Isolation of extracellular enzyme

The organism was grown using optimum condition for 20 h in a shaker. The culture was then centrifuged at 5,000 rpm at 4.0°C for 10 min. The clear supernatant was used as the source of extra-cellular enzyme.

Assay of amylase

The assay followed a standard protocol for sugar estimation (Bernfeld, 1955). The assay was carried out using 1.0 ml of 4 different substrates (1% soluble starch, potato, rice, and wheat starch), 0.5 ml of 100 mM phosphate buffer and 0.2 ml of extra-cellular enzyme. The final volume of the reaction mixture was adjusted to 2.0 ml using requisite amount of distilled water (Sen and Oriel, 1989a) for 15 min at optimum temperature. The reducing sugar which was produced in the assay condition was estimated using a colorimeter at $\lambda=580$ nm. One unit of enzyme activity was described as µmole of reducing sugar (as glucose) produced per ml of the extra-cellular enzyme per min. Effects of pH, temperature, prolongation of time of incubation, substrate concentration and effect of different metal ions on extracellular amylase activity against four substrates were also determined. PH optimum was determined using buffer with variable pH like 1.0, 3.0, 4.0, 5.0, 7.0, 9.0 and 11.0, respectively. The buffers were prepared according to Gomori (1955).

Different temperatures like 10.0, 17.0, 27.0, 37.0, 47.0, 57.0, 67.0 and 77.0°C were used to determine temperature optimum. Effect of prolongation of time of incubation was carried out by incubating the assay mixture at various time periods. The enzyme activity was also recorded using various substrate concentrations. The concen-trations were 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 and 50.0 mg, respectively. K_m and V_{max} value against four substrates were also determined. Different monovalent and divalent

Table 1. Growth of selected bacterial isolates in presence of natural substrates.

Strain number	Potato	Pumpkin	Tomato	Cucumber	Bottle-gourd
AB-13	0.27	0.3	0.07	0.19	0.16
AB-27	0.27	0.26	0.25	0.19	0.09
AB-55	0.28	0.25	0.17	0.2	0.04
AB-56	0.56	0.52	0.41	0.29	0.08
AB-63	0.35	0.51	0.36	0.28	0.08
AB-65	0.37	0.44	0.37	0.21	0.10
AB-66	0.23	0.34	0.26	0.14	0.06
AB-67	0.28	0.32	0.26	0.23	0.07
AB-88	0.41	0.21	0.22	0.21	0.08
AB-98	0.37	0.38	0.26	0.29	0.19

cations like Na^+, K^+, Mn^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} were used to determine the effect of concentration of metal ions on enzyme activity.

Molecular cloning and expression of the 'amy' of the bacterium in an *E. coli* host

Molecular cloning and expression of amy gene of the organism in *E. coli* host were carried out following standard procedures with a little modification (Sen, 2006). The genomic DNA of the strain was isolated and purified using standard procedure (Green and Sambrook, 2012). Approximately, 50.0 µg of genomic DNA was completely digested by using 10 units of Hind III restriction endonuclease for 8 h at 37.0°C. Then pBR322 plasmid DNA from *E. coli* HB101 strain was isolated and purified. About 20.0 µg of purified plasmid DNA was digested with 5.0 units of Hind III restriction endonuclease for 8 h at 37.0°C. It was extracted with TE saturated phenol, chloroform and precipitated with ethyl alcohol. The plasmid was then treated with 5.0 units of alkaline phosphatase for 8 h at 37.0°C. The ligation of Hind III digested genomic DNAs and alkaline phosphatase treated plasmid DNA was carried out using equal molar concentrations of plasmid and genomic DNAs in 50.0 µl of ligation mixture. The ligation was carried out by using 4.0 units of T4 DNA ligase at 21.0°C for 48 h. The transformation was carried out using 100.0 µl of competent cells (*E. coli* DH5α) and 10 µl of ligated mixture. After the transformation, the cells were incubated using 1.0 ml of Luria broth for 24 h. Then cells were plated using starch agar plate containing 35.0 µg/ml of ampicillin.

Data analysis

Scientific data analysis and graphing was done following Sigma plot 12 scientific graph system. Microsoft office excel 2007 was also used to make various graphs. Other results were expressed as mean value.

RESULT

Isolation of an amylolytic bacterium from soil sample

Starch degrading, extracellular amylase producing bacteria were isolated by spread plate method. To detect starch degradation, plates were flooded with I_2 and KI solution. The colonies that showed higher zone of starch hydrolysis were selected. These were then grown in 1% starch containing broth and growth was measured at 540 nm. The isolates which showed higher growth rate were grown in presence of different natural starchy materials like potato, pumpkin, tomato, cucumber and bottle-gourd (5%) and incubated at 37.0°C in shake condition for 20 h. It showed that the ability of using natural starches was highest for the strain AB-56 (Table 1).

Partial characterization of the strain AB-56

The strain AB-56 showed highest growth in presence of natural carbon sources and it utilized potato starch more conveniently. Morphological characters were observed by Gram staining (Figure 1c) and also by scanning electron microscope (Figure 1a). Endospore staining (Figure 1d) was carried out for conformation of spore formation. Electron microscopic study of starch granules was also carried out (Figure 1b). Many biochemical tests were performed to identify the organism (Table 2). From the above tests and other associated experiments, the organism was partially characterized as *Bacillus* sp., utilization of different carbon sources, extracellular and intracellular amylase activity in presence of those carbon sources is presented in Table 3.

Determination of optimum growth conditions of the strain AB-56

Standardizations of pH, temperature and substrate concentration for optimum growth of the organism were carried out. It showed that the maximum growth of the organism was at pH 7.0 (Figure 2a) above or below this pH it showed inhibition of the growth of the organism. Determination of optimum temperature was carried out by incubating the culture medium at various temperatures

a.

b.

c.

d.
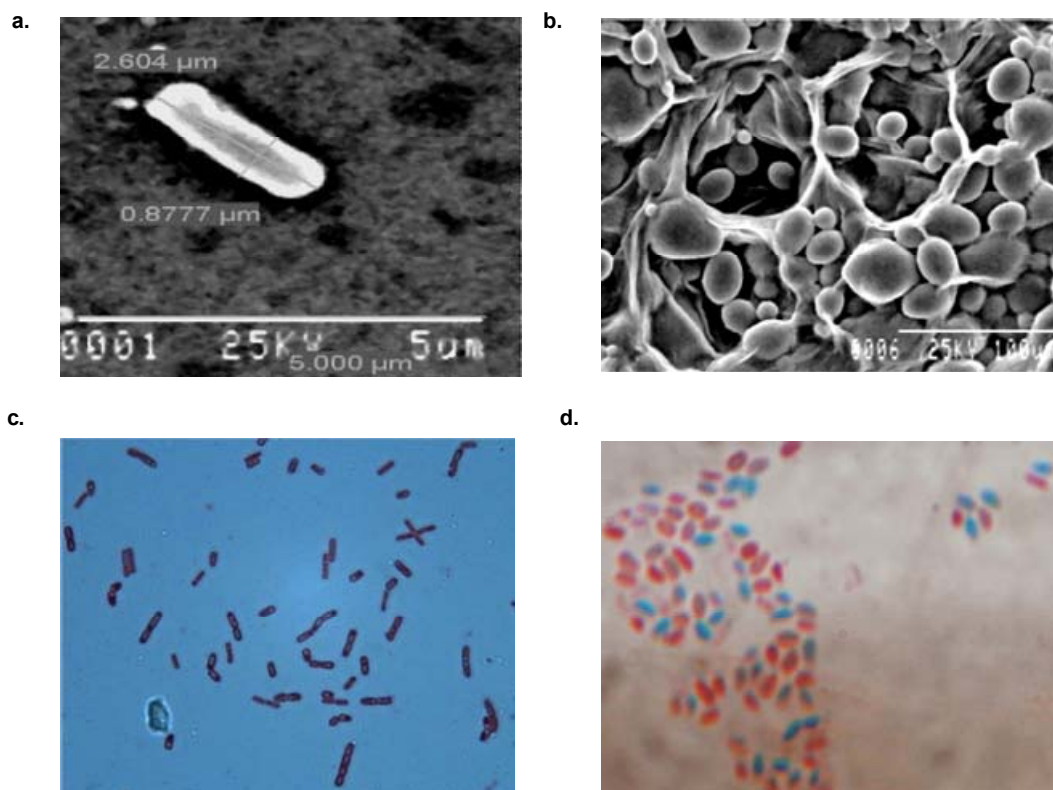
Figure 1. Morphological study of the strain AB-56 and potato starch granules. **a)** Scanning electron microscopic view of the organism. **b)** Scanning electron microscopic view of potato starch granules used to grow the bacterial strain. **c)** Light microscopic view of the organism after gram staining. **d)** Green colours spores after endospore staining.

Table 2. Characteristics of the strain AB-56.

Test	Result
Strain characteristics	
Shape of the colony	Rod shape with irregular edge
Colour	Slightly yellowish orange
Gram character	+
Spore	+
Size	2.6 µm × 0.9 µm
Biochemical characteristics	
IMViC test	–
Nitrate reduction test	–
Triple sugar iron test	–
H_2S production test	–
Catalase test	+
Urease test	–
Mannitol motility test	–
Phenylalanine degradation test	+
Tolerance of NaCl	4%
Oxidation fermentation test	+
Gelatin hydrolization	++

Table 3. Effect of different carbon sources on growth of AB-56 and induction of extracellular or intracellular amylase by the same.

Carbon source	Growth	Units of extracellular amylase activity	Units of intracellular amylase activity
Glucose	1.33	Nil	Nil
Ribose	0.67	Nil	3.7
Sucrose	0.83	Nil	4.65
Lactose	0.71	Nil	3.7
Maltose	1.08	Nil	752.
Mannitol	1.52	15.7	3.7
Starch	1.66	17.6	6.48
CMC	0.28	13.88	Nil

1% carbon source was used in the medium. 100 µl of cell lysate was used for intra-cellular amylase assay.
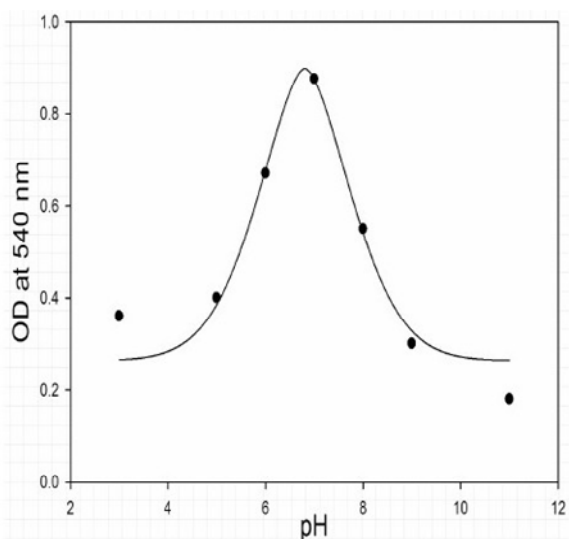
a.

b.

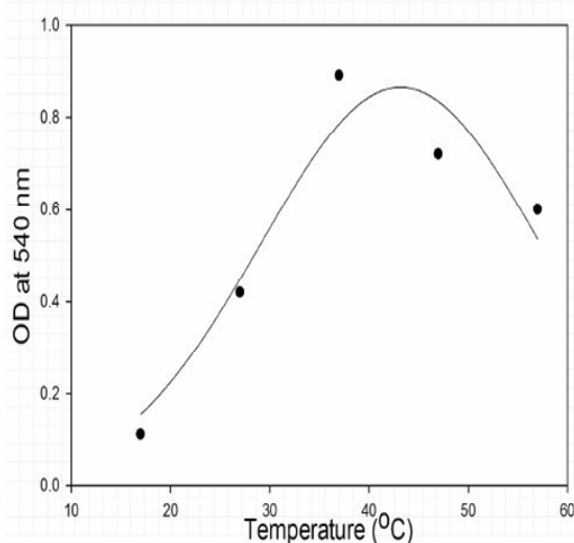**Figure 2.** Determination of optimum pH and temperature for the growth of the strain. **a)** Effect of pH on the growth of AB-56. **b)** Effect of temperature on the growth of AB-56.

Table 4. Effect of potato starch concentration on the growth of AB-56.

Potato starch (%)	Growth at 540 nm
0.5	0.24
2.0	0.30
4.0	0.48
6.0	0.69
8.0	0.88
10.0	0.40
12.0	0.34

with pH 7.0. It showed the maximum growth of the organism at 37.0°C (Figure 2b). Growth of the organism was also recorded at 57.0°C where the O.D was 0.60 which represents its capability to grow at high temperature. The strain AB-56 was grown in presence of variable starch concentrations where potato starch was used as sole carbon source (Table 4). It showed that the optimum growth occurred when potato starch concentration was 8.0% and above or below this concentration there were reduction of growth.

Extra-cellular amylase activity of the strain AB-56

Effect of pH on amylase activity

The optimum pH for amylase activity was carried out using four different substrates. The result showed that when soluble starch was as substrate, the optimum pH was 4.0 while for other substrates it was 5.0 (Figure 3a).

Effect of temperature on amylase activity

The optimum temperature for amylase activity was also determined using those substrates. When soluble starch
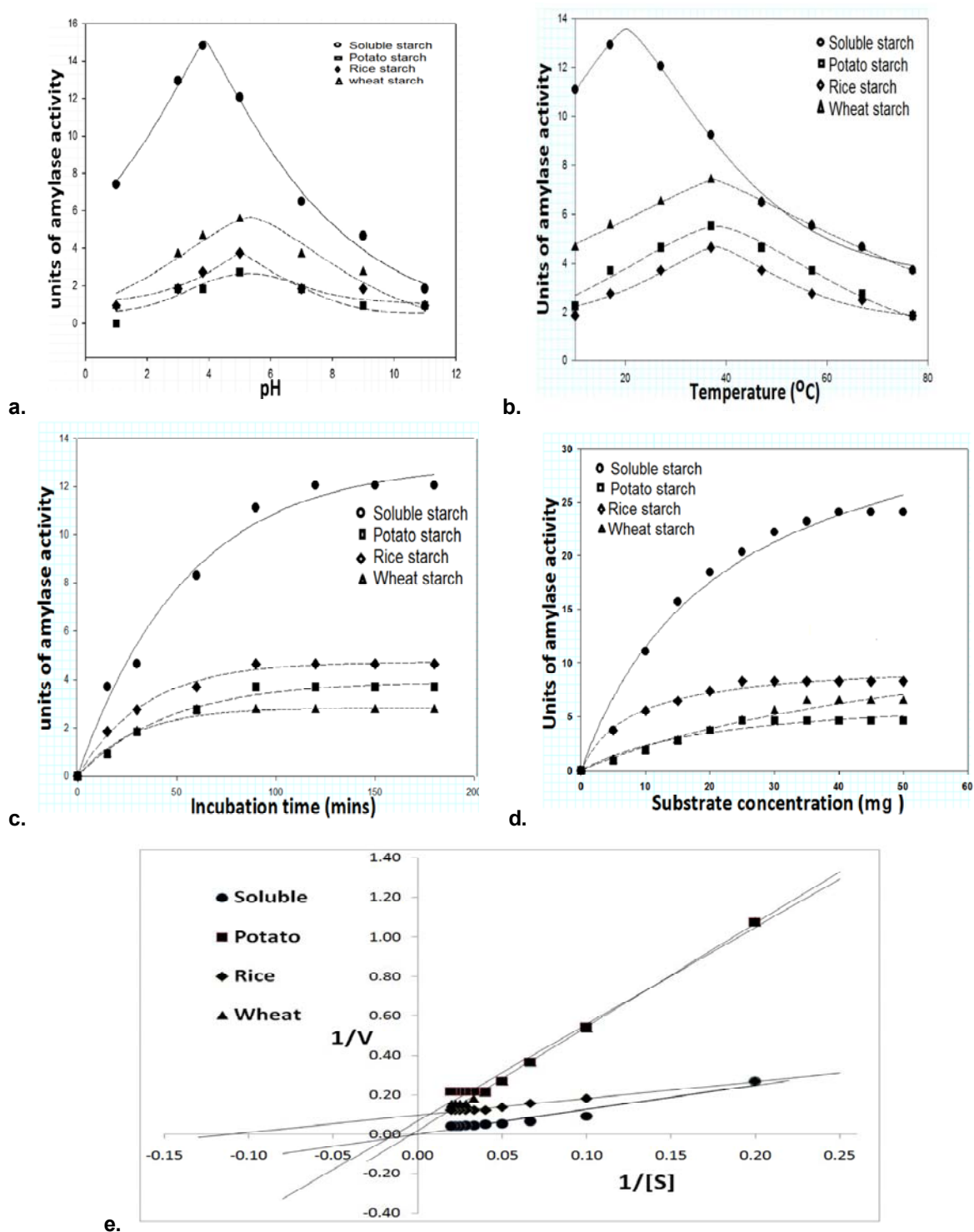
Figure 3. Effect of different parameters on extracellular amylase of the strain AB-56. **a)** Effect of pH on amylase activity. Four different substrates were used. 100 mM buffer of different pH were used in these assays. **b)** Effect of temperature on amylase. Different temperatures were used and other parameters were remained same as described before. **c)** Effect of prolongation of time of incubation period on amylase activity. The reaction mixture was incubated in various time periods using standard assay conditions. **d)** Effect of substrate conc. on amylase activity. Various concentrations of substrate and standard assay conditions were used. **e)** Double reciprocal plot of substrate concentration. [S] v/s (V) plot of amylase showing the effect of substrate concentration on the velocity of amylase activity.

was used as substrate it showed optimum temperature at 17.0°C while for other substrates it was at 37.0°C (Figure 3b).

Effect of prolongation of time of incubation

Amylase activity was measured against prolongation of time of incubation. These assays were carried out using optimum pH and temperature. When soluble starch was used as substrate, the amylase activity was increased upto 150 min of incubation but for other substrates, it was 90 min. Further prolongation of incubation time showed no change in enzyme activity (Figure 3c).

Effect of substrate concentration on amylase activity

Effect of substrate concentration on amylase activity was measured using various substrate concentrations. Hydrolysis of starch was increased linearly with increasing substrate concentrations and reached a plateau at 45.0 mg concentration for soluble starch. Similarly for potato, rice, wheat starches it was 35.0, 25.0 and 40.0 mg, respectively. It produced an apparent Km value of 110.6, 110.6, 38.8 and 313.65 mg/ml, and Vmax of the enzyme were 12.32, 2.45, 3.35, 5.33 µmoles/min/ml for soluble, potato, rice and wheat starches, respectively (Figure 3d). Lineweaver burk plot also produced similar results (Figure 3e).

Effect of various metal ion concentrations on amylase activity

Various concentrations of monovalent, divalent cations like Na^+, K^+, Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} were used in these assays. These ions were added in the form of salts of chloride or sulphate. The concentrations of cations used in the reaction mixture were 1.0, 5.0, 10.0, 20.0, 40.0 and 60.0 mM, respectively. But in case of Zn^{2+} the concentrations were 1.0, 2.0, 3.0, 4.0, 5.0 mM, because higher concentrations produced precipitation. In the presence of Na^+ ion maximum amylase activity was at 1.0 mM concentration for all substrates. Above this concentration, enzyme activity was decreased (Figure 4a). 5.0 mM K^+ was the optimum concentration for soluble starch and for other substrates it was 1.0 mM (Figure 4b). In presence of Ca^{2+} ion the optimum enzyme activity was at 1.0 mM concentration for soluble starch, but for others the maximum enzyme activity was obtained at 10.0 mM concentration (Figure 4c). When Mn^{2+} was used in the reaction mixture, for soluble starch enzyme activity gradually increased but the optimum concentration for potato starch was 40.0 and 30.0 mM, respectively (Figure 4d). The optimum concentration of

Mg^{2+} was 1.0 mM for all substrates however in presence of potato starch it showed no effect (Figure 4e). The maximum enzyme activity was obtained at 1.0 mM concentration in presence of all four substrates for Zn^{2+} ion. Above this concentration it showed inhibitory effects (Figure 4f).

Molecular cloning and expression of the 'amy' of AB-56 in an *E. coli* host
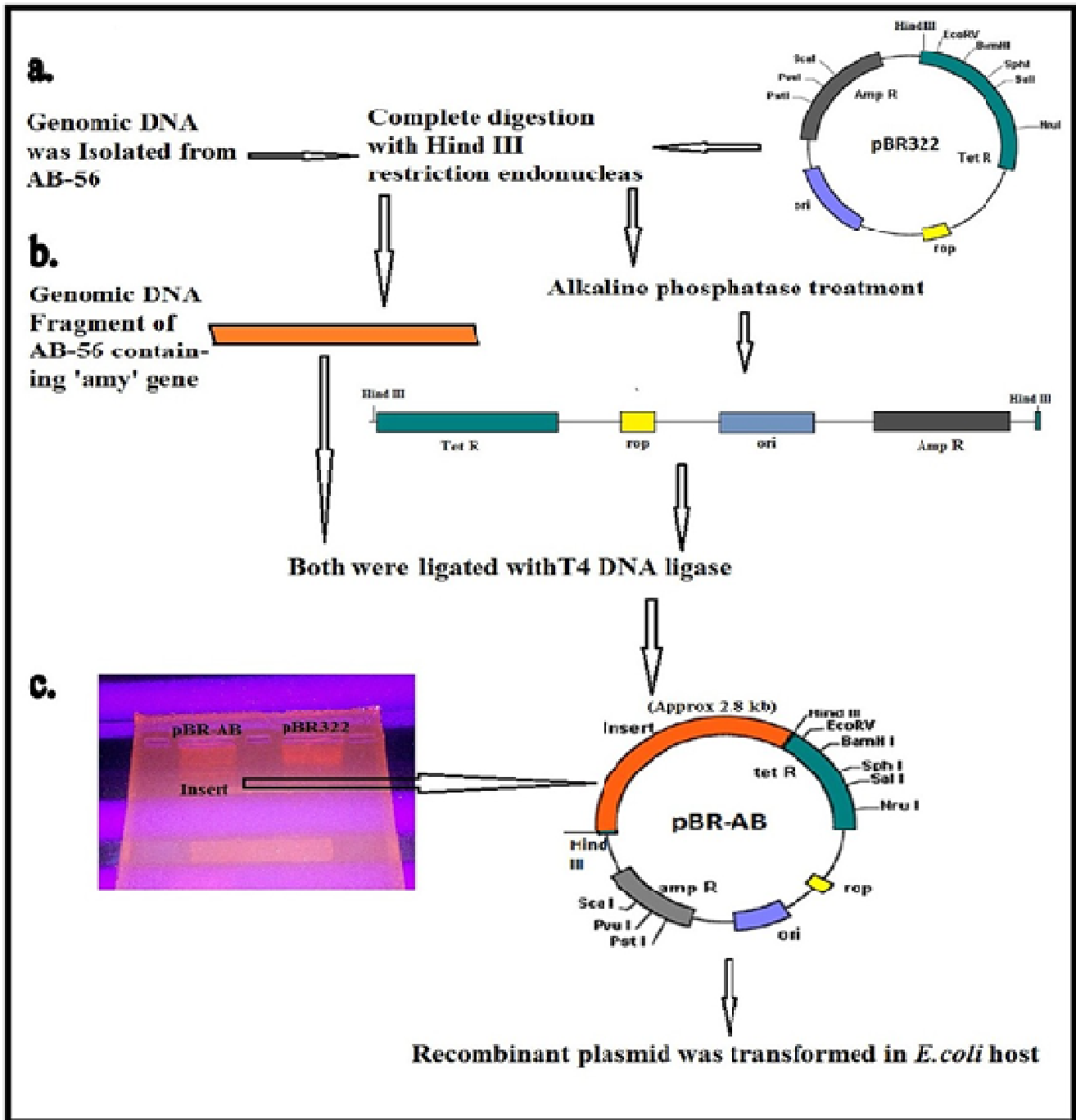
The genomic DNA was isolated, purified and cleaved with Hind III restriction endonuclease. The Hind III cleaved and dephosphorylated pBR322 was ligated with genomic DNA. Ligated DNA sample was used to transform *E. coli* DH5α (Figure 5). The transformants were selected using starch agar plates. 10 transformant were grown separately in liquid medium containing 1.0% soluble starch as carbon source. Recombinant plasmid was isolated and digested with Hind III restriction endonuclease. In all cases, it showed presence of an insert DNA of variable sizes. Approximately, the sizes of the inserts were 2.8 to 3.2 kb. The Petri plates containing transformants were stained with I_2 and KI solution and showed clear starch hydrolyzing zone around the colony. The insert was with promoter and operator sites of the wild strain therefore helped in the expression of the cloned amy of AB-56. One of the transformants which showed maximum starch hydrolyzing zone was selected and was grown in potato starch. The extracellular amylase activity indicates positive cloning and expression of the amy of AB-56 in *E. coli* host. The transformant can be grown in potato starch medium for long time and after cellular lysis, the cell lysate can be used as single cell fertilizer.

DISCUSSION

The use of bio-fertilizer is pivotal to ensure sustainable soil fertility and to restore soil structure. Bio-fertilizers are non toxic and ecologically suitable. Therefore, agricultural scientists suggest using much amount of bio-fertilizers (Mohammadi and Sohrabi, 2012). However, living cells of bio-fertilizers incipiently compete for food and nutrition with the crop plants in an agricultural land (Sen, 2007). After the death and decomposition of a living cell, the cellular molecules will be freed and easily available to the crop plant as the source of nutrients. Due to this reason bio-fertilizers show a slow and antagonistic effect on cultivated plants (Sen, 2007) even though, many reports provided accelerated growth and yield by using various bio-fertilizers (Rao et al., 1983). To overcome this problem, the work focuses on the use of cellular molecules after the lyses of a cell as bio-fertilizer (Sen et al., 2013). The present study includes isolation of a
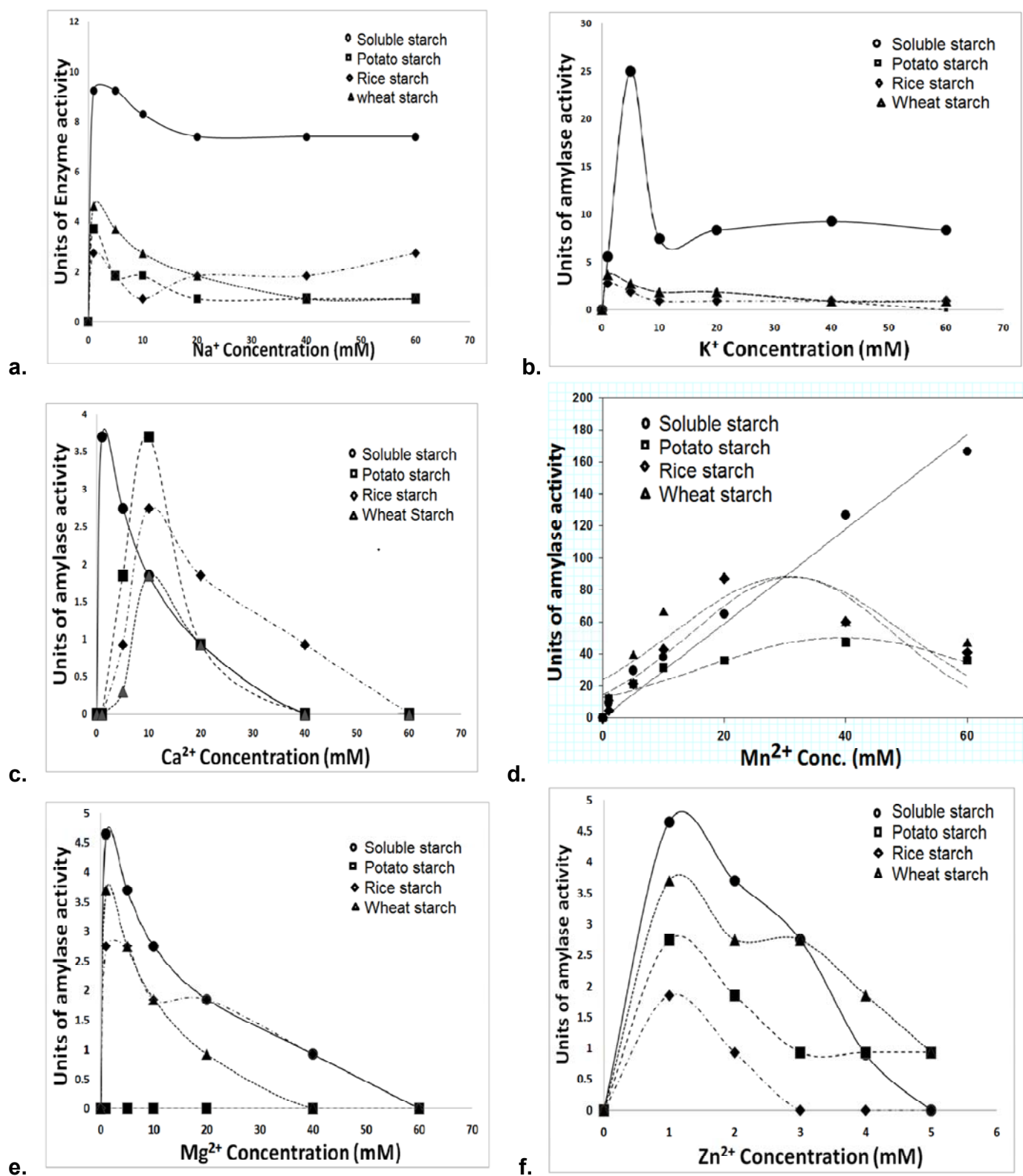
Figure 4. Effect of different metal ions on amylase activity. **a)** Effect of Na$^+$ concentration on amylase activity. Four various substrates were used   separately in the assay. **b)** Effect of K$^+$ concentration on amylase activity. **c)** Effect of Ca^{2+} conc. on amylase activity. **d)** Effect of Mn^{2+} conc. on amylase activity. **e)** Effect of Mg^{2+} conc. on amylase activity. **f)** Effect of Zn^{2+} conc. on amylase activity. Other parameters in all the assays were remained same as described in Materials and methods.

Figure 5. Strategy of cloning of 'amy' gene of AB-56. **a)** Genomic DNA and vector pBR322 isolated, purified and cleaved with Hind III; pBR322 was dephosphorylated. **b)** These DNAs were ligated with T4 DNA ligase. Ligated DNA was used to transform DH5α competent cells. **c)** Recombinant vector DNA was isolated and cleaved with Hind III. Figure shows the construct and the agarose gel containing the insert.

bacterium that uses potato starch as carbon source for its growth. Undefined starches are readily available with low cost in comparison to the more expensive industrially prepared starches (Nwagu and Okolo, 2011) and native starch sources significantly stimulated enzyme production (Nguyen et al., 2000). Therefore, the present study utilized potato starch as a suitable carbon source which can minimize the production cost (Kidd and Pemberton, 2002). On the basis of different biochemical tests, the organism was partially characterized as *Bacillus* sp. Impor-

tantly, starch, mannitol and carboxymethylcellulose induce extracellular amylase production of the organism whereas very low amount of intracellular amylase production in presence of various carbon sources indicates that amylase of the organism is mainly extracellular. The optimization of growth condition using different parameters like pH, temperature, potato starch concentration were carried out to get maximum growth of the organism. The organism grows above 50.0°C, therefore, it can be categorized as a thermo-tolerant bacterium. The analysis of amylase activity on different parameters showed some interesting results. Many reports support that the fungal amylase is optimally active at low pH whereas bacterial amylase is active in neutral pH (Patel et al., 2005; Nwagu and Okolo, 2011). The enzyme produced by the organism is active at low pH which is in agreement with the work of Demirkan (2011). This is probably due to the structure of the functional group in the active site which changed in basic condition and cause deformation (Al-Qodah et al., 2007). When soluble starch was used as substrate, optimum temperature for enzyme activity was at 17.0°C. This may be due to the better accessibility of the substrate to the active site at low temperature (Amico et al., 2003). K_m and V_{max} values were determined which are almost similar with the data obtained from the double reciprocal plot of substrate concentration versus velocity of enzymatic reaction (Al-Qodah et al., 2007; Demirkan, 2011). The enzymatic activity was increased upto 10 fold in presence of Mn^{2+} (Patel et al., 2005; Al-Qodah et al., 2007; Demirkan, 2011). According to some investigators Ca^{2+} is the main activator of amylase (Heinen and Lauwers, 1976; Bush et al., 1989; Demirkan, 2011). But here it was different. The molecular cloning of amy gene results improved production of amylase.

There are several reports on amy gene cloning and expression using Hind III restriction endonuclease (Mielenz, 1983; Sen and Oriel, 1989a; Sen, 2006). By growing a transformant using potato starch in a fermenter, huge amount of biomass can be produced in a short period which can be used as bio-fertilizer. The long term incubation of the culture caused cellular lyses after that it was used as bio-fertilizer to cultivate rice in field condition (data was not shown). However, liquid bio-fertilizer is more acceptable for farmers as it has more advantages over carrier based bio-fertilizer and can be considered as advanced bio-fertilizer production technology (Sheraz Mahdi et al., 2010).

Conclusion

Use of bio-fertilizer to improve structure and texture of agricultural land is essential because cellular molecules are organic molecules and eco-friendly. Production and supply of water-soluble nutrients as readymade food has

a logistic prospect regarding the future aspect of bio-fertilizer. Encouraging the development of low-cost indigenous bio-fertilizer production is the main theme of the work by using a recombinant bacterium of this kind.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Influence of storage of refrigerated milk on yield and sensory characteristics of queso fresco

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The aim of this study was to evaluate the quality of milk stored at different temperatures and storage times and the influence on yield and sensory characteristics of queso fresco. The milk collected was stored at temperatures of 3 and 7°C for times of 24, 48, 72 and 96 h for analysis and processing of queso fresco. Cheeses were stored at 3°C for times of 24 h, 5, 9, and 13 days and were evaluated for yield, production and sensory profile by the acceptability test. Cheeses stored for up to 13 days were analyzed for pH and titratable acidity. The mean results of the analyses of milk and sensory profile of queso fresco were compared by the Tukey test at 5% significance. It was observed that most results of refrigerated milk showed significant differences. Yield, production and sensory profile of queso fresco showed no significant differences. Regarding the coefficient of agreement of the sensory analysis of queso fresco, the mean values were between three and five points. Quesos frescos showed variations in titratable acidity and pH. Storage influenced the quality of refrigerated milk; however, it showed no changes on yield and sensory profile of queso fresco.

Key words: Fresh milk, storage, processing, acceptance testing, coefficient of agreement.

INTRODUCTION

The storage of milk under refrigeration for short time aims to preserve the microbiological and physicochemical characteristics of the raw material. However, even if stored at low temperatures, milk can present the growth of psychrotrophic microorganisms and produce low-quality dairy products. It is extremely important that raw milk is obtained in adequate sanitary conditions and stored at low temperatures, so you can maintain the microbiological count at low levels (Fagundes et al., 2006). The milk cooling controls the multiplication of mesophilic aerobic, but around 4 to 7°C allows the growth of psychrotrophic microorganisms that multiply well at

temperatures (Saeki and Matsumoto, 2010). Casarotti et al. (2009), will state that psychotropic bacteria are important in deteriorating milk and dairy products.

Among dairy products, cheeses are the most hampered in relation to the quality of refrigerated milk. In addition to the presence of microorganisms, chemical composition is another factor that influences the yield and sensory characteristics of cheeses. The yield of cheese is not only influenced by volume of milk, but also by proportion of protein in milk, being an important economic factor of the dairy industry (Wedholm et al., 2006). Lilbaek et al. (2006), report that maximizing yield without compromising quality is a key concern for manufacturers of industrial cheeses.

The economic losses associated with reductions in manufacturing yield due to the activity of proteases and lipases from psychrotrophic bacteria in refrigerated milk can be significant for industries of cheeses (Barbosa et al., 2009).

Studies aimed at assessing the quality of milk and yield of dairy products obtained from raw materials stored for long periods even at proper temperatures can result in significant information for milk and cheese processing industries. Thus, this study aims to evaluate the quality of milk stored at different times and temperatures and the influence on yield and sensory characteristics of queso fresco.

MATERIALS AND METHODS

This project was submitted to the Ethics Research Committee of the Federal Institute of Education, Science and Technology of Goiás, Brazil and approved under Protocol n°. 006/2013. Fresh milk was obtained from isothermal tank of a Dairy Industry at temperature of 16°C. To assess the quality of milk before collection, preliminary analyses on industrial platform were carried out. The results of milk samples indicated characteristic milk color and odor; the alizarol test showed pink-purple color and no lumps, negative result for antibiotics and fraud and titratable acidity (16°Dornic) within limits required by Brazilian legislation (Brasil, 2011) for processing of queso fresco.

The milk was packaged in milk cans with capacity of 50 L, properly cleaned and sanitized. The milk was filtered to remove any dirt, following storage in cold chamber at temperatures of 3°C and 7°C, using storage times of 24, 48, 72 and 96 h resulting in eight treatments. Six litres of milk at two temperatures and three replicates per treatment were used, where the analyses and processing of queso fresco were carried out. Milk samples refrigerated at 3 and 7°C were studied and assessed from the storage time of 24 h; after collection, milk showed no differences in physicochemical characteristics at 3 and 7°C.

Analysis of refrigerated milk

Electronic analyses were performed at the Laboratory of Milk Quality - Center for Food Research, School of Veterinary and Animal Science, Federal University of Goiás, Brazil. Contents of fat, protein, lactose, total dry extract (TDE) and degreased dry extract (DDE) were assessed using the IDF methodology (2000). Analysis of somatic cells count (SCC) was determined according to IDF (2006) and total bacterial count (TBC) by IDF (2004). The pH analyses were determined as IAL (2008) and titratable acidity by AOAC n°. 947.05 (1998), both held at the Laboratory of Animal Products - Campus Rio Verde, Goiás, Brazil.

Processing of queso fresco

Quesos frescos were processed according to good manufacturing practices for queso fresco in every storage time and temperature of refrigerated milk, according to methodology of Behmer (1999). The milk was pasteurized by slow heating to a temperature of 65°C/30 min under constant shaking in a previously sanitized container, followed by immediate cooling to a temperature of 32°C and was added of lactic acid and calcium chloride. For the addition of rennet to milk temperature was raised to 38°C.

For the manufacture of cheeses, calcium chloride was added at a ratio of 40 to 100 L of milk, lactic acid with industrial purity of 85% in the proportion of 25 mL per 100 L of milk diluted in 2.5 L of water and 9 mL liquid curd to 10 milk liters. After coagulation, the dough was cut into grains of approximately two inches of edge and left to stand for three minutes. Followed by discontinuous and slow stirring for 20 min and finally held at shaping.

The first turning of the cheeses was performed in 15 min, followed by the addition of sodium chloride on the cheese after resting for 30 min, and again turning the second addition of sodium chloride on the surface of the cheese (1.5% compared the total weight of the cheese). After the last turning, the cheeses were taken to a cold room at 3°C for 24 h (control), unmolded, packed in polyethylene bags and the cheeses were stored in refrigerated incubator for 13 days at temperature of 3°C.

Analyses of queso fresco

Quesos frescos were analyzed at Laboratory of Animal Products. During the storage time of queso fresco (24 h, five, nine, and 13 days), pH was analyzed by IAL methodology (2008) and titratable acidity by AOAC n°. 920,124 (1995).

The crude yield of cheeses was calculated as the difference between the milk volume used and the weight of cheeses after one day of storage (Equation 1).

$$\text{Crude yield (L/Kg)} = \frac{V}{Pq} X\,100 \qquad (1)$$

Where, V= milk volume, Pq = weight of cheese after one day of storage

Cheese production was determined by the weight of cheeses after one day of storage. Adjusted yield (Equation 2) and adjusted production (Equation 3) were obtained as Furtado (1999).

$$\text{Adjusted yield (L/Kg A)} = \frac{V \times (100 - Up)}{Pr \times ST} \qquad (2)$$

Where, V= milk volume, Up = desired percentage of moisture, Pr = cheese production (kg), ST = total solid content of cheese.

$$\text{Adjusted production (Kg)} = \frac{Pr \times ST}{100 - Up} \qquad (3)$$

Where, Up = desired percentage of moisture, Pr = cheese production (kg), ST = total solid content of cheese.

Table 1. Average values of fat, protein, lactose, total dry extract (TDE),degreased dry extract (DDE), pH, titratable acidity, somatic cells count (SCC) and total bacterial count (TBC) of refrigerated milk stored at 3 and 7°C/24, 48, 72 and 96 h.

Variable	Temperature (°C)	Storage time of milk (h)			
		24	48	72	96
Fat (%)	3	3.12^{bA}	3.04^{aA}	3.01^{aA}	3.04^{aA}
	7	3.47^{aA}	3.01^{aB}	2.58^{bC}	2.42^{bC}
Protein (%)	3	3.10^{aB}	3.11^{aB}	3.11^{aB}	3.18^{aA}
	7	3.10^{aB}	3.11^{aB}	3.13^{aB}	3.21^{aA}
Lactose (%)	3	4.55^{aA}	4.56^{aA}	4.55^{bA}	4.56^{bA}
	7	4.54^{bB}	4.55^{bB}	4.57^{aA}	4.58^{aA}
TDE (%)	3	11.74^{bA}	11.66^{aA}	11.62^{aA}	11.77^{aA}
	7	12.06^{aA}	11.62^{aB}	11.23^{bC}	11.19^{bC}
DDE (%)	3	8.61^{aB}	8.62^{aB}	8.61^{aB}	8.74^{aA}
	7	8.60^{aB}	8.62^{aB}	8.65^{aB}	8.77^{aA}
pH	3	6.94^{aA}	6.93^{aA}	6.93^{aA}	6.92^{aA}
	7	6.93^{aA}	6.91^{bAB}	6.92^{aA}	6.89^{bB}
Titratable acidity (% lactic acid)	3	16.92^{aA}	17.42^{aA}	17.66^{aA}	18.16^{bA}
	7	17.20^{aB}	18.53^{aB}	18.53^{aB}	20.75^{aA}
TBC (UFC/mL)	3	$7.9x10^{5aAB}$	$4.2x10^{5aB}$	$3.1x10^{5aB}$	$2.9x10^{6bA}$
	7	$6.2x10^{5aB}$	$6.2x10^{5aB}$	$7.1x10^{5aB}$	$5.1x10^{6aA}$
SCC (CS/mL)	3	$3.1x10^{5aA}$	$2.9x10^{5aA}$	$2.9x10^{5aA}$	$2.8x10^{5aA}$
	7	$4.1x10^{5aA}$	$3.0x10^{5aAB}$	$1.8x10^{5bBC}$	$1.5x10^{5bC}$

Means followed by different letters, uppercase in the line and lowercase in the column differ by the Tukey test at 5% probability.

Sensory analysis of queso fresco

Quesos frescos processed and stored under refrigeration at 3°C for the period of 24 h were evaluated through the acceptability test for attributes of color, odor, sour taste, bitter taste and texture. Sensory analysis consisted of individual evaluation of cheese samples using the five-point hedonic scale, whose extremes were: 1 - dislike very much and 5 - liked very much, being conducted with 17 adult, untrained volunteer panelists, consumers of queso fresco, selected based on interest and availability to participate in the sensory testing throughout the experiment.

Statistical analysis

Milk analyses were performed using a completely randomized design in triplicate for each replicate of the treatment, totaling nine results. The results were evaluated in a split-plot (2 x 4 x 4) scheme, in which curves were storage temperatures, subplots were the storage time of milk and sub subplots the storage time of cheese. The average results were compared by the Tukey test using the Sisvar Software (Ferreira, 2003) with significance level of 5%. pH and titratable acidity of quesos frescos were expressed as regression curves using Excel software, version 2007, from Microsoft Office.

The results of the sensory analysis of cheeses were compared by the Tukey test at 5% probability through the Sisvar Software (Ferreira, 2003). To complement the sensory analysis results, the percentage of the coefficient of agreement among judges with respect to the scores given was analyzed using the Consensor software (Silva et al., 2010a).

RESULTS AND DISCUSSION

Analysis of refrigerated milk

Table 1 shows the average values of the analyses of refrigerated milk stored at 3 and 7°C for 24, 48, 72 and 96 h. The average fat content of refrigerated milk stored at temperature of 3°C showed similar results (p>0.05); however, for storage at 7°C, results show significant reduction (p<0.05). In the interaction between storage temperature and time of refrigerated milk, it was observed that the fat results were different (p<0.05), except for the storage time of 48 h, showing similar results (p>0.05).

The mean fat results of milk refrigerated at 7°C/72 and 96 h showed values outside the standards required by Brazilian legislation (Brasil, 2011), which recommends at least 3%. According to Silva et al. (2010b), variations in fat content are influenced by several factors such as nutritional management, heat stress and inadequate mixing during milk collection.

The mean fat results for milk stored at 7°C/72 and 96 h were lower than the other storage times. To conduct this study, milk was stored in milk cans without constant stirring, causing fat separation.

During milk collection, the lack of adequate homogenization may have led to underestimation of fat results. It is known that the main milk component is fat and its reduced content by inadequate mixing may result in changes in the other milk components such as increased protein and lactose contents.

Samples of refrigerated milk stored for 96 h at temperatures of 3 and 7°C showed higher levels of protein, with a significant difference (p<0.05) from the other storage times. However, when evaluating tem-perature versus storage times of milk, the results were similar (p>0.05).

For the average levels of lactose, samples refrigerated at 3°C showed no difference in results (p>0.05). However, at temperature of 7°C, milk samples showed increased lactose content at the end of the storage period (72 and 96 h) (p<0.05).

In evaluating the temperatures in each storage time of refrigerated milk, it was observed that the lactose content differed significantly (p<0.05), with higher results in storage times of 24 to 48 h for temperature of 3°C, and higher mean values at 7°C at the end of the storage period.

In the work of Forsbäck et al. (2011), it was observed that milk samples stored for up to five days showed high levels of protein and lactose due to the reduced fat content in milk. In the present study, the low fat content in milk samples stored up to 96 h evidenced the high protein and lactose values in refrigerated milk samples.

The TDE value of refrigerated milk samples stored at 3°C showed similar results (p>0.05), but for temperature of 7°C, the TDE values decreased when the storage time increased (p<0.05). Interactions between temperature and storage time of refrigerated milk, with the exception of the storage time of 48 h (p>0.05), showed significant differences (p<0.05) between TDE results.

Refrigerated milk stored at temperatures of 3 and 7°C for 96 h showed higher DDE values (p<0.05). However, when evaluating storage temperature and time, the mean DDE values showed no significant differences (p>0.05).

According to Oliveira et al. (2012), the high mean TDE value is due to the fat content, which is similar to the DDE value, since this variable is obtained from the difference between TDE and the fat content. In this study, refri-gerated milk samples stored at 7°C/24 h showed higher TDE values due to the high average fat contents present in milk, since TDE is obtained from the sum of all components but water.

In contrast, the high fat levels provided lower DDE levels for refrigerated milk stored at 3 and 7°C for up to 72 h, since DDE is the sum of all components but water and fat.

The pH of milk samples stored at 3°C for 96 h showed significant similarities (p>0.05). For milk refrigerated at 7°C/24 h, the pH was similar for storage times of 48 and 72 h (p<0.05), but the storage time of 96 h differed from storage times of 24 and 72 h (p<0.05). Regarding the interaction between temperature and time, the pH of milk stored at temperature of 3°C was higher than milk stored for 48 and 96 h (p>0.05).

The mean titratable acidity value of milk refrigerated at 3°C for 96 h did not differ significantly (p>0.05). When stored at 7°C, the titratable acidity was proportion to the increased storage time of refrigerated milk (p<0.05). When evaluating storage temperature and time, refrigerated milk stored for 96 h at 7°C showed higher titratable acidity when compared to temperature of 3°C (p<0.05). To meet the quality standard of milk required by instruction normative 62/2011 (Brasil, 2011), the minimum and maximum limits for titratable acidity of milk should be between 0.14 and 0.18 g of lactic acid /100 mL.

The high average titratable acidity value of milk stored at 7°C for 96 h was influenced by increasing storage time and temperature. Since pH and titratable acidity are inversely proportional, it was expected that milk refrigerated at 3 and 7°C for 96 h with higher titratable acidity levels showed low mean pH values.

The mean TBC values for storage time of 96 h of milk refrigerated at 3°C were similar to the storage time of 24 h (p>0.05). However, there was an increase in the TBC values for refrigerated milk stored at 7°C for 96 h and in the interaction with temperature of 3°C (p<0.05).

According to Vallin et al. (2009), the variation of temperature and storage time of milk and inadequate sanitation are factors that influence the quality of refrigerated milk. In the present study, the processes of sampling, temperature raise and storage time of milk refrigerated at 3°C in times of 24 and 96 h and 7°C for 96 h resulted in increased TBC values.

The SCC value was similar for milk samples stored at 3°C for up to 96 h (p>0.05). Storage at 7°C for times of 24 h indicates higher mean SCC versus times of 72 and 96 h (p<0.05). The interaction of temperature and storage time was similar in times of 24 and 48 h (p>0.05) for variable SCC. In the work of Paula et al. (2004), it was verified that from the first to the fourth day of storage, there was a reduction in the average SCC, which may be attributed to cell breakdown. In the present study, the decrease SCC in samples refrigerated at 7°C was due to the high storage time and temperature of refrigerated milk

Table 2. Mean values of crude yield, adjusted yield, production and adjusted production of queso fresco stored for 24 h produced with milk refrigerated at 3 and 7°C/24, 48, 72 and 96 h.

Variable	Temperature (°C)	Storage time of milk (h)			
		24	48	72	96
Crude yield (L/Kg)	3	5.01[aA]	4.88[aA]	5.28[aA]	5.09[aA]
	7	4.88[aA]	4.93[aA]	5.02[aA]	5.07[aA]
Adjusted yield (L/Kg)	3	5.08[aA]	4.88[aA]	5.30[aA]	5.29[aA]
	7	4.98[aA]	4.99[aA]	5.11[aA]	5.23[aA]
Production (Kg)	3	1.20[aA]	1.23[aA]	1.14[aA]	1.18[aA]
	7	1.23[aA]	1.22[aA]	1.20[aA]	1.19[aA]
Adjusted production (Kg)	3	1.18[aA]	1.23[aA]	1.13[aA]	1.14[aA]
	7	1.21[aA]	1.20[aA]	1.18[aA]	1.15[aA]

Means followed by different letters, uppercase in the line and lowercase in the column differ by the Tukey test at 5% probability.

and the use of preservative bronopol to preserve samples prior to analysis, these factors were essential for cell degradation in refrigerated milk during storage.

Analyses of queso fresco

Table 2 shows the mean values of crude yield, adjusted yield, production and adjusted production of queso fresco produced with milk refrigerated at 3 and 7°C stored for 24, 48, 72 and 96 h. The mean crude yield, adjusted yield, production and adjusted production of queso fresco values were similar (p>0.05) in both storage temperature and time and interaction between them.

Several factors can affect the yield of cheeses such as milk composition, amount of components lost in whey, amount of salt added to cheese and amount of water retained in the cheese (Emediato et al, 2009). In the study by Pretto et al. (2012), the levels of fat, protein and casein were positively and strongly correlated with cheese yield. Silva et al. (2012) observed that the reduction in cheese yield was due to the high SCC in milk.

In the present study, differences in temperature (3 and 7°C), storage time (24, 48, 72 and 96 h), low SCC, high protein content and decreased fat content of milk refrigerated at 7°C did not affect the yield and production of queso fresco. It is known that milk with high protein content is valuable for the dairy industry because protein is the component responsible for the increase in the cheese yield, in addition to the low SCC. Thus, in this study, these factors were essential for the absence of significant variation in yields and production of queso fresco produced with milk refrigerated at 3 and 7°C stored for up to 96 h.

The similarities observed in the production and yield of queso fresco could be attributed to the dissolution of fat during milk pasteurization, to the high protein content found and the average low SCC, within the maximum limits (6.0 x10^{5}SC /mL) allowed by instruction normative 62/2011 (Brasil, 2011).

Figure 1 shows the regression curve for titratable acidity of queso fresco stored for 24 h, five, nine and 13 days.

Cheeses stored for up to 13 days and processed with milk stored at 3°C/48 h (R^2 = 0.9321) and 72 h (R^2 = 0.9808) showed significant differences for titratable acidity. However, for time of 72 h, higher acidity after 13 days of storage was observed. Cheeses manufactured with milk stored at 3°C/24 h (R^2 = 0.1558) and 96 h (R^2 = 0.7906) showed similar variations for titratable acidity levels. Figure 2 shows the regression curve for titratable acidity of queso fresco stored for 24 h, five, nine, and 13 days. Cheese processed with milk stored at 7°C/24 h (R^2 = 0.4464), 48 h (R^2 = 0.9968) and 72 h (R^2 = 0.9600) showed different behaviors for the mean titratable acidity values during storage for up to 13 days.

For time of 24 h, the acidity of cheeses increased with nine days and decreased after 13 days of storage, but in the storage time of 48 h, acidity was constant from the fifth day on. However, in the storage time of 72 h, the titratable acidity showed increasing behavior. Cheese samples stored for up to 13 days made with milk stored at 7°C for 96 h (R^2 = 0.8359) showed linear increasing titratable acidity.

In the study by Cunha et al. (2002), the titratable acidity of Minas cheese increased during 30 days of refrigerated storage. Queiroga et al. (2009) reported that the release of whey after packaging can eliminate the lactose content and influence the acidity values. Changes in titratable
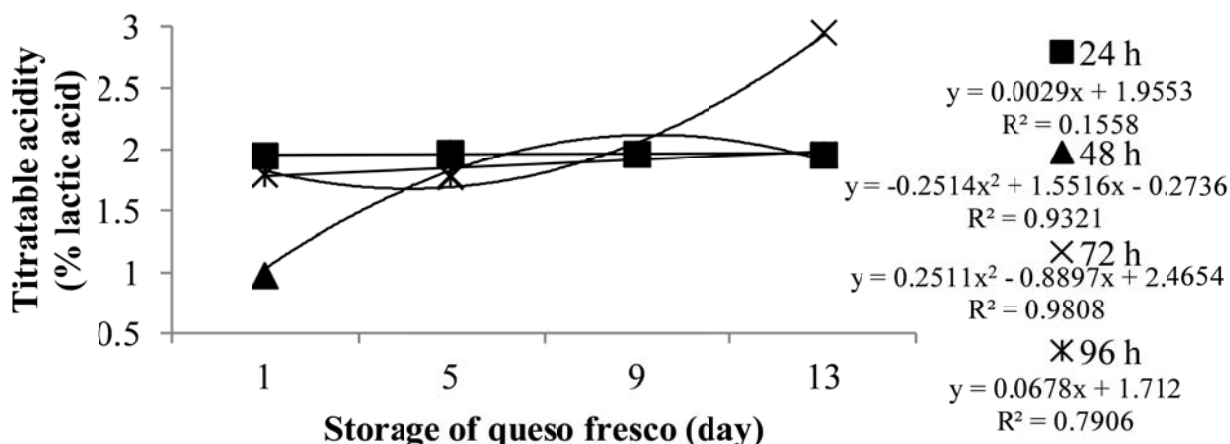
Figure 1. Regression curve for titratable acidity of queso fresco stored for 24 h, five nine and 13 days processed with milk refrigerated at 3°C/24, 48, 72 and 96 h.

Figure 2. Regression curve for titratable acidity of queso fresco stored for 24 h, five, nine and 13 days processed with milk refrigerated at 7°C/24, 48, 72 and 96 h.
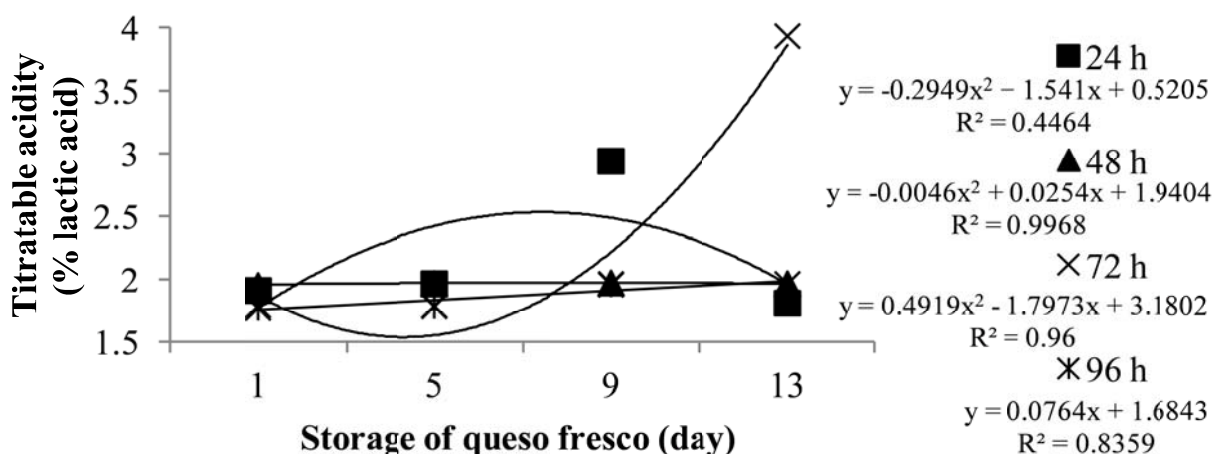
acidity values found in this study for cheese processed with milk refrigerated at 3 and 7°C up to 96 h may be due to the release of whey after packaging throughout the shelf life, since the lower the release of whey, the greater the retention of lactose, with consequent production of cheese with high acidity.

The results of the regression curve for the pH of queso fresco stored for 24 h, five, nine, and 13 days are shown in Figure 3. Cheese processed with milk stored for 24 h (R^2 = 0.9333), 72 h (R^2 = 0.9926) and 96 h (R^2 = 0.6429) showed different behaviors during the storage time. For time of 48 h, the pH of cheeses was constant throughout the shelf life; therefore, it was not possible to generate the regression curves and determine the R^2 value.

Figure 4 shows the results of the regression curve for

the pH of quesos frescos stored for 24 h, five, nine, and 13 days. Cheese processed with milk stored for 24 h (R^2 = 0.9953), 48 h (R^2 = 0.8364) and 72 h (R^2 = 0.8077) showed no similarity in pH values of cheese samples stored for up to 13 days. However, in the storage time of 96 h (R^2 = 0.02), pH showed linear behavior with respect to storage time of 13 days.

Gigante et al. (2006) found that storage temperatures of 4 and 20°C for up to 28 days did not affect the pH of samples. Pereira et al. (2003) reported that the higher average pH value found for Minas cheese was due to the use of lactic acid in cheese processing. Comparing Figures 3 and 4, it could be observed that the pH of cheeses stored for 13 days and processed with milk at 3 and 7°C for up to 96 h showed similar values during the
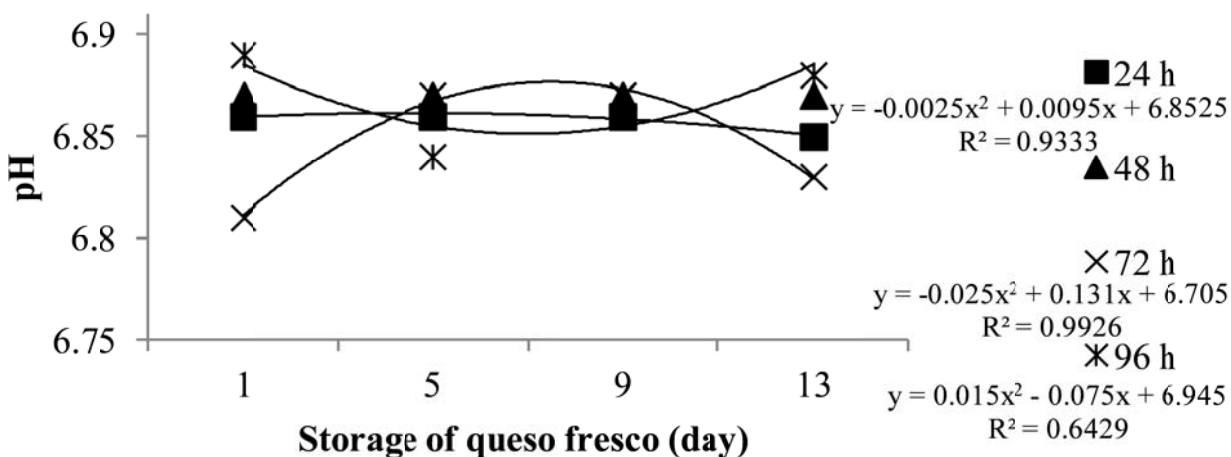
Figure 3. Regression curve for pH of queso fresco stored for 24 h, five, nine and 13 days processed with milk refrigerated at 3°C/24, 48, 72 and 96 h.
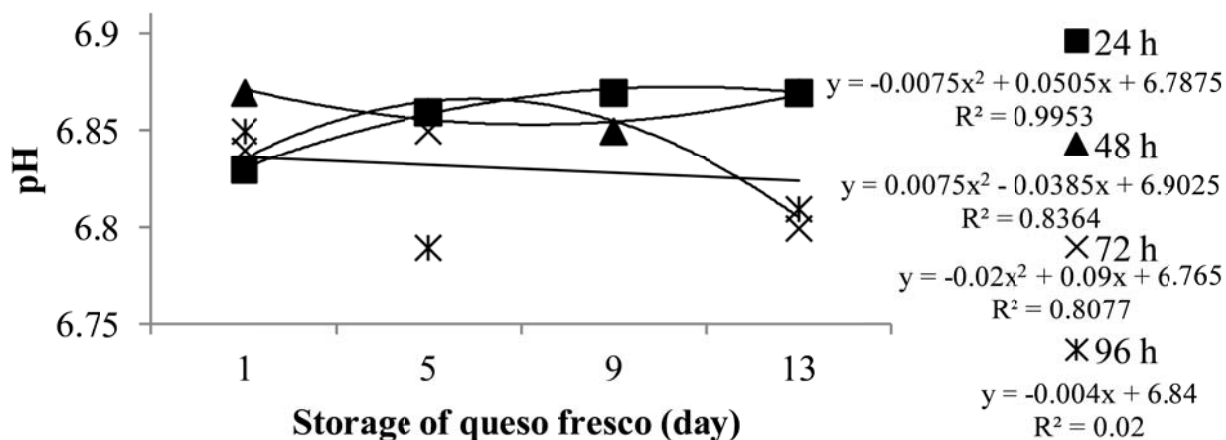
Figure 4. Regression curve for pH of queso fresco stored for 24 h, five, nine and 13 days processed with milk refrigerated at 7°C/24, 48, 72 and 96 h.

storage time, since the use of lactic acid for the processing of queso frescos is critical for adjusting the mean pH values.

Sensory analysis of queso fresco

The sensory analysis of quesos frescos stored for 24 h and processed with milk refrigerated at 3 and 7°C for up to 96 h of storage, through acceptance test, was performed in order to evaluate if panelists were able to identify and inform the presence of significant difference between samples (Table 3). Table 3 shows the average values of the sensory analysis for color, odor, sour taste, bitter taste and texture of quesos frescos stored for 24 h

and processed with milk refrigerated at 3°C and 7°C/24, 48, 72 and 96 h. The mean values of the acceptance test of queso fresco stored for 24 h and processed with milk refrigerated at temperatures of 3 and 7°C for up to 96 h were similar in all variables (p>0.05) both in the interaction between time and temperature and the evaluation of temperature and storage time.

Antonello et al. (2012) reported that the high microbial count may indicate a potential risk to the health of consumers and suggest possible economic losses arising from the rejection of cheeses, which is caused by sensory changes caused by microbial action. Schuster et al. (2006) demonstrated that pasteurization was effective in eliminating pathogenic microorganisms which might occasionally be present in raw milk.

Table 3. Average values of the sensory analysis for color, odor, sour taste, bitter taste and texture of quesos frescos stored for 24 h and processed with milk refrigerated at 3 and 7°C/24, 48, 72 and 96 h.

Variable	Temperature (°C)	Storage time of milk (h)			
		24	48	72	96
Color	3	4.47[aA]	4.76[aA]	4.88[aA]	4.53[aA]
	7	4.24[aA]	4.82[aA]	4.82[aA]	4.71[aA]
Odor	3	3.94[aA]	4.00[aA]	3.82[aA]	4.24[aA]
	7	3.94[aA]	4.12[aA]	4.29[aA]	4.41[aA]
Sour taste	3	3.41[aA]	3.88[aA]	4.06[aA]	3.53[aA]
	7	3.59[aA]	4.06[aA]	4.06[aA]	3.88[aA]
Bitter taste	3	3.18[aA]	4.00[aA]	3.88[aA]	3.53[aA]
	7	3.18[aA]	3.88[aA]	3.71[aA]	3.82[aA]
Texture	3	3.82[aA]	4.35[aA]	4.35[aA]	4.47[aA]
	7	4.00[aA]	4.41[aA]	4.47[aA]	4.53[aA]

Means followed by different letters, uppercase in the line and lowercase in the column differ by the Tukey test at 5% probability.

Table 4. Means and coefficient of agreement (CA) of panelists in the sensory evaluation for color, odor, sour taste, bitter taste and texture of quesos frescos stored for 24 h processed with milk refrigerated at 3 and 7°C/24, 48, 72 and 96 h.

Milk temperature (°C)	Storage time of milk (h)	Color		Odor		Sour taste		Bitter taste		Texture	
		Mean	CA (%)	Mean	CA (%)	Mean	CA (%)	Mean	CA (%)	Mean	CA (%)
3	24	4.47	62.11	3.94	51.28	3.41	27.28	3.18	15.00	3.82	34.30
	48	4.76	74.18	4.00	38.02	3.88	35.54	5.00	44.18	4.35	56.78
	72	4.88	86.05	3.82	35.54	4.06	39.02	3.88	43.22	4.35	55.49
	96	4.53	62.11	4.24	50.60	3.53	19.95	3.53	33.02	4.47	58.53
7	24	4.24	51.45	3.94	41.21	3.59	29.28	3.18	11.77	4.00	37.89
	48	4.82	79.79	4.12	44.22	4.06	37.89	3.88	34.54	4.41	53.91
	72	4.82	79.79	4.29	49.74	4.06	39.02	3.71	25.64	4.47	59.26
	96	4.71	69.35	4.41	54.71	3.88	46.13	3.82	34.30	4.53	64.50

In this study, queso fresco processed with milk stored at 7°C for up to 96 h showed no unfavorable sensory characteristics in relation to time and high temperature of the refrigerated milk, which may be explained by the maintenance of stable temperature used in cold chambers for refrigerated milk storage and used in the processing of queso fresco. These factors were essential to maintain the sensory characteristics of cheeses, since the high count of microorganisms in refrigerated milk used for cheese processing is partially reduced during pasteurization, which probably will produce dairy products with characteristics similar to those processed with refrigerated milk of low microbial count.

To better assess the agreement between panelists on the correlation between scores given by the five-point hedonic scale and the average scores of all panelists, data were evaluated by the coefficient of agreement shown in Table 4.

Cheese processed with milk at 3°C/72 h showed greater coefficient of agreement and average scores given by panelists regarding color. For odor, the queso fresco sample showing the highest average scores and coefficient of agreement was that processed with milk at 7°C/96 h.

The highest percentage of agreement for the acid taste was for cheese processed with milk stored at 7°C for 96 h. However, the best means were for quesos frescos processed with milk refrigerated at 3°C/72 h and 7°C/48 and 72 h. For bitter taste, queso fresco processed with milk stored at 3°C/48 h showed greater coefficient of agreement and mean score of 5.00 in the hedonic scale.

Queso fresco processed with milk stored at 7°C for 96

h showed the highest coefficient of agreement and average texture.

Machado et al. (2004) performed sensory analysis of Minas cheese from the region of Serro and found average score of 6.00, which attributed the rating: liked slightly. Due to the higher coefficient of agreement and average scores given by panelists to queso fresco processed with milk refrigerated at 3 and 7°C for up to 96 h, the results of this study indicated that the mean scores ranged from did not like / nor disliked and liked very much for all variables. The agreement index among panelists for parameters color, odor, sour taste, bitter taste and texture for cheese processed with milk refrigerated at 3 and 7°C for 24, 48, 72 and 96 h, showed good acceptability in relation to the mean values of scores given by panelists.

Conclusion

The sampling and storage of milk refrigerated at 3 and 7°C for up to 96 h influenced the quality of refrigerated milk. Yield, production and sensory profile of queso fresco were not influenced by the use of milk refrigerated at 3 and 7°C for up to 96 h. The acidity of queso fresco was influenced by the release of whey after packing throughout the shelf life. The constant pH of queso fresco was due to the use of lactic acid during processing.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

In vivo evaluation of the hypoglycemic effect of wolf-apple flour (*Solanum lycocarpum* A. St.-Hil)

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The prevalence of diabetes has increased at alarming rates worldwide, and has become a serious health problem in modern society, highlighting the need for adjuvants to assist in its treatment. The starch from wolf-apple is a product extracted from the pulp of the unripe wolf-apple (*Solanum lycocarpum* A. St.-Hil), which has been used empirically by the population due to various therapeutic effects, among them, its hypoglycemic action. The objective of this study was to evaluate the hypoglycemic effect of the administration of wolf-apple starch on diabetic Wistar rats, during five weeks. The animals were randomly divided into three groups: normal control, diabetic control and treated diabetic (received 100 mg/day of wolf-apple flour by gavage), and diabetes was induced with streptozotocin (40 mg/kg rat). The following parameters were evaluated: glycemia, animal weight, food intake, diuresis, water intake and histopathological analyses of liver and pancreas. The results show that the flour presented a hypoglycemic effect of 19.76%, and there was no significant difference in food consumption, water consumption and weight gain among the evaluated groups. On the other hand, the treated diabetic group showed a urine volume significantly higher than the other groups. The treated animals did not show toxicity in the liver and pancreas. It is concluded that the starch from wolf-apple has hypoglycemic potential.

Key words: Wolf-apple, flour, hypoglycemic effect, diabetes.

INTRODUCTION

Currently, diabetes represents a serious public health problem due to its high frequency in the population, complications, mortality, high financial and social costs involved in the treatment and a significant decrease in quality of life (Santo et al., 2012). Diabetes mellitus is a metabolic disease of multifactorial cause, characterized

by the relative deficiency in insulin production or a decrease in its action, causing hyperglycemia (Gallego, 2005; American Dietetic Association (ADA), 2009). The disease has an increasing prevalence worldwide and could become a pandemic in the next decades, due to an increasingly aging population, but mainly due to the increasing prevalence of obesity and physical inactivity (Murussi et al., 2003; Rato, 2010). Facing that reality, the search of new alternatives in the treatment of diabetes becomes indispensable.

Although there is no cure for the disease, its control is viable by the adhesion of the diabetic patient to a series of care behaviors: diet, regular physical exercises, monitoring of glucose levels and appropriate use of hypoglycemics and/or insulin when necessary (Cruz, 2005). Studies on new hypoglycemic drugs have been conducted with special focus on the medicinal plants used in traditional medicine, because data in the literature shows that it is much more probable to find biological activity in plants oriented for use in traditional medicine than in plants chosen at random (Barbosa-Filho et al., 2006).

Solanum lycocarpum A. St. Hil is a plant species of the Solanaceae family that can be found throughout the entire Brazilian territory, mainly in savannas (Santos and Coelho, 2002). Its fruit is referred to as wolf-apple and presents a slightly flattened bulbous form (8 to 12 cm diameter) and could weigh up to 500 g (Rocha et al., 2012). Sedative, soothing, hypocholesterolemic and hypoglycemic properties have been attributed to wolf-apple (Dall and Von Poser, 2000; Vieira et al., 2003; Clerici et al., 2011). In the Southeast and Central-West regions of Brazil, the wolf-apple flour is widely used as an oral hypoglycemic. It is a powder extracted from the pulp of the unripe wolf-apple, which can be elaborated domestically or acquired in drugstores specialized in herbal medicines in the form of capsules (Clerici et al., 2011; Rocha et al., 2012). Given the above, the objective of this study was to evaluate the hypoglycemic effect of the wolf-apple flour on diabetic Wistar rats.

MATERIALS AND METHODS

Plant material

The fruits of the polant species *Solanum lycocarpum* A. St. - Hil were collected in the pasture area of Departamento de Zootecnia at Universidade Federal de Lavras. A voucher specimen is on deposit in Esal herbarium 00836. The wolf-apple flour was obtained according to Rocha et al. (2012). Sixty (60) wolf-apples were collected, weighing an average of 350 g each, and taking into account the degree of maturation, excluding mature and very unripe fruit.

The fruits were weighed, washed with distilled water and peeled. The seeds were removed and the pulp was chopped into pieces, weighed, crushed in an industrial blender with distilled water, at the ratio 2:1 (pulp/distilled water), for two minutes. The homogenized pulp was filtered on a cotton cloth, the filtrate was taken to a refrigerator (4°C) for 6 h, to decant. After decanting, the supernatant

was discarded and the precipitate was washed with distilled water and put back in the refrigerator to decant for 12 h. On the next day, the sample was washed and decanted again. The supernatant was discarded and it was possible to observe the formation of a light fraction in the bottom of the beaker, and a dark fraction above that one, which was discarded. The light fraction was placed in a tray and brought into a ventilated oven, at 30°C, for three days. The dry light fraction was called starch, which was crushed, weighed and then stored in a hermetically sealed glass container protected from light, at room temperature.

Animals and experimental conditions

24 adult male Wistar rats were used, (*Rattus novergicus*), in growth phase and with an initial average weight of 193±25 g, from the Biotery of Departamento de Medicina Veterinária da Universidade Federal de Lavras. The animals were randomly divided into three groups: normal control (NC), diabetic control (DC) and treated diabetic (TD), with eight animals in each group. The animals were maintained in individual cages, at a room temperature of 25±3°C (12 h light/dark cycle) and with access to food (food suitable for this species, Nuvilab CR1®) and water *ad libitum* for five weeks. This study was examined and approved by the Ethics Committee on Animal Use of Universidade Federal de Lavras-UFLA (Ruling № 006/2009).

Diabetes induction

The animals of the diabetic control and treated diabetic groups, after anesthesia with 35 mg/Kg thiopental, received 40 mg/kg streptozotocin (Sigma®), diluted in a 0.01 mol/L citrate buffer pH 4.5 via intravenous administration, using the penile vein as access (Cunha et al., 2009).

A week after the application of the diabetogenic agent, blood glucose in the animals was measured. The rats with glycemic levels equal or superior to 120 mg/dL (Delfino et al., 2002) were considered as diabetic, and the remaining ones, which did not reach the levels, were discarded. Soon afterwards, these animals were randomly divided into the diabetic control and treated diabetic groups.

Determination of glycemia

Blood glucose was determined weekly, using the glucose oxidase method, with the use of glucose test strips, and reading was conducted in the Optium Xceed® model apparatus (Abbott). The blood samples were collected from the tail of the animals, after an 8 h fast.

Treatment with the flour

The group diabetic treated received 100 mg/day of wolf-apple flour in a single dose, which is equivalent to approximately 10 times the recommended dose for adult humans (1,500 mg/day). An orogastric tube was used (gavage) for delivery and the flour was dissolved in 1 mL of filtered water. In the control and diabetic control groups, 1 mL of water was administered by the same procedure.

Biological evaluation

Food consumption

The animals were placed in individual metabolic cages and fed with

Table 1. Weekly glucose levels (mg dL^{-1}), during the five weeks of experiment, for the groups normal control, diabetic control and treated diabetic.

Treatment/week	0	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Normal control	99.0±7.6C	91.12±1.8C	103.5±5.8C	113.3±5.3C	101.6±3.3C	108.2±7.7C
Diabetic control	121.0±6.8B	94.0±2.6B	121.5±1.4A	131.8±3.4A	129.1±10.4A	129.8±2.9A
Treated diabetic[1]	156.0±16.9A	114.3±3.7A	118.2±2.2B	124.0±3.3B	109.5±5.7B	125.3±1.3B

Data are the mean of eight replicates ± standard deviation. Averages followed by the same letter in the columns do not differ by the Tukey test (p≤0.05). [1]Treated diabetic: received 100 mg/day of wolf-apple flour.

25 g food/day. The consumption was recorded daily and calculated based on the remainder verified on the following day.

Water consumption

Water consumption was verified every two days, through the difference between the placed and the remaining volume.

Weight of animals

The animals were weighed weekly, on a Bel Engineering® digital scale.

Urine volume

The urine of the animals was collected in a graduated beaker and volume readings were taken every other day.

Euthanasia of animals

At the end of the experiment, the animals were anesthetized with 35 mg/Kg sodium thiopental, intraperitoneally. A median laparotomy was carried out in the pelvic-cranial direction of the abdominal and thoracic cavities. Soon afterwards, the exsanguination was performed to sacrifice the animals, and the liver and the pancreas of the animals were removed.

Histopathological analyzes

The liver and the pancreas of each animal were collected, weighed and fixed in a formalin solution. The organs were transversely sectioned, dehydrated and diaphanized at room temperature. 4 μm cuts were made in a rotary microtome, stained with hematoxylin-eosin, mounted on slides/coverslips and analyzed under light microscopy (100x magnification) for the qualitative analysis.

Statistical analysis

The data are the average of eight replicates ± standard deviation analyzed by Sanest program and when this analysis showed a significant difference, the Tukey test (P≤0.05) was used for the comparison of means. For the variance analysis, the design used was completely randomized, in a 3 x 5 (groups x weeks) factorial outline, with eight repetitions, for the parameters glycemia, food consumption, weight gain, water consumption, urine volume and liver weight.

RESULTS AND DISCUSSION

It was observed (Table 1) that there was a significant difference in blood glucose levels between the evaluated groups of animals. The group treated with wolf-apple starch showed a decrease of 19.76% in their glycemic levels over the five weeks of experiment, whereas in the diabetic group, there was an increase of 6.61% and, in the normal control group, an increase of 9.29%. At the beginning of the experiment (week 0), the treated diabetic group had glycemic levels higher than those of the normal control and diabetic control groups. However, throughout the experiment, the glycemic levels of the treated diabetic group decreased, while those of the normal control and diabetic control groups showed a small increase, demonstrating the hypoglycemic activity of wolf-apple starch.

Martha et al. (2000) demonstrated that a drug is considered effective when it reduces the glucose levels by at least 15% of the initial values. Therefore, it can be suggested that the wolf-apple flour is effective in the reduction of blood glucose levels in Wistar rats.

Studies show the presence of dietary fiber and alkaloids in wolf-apple starch (Dall-Agnol and Von-Poser, 2000; Rocha et al., 2012), and these substances may be responsible for the reduction in glycemia observed in this study. Dietary fiber delays gastric emptying, decrease in absorption of carbohydrates by the inclusion of sugars in the fiber matrix and modification in hormone secretion. On the other hand, alkaloids present in the Solanaceae family can stimulate the release of insulin by the beta cells of the pancreas (Dall-Agnol and Von-Poser, 2000), therefore reducing glycemia.

Table 2 shows the consumption of water, food, weight gain and urine volume of the experimental animals, with no statistical difference (p≤0.05) between treatments for the consumption of water, food and weight gain. The treated diabetic group presented a urine volume significantly higher than the other groups, showing that the reached glycemic levels were enough to lead to polyuria in those animals.

Regarding food consumption, water and weight gain, there was no significant difference, probably due to the fact that streptozotocin led to mild diabetes in the rats. If the disease were more severe, more severe differences would probably have been observed in those parameters.

There was no significant difference (p≤0.05) in the three studied groups, when evaluating the weights of the livers of the animals and the LW/BW ratio (Table 3), probably due to the fact that the animals did not present high glycemia.

Table 2 Average weekly consumption of water, food, weight gain and urine volume during the five weeks of experiment, for the groups normal control, diabetic control and treated diabetic.

Parameter	Treatment		
	Normal control	Diabetic control	Treated diabetic
Water consumption (mL)	180.6±73.0A	203.9±63.1A	208.1±61.0A
Food intake (g)	162.0±43.4A	151.7±26.3A	151.9±32.9A
Weight gain (g)	19.7±7.5A	17.5±23.2A	13.6±16.5A
Urine volume (mL)	10.4±3.3B	18.5±10.2B	57.3±24.1A

Data are the mean of eight replicates ± standard deviation. Averages followed by the same letter in the lines do not differ by the Tukey test (p≤0.05). [1]Treated diabetic: received 100 mg/day of wolf-apple flour.

Table 3. Average liver weight and liver weight/body weight (LW/BW) for the groups normal control, diabetic control and treated diabetic, after five weeks of experiment.

Treatment	Liver weight (g)	LW/BW (%)
Normal control	10.06±1.7[A]	3.89±0.45[A]
Diabetic control	11.00±2.05[A]	4.13±0.34[A]
Treated diabetic	10.52±2.04[A]	4.50±0.52[A]

Data are the mean of eight replicates ± standard deviation. Averages followed by the same letter in the columns do not differ by the Tukey test (p≤0.05). [1]Treated diabetic: received 100 mg/day of wolf-apple flour.
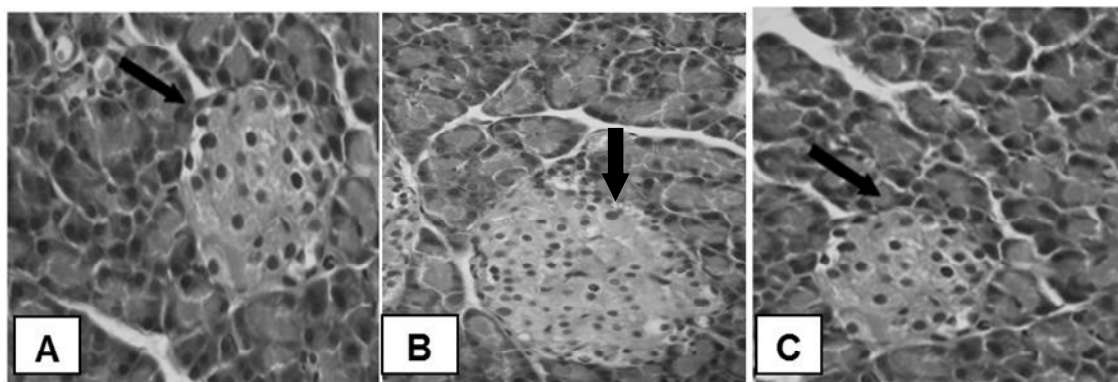
Figure 1. Histological section of the pancreas (islet of Langerhans is indicated by black arrow) stained with hematoxylin-eosin, for the groups normal control (A), diabetic control (B) and treated diabetic - received 100 mg/day of wolf-apple flour (C), for six weeks. 40 X magnification.

The histological sections of the pancreas and liver of the animals are presented in Figures 1 and 2, respectively. No macroscopic changes were observed in the organs analyzed, and neither were injuries, or significant microscopic changes.

Observing the pancreas and liver slides from all of the studied groups, it was noticed that there was no difference among the groups under optical microscopy analysis. This is probably due to the fact that the animals presented mild diabetes, not leading to lesions in those organs, observable by this technique.

The wolf-apple flour did not present any toxic effect to the liver, under the tested conditions. To affirm the non-hepatic toxicity of the wolf-apple flour, other analyses would be necessary, such as the plasmatic analyses of aminotransferases: aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Conclusions

Although the wolf-apple flour did not normalize the

Figure 2. Histological section of liver stained with hematoxylin-eosin, for the groups normal control (A), diabetic control (B) and treated diabetic - received 100 mg/day of wolf-apple flour (C), for six weeks. 40 X magnification.
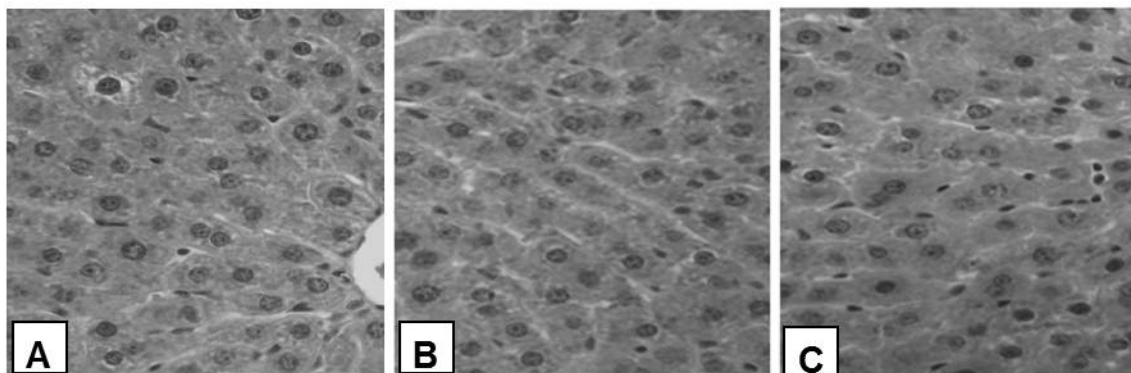
glycemic levels of the treated rats, there was a relevant reduction of 19.76% in glycemia. However, in the non-treated group, the glycemia practically did not change, and it could be inferred that the wolf-apple flour can aid in the glycemic control of diabetics.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effects of aqueous leaves extract of *Waltheria indica* Linn on reproductive indices of male albino rats

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Wide usage of *Waltheria indica* plant has been reported but its effects on the reproductive indices of male albino rats have not been evaluated. This study investigates the effects of *W. indica* leaf extract on the sperm counts, motility, live-dead ratio and sperm morphological abnormality of male albino rats. Thirty six (36) healthy white male albino rats were randomly selected and divided into six groups. Each of the treated groups was administered with different concentrations of aqueous leaf extract for 21 days after which the semen samples were taken for analysis of reproductive parameters. The testicles were also removed for histological study. There was significant decrease (p<0.01) in sperm motility and sperm counts at doses 800, 1600 and 2000 mg/kg. The live-dead ratio was also significantly decreased (p<0.01) at 2000 mg/kg dose. There was also significant increase in total abnormal cell for all the treated groups. The extract induced periportal cellular infiltration and interstitial congestion on the testes of the treated rats.

Key words: *Waltheria indica,* motility, sperm counts, live-dead ratio, abnormal cell, histology, rats.

INTRODUCTION

Waltheria indica L. also known as sleepy morning and many other names (Burkill, 2000), belongs to the family Sterculiaceae. It is widespread in West Africa (Akobundu and Agyakwa, 1998). Locally, the plant is called 'hankufah' in Hausa, 'korikodi'' in Yoruba and 'efu-abe in Nupe (Hutchinson and Dalziel, 1958; Irvine, 1961). The uses of the plant are diverse; the plant has been used as an infusion or decoction where febrifugal, purgative, emollient, tonic, analgesic and astringent action is sought (Burkill, 2000). It is used in Northern Nigeria by the Hausas for the treatment of skin diseases, impotence, and infertility, as an aphrodisiac, and as children's medicine at birth and during teething (Mohammed et al.,

2007). In the Fulani community, the aqueous extract of the root is used in relieving aches and pains during the 'Sharo' festival. Among the Yorubas, the aqueous extract of the root and stem is used in treating syphilis, internal haemorrhage, and as a restorative during the labours of harvesting (Mohammed et al., 2007).

The wide usage of *W. indica* in folk medicine has been corroborated by scientific evidences in recent time. The antioxidant effects (Saidu et al., 2012), antibacterial effects (Olajuyigbe et al., 2011), antimalarial (Clarkson et al., 2004) anti-inflammatory effects (Yerra et al., 2005) anticonvulsants effects (Hamidu et al., 2008) and trypanocidal effects (Bala et al., 2010) of *W. indica* have

been reported. It has also been reported that medicinal plants with antimicrobial effects have tendency to adversely affect male fertility (Olayemi, 2010). Many antimalarial drugs have been implicated in male infertility. For instance chloroquine, quinine and quinacrine have been reported to inhibit Leydig cell steroidogenesis and fertility in male (Sairam, 1978). Hence, this study was designed to investigate the effect of *W. indica* leaf extract on the reproductive indices of male albino rats.

MATERIALS AND METHODS

Experimental animals

Thirty six (36) healthy white male adult albino rats (100 to 190 g) obtained from the Animal House, Faculty of Veterinary Medicine, University of Ibadan, were used for the study. The rats were fed with rat cubes (Ladokun feeds limited, Ibadan, Nigeria) and water *ad libitum*. The rats were kept at the Experimental Animal House of the Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Ibadan, Ibadan. They were acclimated to their new environment for two weeks before the commencement of the experiment. All experimental protocols were in compliance with University of Ibadan Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

Plant material

The *W. indica* plants were obtained from a farm land at Moniya in Akinyele area Council of Ibadan, Oyo state, Nigeria and identified at the Herbarium, Department of Botany, University of Ibadan with voucher number UIH-22371.

Extract preparation

The leaves of the plant were separated from the whole plant and air dried at room temperature for two weeks. A total of 200 g of the ground powder was soaked in 1 L of distilled water for 24 h at room temperature. The mixture was filtered into conical flask with Whatman filter paper. The filtrate was concentrated *in vacuo* using a rotary evaporator at 40°C to produce a gel-like extract, which weighed 43 g (21.5% yield). Appropriate concentration of the extract was then subsequently made by dilution with distilled water into graded doses and administered to the rats.

Experimental design

Thirty six (36) male albino rats were randomly divided into six groups (n = 6), labelled A to F; where, group A served as the control while the animals in the groups B, C, D, E and F served as the treated group. The treated groups were then orally administered with 200, 400, 800, 1600 and 2000 mg/kg body weight of the extract, respectively for 21 days.

  After 21 days of extract administration, the rats were sacrificed and the testicles were surgically removed through a lower abdominal incision for histological study. Semen samples were collected from the epididymides for the following andrological analyses:

Motility

The percentage of sperm cells in a unidirectional progressive move-

ment over a field on a slide was observed, using a light microscope as described by Zemjanis (1970). Briefly, a small drop of semen was placed on a warmed slide mixed with one drop of warm sodium citrate covered with a glass slip. Sperm cells moving in a straightforward unidirectional motion were counted while sperm cells moving in circles or in backward direction were excluded.

Sperm count

Epididymal sperm count was obtained by mincing the cauda epididymis in distilled water and filtering through a nylon mesh. The spermatozoa were counted by haemocytometer using improved Neubauer chamber (Deep 1/10mm, LABART, Germany) described by Pant and Srivastava (2003).

Live - dead ratio/percentage liveability

One drop of semen was mixed with one drop of eosin-nigrosin stain on a warm slide. A thin smear was then made from the mixture of semen and stain. The smear was then air-dried and observed under the microscope. The live and the dead sperm cells were separately counted and the ratio of the live to dead sperm cells was calculated (Zemjanis, 1970).

Sperm morphological abnormalities

On a clean, warm glass slide, a drop of semen was placed as well as two drops of Wells and Awa stain as reported by Hammer (1970). The semen and stain were thoroughly mixed together with a smear made on another clean and warm slide. The smear was air-dried and observed using the light microscope starting with low power to high magnification. The presence of abnormal cells out of at least 400 sperm cells from several fields on the slide was counted and their total percentage was estimated.

Histology

All the animals from each of the treated groups B, C, D, E, F and the control were sacrificed 24 h after their respective daily doses. The rats were thereafter quickly dissected to remove the testes and then transferred into 10% buffered formalin. The organs were dehydrated in ethanol (70 to 100%), cleared in xylene and embedded in paraffin. Tissue sections (5 μm thickness) were examined under a light microscope after staining with haematoxylin and eosin (H and E) (Culling, 1963; Lillie, 1965).

Statistical analysis

The data obtained from the experiment were presented as mean ± standard error of mean (S.E.M) and analysed using the one-way analysis of variance (one-way ANOVA). The group means were separated by Duncan Multiple range Tests at 95% confidence interval using GraphPad Instat® software.

RESULTS

Effect of the aqueous extract of *Waltheria indica* on sperm parameters of rats

The result of the effect of graded doses of *W. indica* on sperm parameters of rats is presented in Table 1. There

Table 1. Effect of the aqueous extract of *Waltheria indica* on sperm parameters of rats (n=6).

Parameter	Control	B	C	D	E	F
Motility (%)	088±2.55	073±2.0[a]	074±5.10[a]	065±2.24[b]	052±3.74[b]	038±4.89 [b]
Live-dead ratio (%)	097.4±0.60	094.2±1.80	095.2±1.46	089.6±3.27	089.6±2.27	086.0±2.92[a]
Sperm count (×10^{6})	136.6±3.74	113.2±4.99[a]	110.0±5.05[b]	096.2±4.06[b]	082.6±7.63[b]	082.0±5.45[b]
Volume (ml)	005.18±0.02	005.14±0.02	005.18±0.02	005.14±0.02	005.18±0.02	005.16±0.02

Thirty six (36) male albino rats were randomly divided into six groups (n = 6), labelled A to F; where, group A served as the control while the animals in the groups B, C, D, E and F served as the treated group. The treated groups were then orally administered with 200, 400, 800, 1600 and 2000 mg/kg body weight of the extract, respectively for 21 days. Superscripted items indicate significant values compared to the control ([a] P< 0.05, [b] P<0.01).

Table 2. Effect of the aqueous extract of *Waltheria indica* on sperm morphological abnormality of male rats (n=6).

Parameter (%)	Control	B	C	D	E	F
Total abnormal cell	11.32±0.13	12.62±0.33[a]	13.62±0.14[b]	13.56±0.25[b]	13.54±0.40[b]	14.99±0.46[b]
Tailess head	1.11±0.14	1.16±0.14	1.14±0.13	1.08±0.15	1.O9±0.15	1.13±0.13
Headless tail	0.75±0.19	1.09±0.13	1.09±0.16	1.18±0.09	1.24±0.15	1.18±0.15
Rudimentary tail	0.40±0.09	0.61±0.10	0.65±0.06	0.64±0.06	0.50±0.08	0.44±0.09
Bent tail	2.08±0.09	2.17±0.13	2.73±0.14a	2.58±0.11	2.58±0.12	3.11±0.29[b]
Curved tail	1.93±0.08	2.34±0.06	2.68±0.08b	2.53±0.10[b]	2.53±0.14[b]	3.00±0.17[b]
Bent midpiece	2.05±0.05	2.29±0.17	2.38±0.133	2.63±0.18[a]	2.58±0.15	2.81±0.19[b]
Curved midpiece	2.18±0.14	2.74±0.07[a]	2.29±0.18	2.33±0.11	2.48±0.11	2.86±0.19[a]
Looped tail	0.49±0.08	0.49±0.08	0.65±0.06	0.55±0.09	0.49±0.11	0.39±0.10

Thirty six (36) male albino rats were randomly divided into six groups (n = 6), labelled A to F; where, group A served as the control while the animals in the groups B, C, D, E and F served as the treated group. The treated groups were then orally administered with 200, 400, 800, 1600 and 2000 mg/kg body weight of the extract, respectively for 21 days. Superscripted items indicate significant values ([a]P< 0.05, [b]P<0.01).

was significant decrease in sperm motility at doses 200 and 400 mg/kg (P < 0.05) and 800, 1600, and 2000 mg/kg (P < 0.01). The sperm count was also significantly decreased at 200 mg/kg dose (P < 0.05) and at doses 400, 800, 1600 and 2000 mg/kg doses (P < 0.01). The percentage of live-Dead sperm was significantly (P < 0.01) decreased at 2000 mg/kg b.w dose.

Effect of the aqueous extract of *Waltheria indica* on sperm morphological abnormality of rats

The result of the effect of *W. indica* on sperm morphological abnormality of rats is shown in Table 2. There was significant increase in total abnormal cell for all the treated groups (P< 0.01). The curved tailed abnormality values were also significantly increased (P< 0.01) at doses 400, 800, 1600 and 2000 mg/kg. The bent tail abnormality values were significantly increased at dose 400 (P< 0.05) and 2000 mg/kg (P< 0.01). The extract doses of 800 and 2000 mg/kg corresponding to groups D and F also caused significant increase in bent midpiece abnormality values. The abnormality values for the tailless head, headless tail, rudimentary tail and looped tail for all the treated groups were not significantly

different from the control group. The curved mid piece abnormality value at 200 mg/kg dose was significantly increased (P< 0.05).

Histological effects

The result of the histological changes induced by aqueous leave extract of *W. indica* is presented in Figures 1 to 3. No visible lesion was observed in the testis (Figure 1) of the control group. Periportal cellular infiltration by mononuclear cells was observed in the testis (Figure 2) of rats at 400 mg/kg. Also, interstitial congestion was observed in the testis (Figure 3) at 1600 mg dose.

DISCUSSION

The analysis of spermiogram of the albino rats in this study shows that the administration of aqueous extracts of *W. indica* significantly reduced sperm characteristics and functions (sperm counts, live-dead ratio, motility and morphology) of male albino rats. Although, the effect of the extract of *W. indica* on male reproductive system has
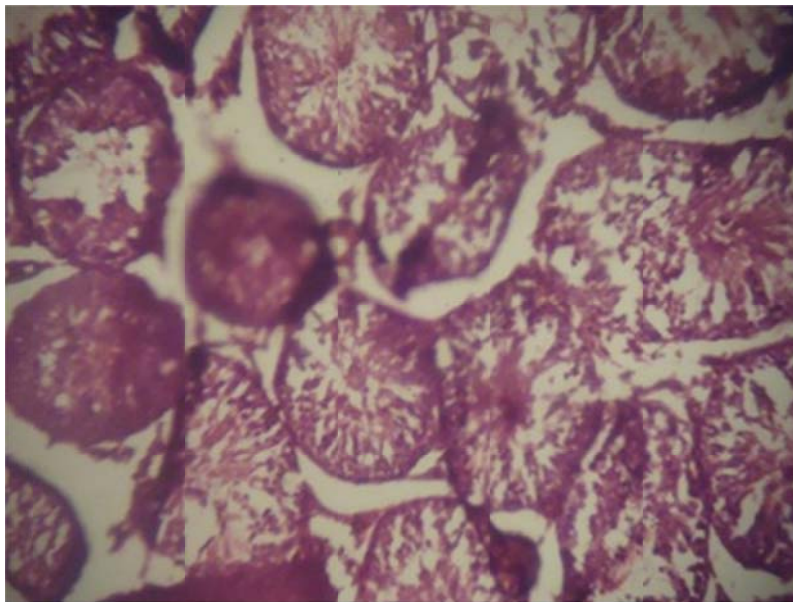
Figure 1. Testis of the control group with no visible lesion (M × 40 H&E).
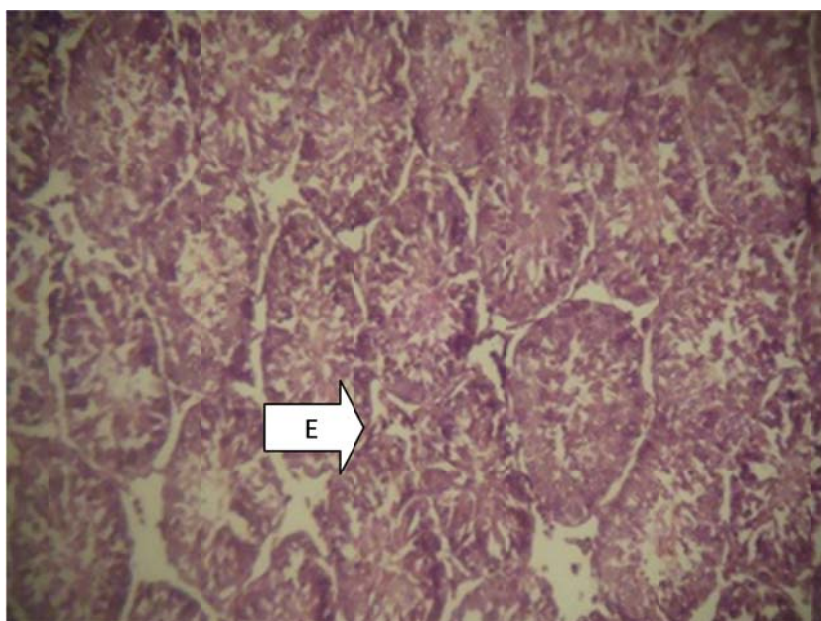
Figure 2. Testis of group C showing periportal cellular infiltration by mononuclear cells E (M × 40 H&E).

not been previously investigated, the result obtained in this study is in agreement with the findings of Raji et al. (2005) who also reported dose-dependent changes in sperm characteristics of rats treated with *Alstonia bonei.* Several other commonly used plants have been reported to adversely affect male reproductive functions in wildlife and humans. The observation with such plants has been attributed to their ability to impact adversely on sperma-

togenesis and steroidogenesis (D'cruz et al., 2010). Other plant extract reported to alter the morphology of sperm or to diminish its motility include *Abrus precatorious* (Adedapo et al., 2007), *Cola nitida rubra* (Adisa et al., 2010), *Croton zambesicus* (Ofusori et al., 2010), and *Kigelia africana* (Adeparusi et al., 2010). The reduction in sperm quality may be subject to the hispathological changes caused by the extract. The
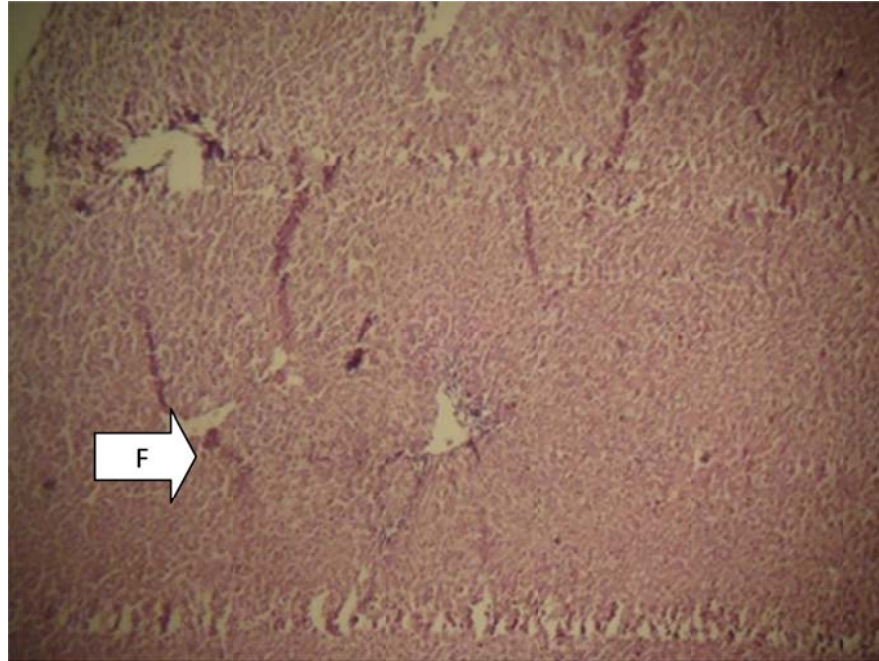
Figure 3. Testis of group E showing interstitial congestion (M × 40 H&E).

aetiology of this pathology has been traced to the presence of alkaloids (Badifu and Ogunsua, 1991). These alkaloids are bioactivated to released reactive metabolites which bind cell molecules and cross-link DNA to cause cellular damage (Cheeke, 1988). The total percentage morphological abnormalities observed increased with increasing dose. More so, the tail abnormalities account for most of these morphological abnormalities, hence significant reduction in sperm motility of all the treated groups. This suggests that treatment with *W. indica* leave extract adversely affect sperm motility. Moreover, sperm capacitation, the series of enzymatic reactions resulting in the release of acrosomal enzymes which allow for fertilization in the female reproductive tract will be adversely affected. The delay in the occurrence of capacitation had been reported to render spermatozoa nonfunctional (Nass et al., 1990).

There is also increase in mid piece abnormalities. The increase in bent mid-piece abnormality was significant at 1600 ($P<0.05$) and 2000 mg/kg ($P<0.01$) doses while the curved mid-piece abnormality was significant at 200 ($P<0.05$) and ($P<0.05$) 2000 mg/kg doses. Occurrence of high number of mid-piece spermatozoa abnormality has been traced to the period of storage in the epididymis (Oyeyemi and Babalola, 2006).

Mid-piece abnormalities had also been traced to the deficiency of zinc. Zinc and folate are involved in the synthesis of DNA and RNA. Although the exact patho-physiology of zinc deficiency leading to clinical symptoms of decreased spermatogenesis and impaired male fertility has not been known but it has been shown to cause

impaired male fertility in the form of reduced sperm motility, reduced percentage motility of sperm, morpho-logical abnormalities and reduced spermatogenesis (Wong et al., 2000).

The study concludes that excessive use of aqueous extract of *W. indica* leaf has adverse effect on repro-ductive parameters of male albino rats; therefore caution should be applied to the use of *W. indica* leaves despite its numerous medicinal values.

Conflict of Interest

The author(s) declared that there is no conflict of interest as regards this paper.

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Full Length Research Paper

Pectinolytic complex production by *Aspergillus niger* URM 4645 using yellow passion fruit peels in solid state fermentation

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The activities of endo-polygalacturonase (endo-PG), exo-polygalacturonase (exo-PG), pectin lyase (PL), and pectin methylesterase (PE), produced by *Aspergillus niger* URM 4645, were studied in solid state fermentation (SSF) using yellow passion fruit peels as substrate. The effect of substrate amount, initial moisture content, and temperature on pectinase production was studied using a full factorial design (2³). Maximum endo-PG, exo-PG, PL, and PE activities were 31.35, 7.98, 551,299.39, and 447.93 U g^{-1} dry substrate, respectively. Optimum activities of the four enzymes were obtained with 5.0 g of the substrate and an initial moisture content of 30% at 34°C with 96 h of fermentation. Optimum endo-PG activity was found at pH 7.5 at an optimum temperature of 40°C; exo-PG and PL at pH 7.0 at an optimum temperature of 80°C; and PE at pH 3.5 at an optimum temperature of 30°C. Endo-PG was stable at pH 7.0 to 8.0 at 40°C, and exo-PG and PL at pH 6.0 to 8.0 and 6.0 to 7.5, respectively at 60 to 70°C. PE was stable at pH 3.5 to 5.0 at 30 to 60°C. The enzyme production optimization clearly demonstrated the impact of process parameters on the yield of pectinolytic enzymes.

Key words: *Aspergillus niger*, residue, pectinolytic activities, solid state fermentation, characterization.

INTRODUCTION

Passiflora edulis f*. flavicarpa*, commonly known as the yellow passion fruit, is cultivated on a large scale in Brazil and is of agronomic importance because of the use of its fruits in nature and in the juice industry. The waste

resulting from passion fruit processing consists of more than 75% of the raw material. The rind constitutes 90% of the waste product and is a source of pectin (20% of dry weight) (Arvanitoyannis and Varzakas, 2008).

Pectinases or pectinolytic enzymes are naturally produced by plants, filamentous fungi, bacteria, and yeasts (Maciel et al., 2013). Conversion of pectin to soluble sugars is possible through enzymatic reactions catalyzed by pectinolytic enzymes that are common in fungi, such as pectin lyase (PL) (EC 4.2.1.10), pectin methylesterase (PE) (EC 3.2.1.11), and polygalacturonases (PG) (EC 3.2.1.15) (Holck et al., 2011). Pectinases are of great importance for clarification and viscosity reduction in fruit juices, which result in increased filtration efficiency. They improve juice extraction, reduce clarification time, and enhance terpene content when employed in wine production (Uenojo and Pastore, 2007).

Filamentous fungi are the most frequently used microorganisms in the enzyme industry because as much as 90% of the enzymes produced by these fungi are extracellular (Blandino et al., 2001; Souza et al., 2003; Sandri et al., 2013). Pectinases can often be produced at high concentrations by strains of filamentous fungi belonging to *Aspergillus* genus. Recently, certain *Aspergillus* species have been characterized by the types of pectinolytic enzymes they produce (Maciel et al., 2011; Fontana et al., 2012; Heerd et al., 2012; Demir and Tari, 2014). PG, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multigene families and may have both endo (Parenicová et al., 2000) and exo activities (Sakamoto et al., 2002).

The microbial production of pectinolytic enzymes can be achieved by solid state fermentation (SSF) or submerged fermentation (SMF) (Maciel et al., 2013). Agroindustrial residues or wastes are generally considered suitable substrates for enzyme production by SSF (Pandey, 2003; Patil and Dayanand, 2006). The application of agro-industrial wastes as a carbon source in enzyme production processes reduces the cost of production and helps in solving problems associated with their disposal (Rodriguez-Leon et al., 2008). SSF systems have generated much interest in recent years because they offer several economic benefits (Fang et al., 2010).

The goal of this study was to evaluate the production of the pectinolytic complex comprising PG (endo- and exo-PG), PL, and PE by *A. niger* URM 4645 in SSF using yellow passion fruit peels as substrate and to partially characterize the enzymes.

MATERIALS AND METHODS

Microorganisms

Twenty-five (25) isolates of *A. niger* obtained from the Micoteca URM culture collection (URM, Recife, Brazil) (Micoteca URM, 2013) were inoculated on malt extract agar (MEA: malt 20 g L^{-1}, glucose 20 g L^{-1}, peptone 1 g L^{-1}, and agar 20 g L^{-1}) and maintained at 28°C.

Screening of pectinolytic fungi

A. niger isolates were subjected to screening in solid medium for selecting isolates with higher potential for pectin degradation. For selection, Petri dishes (diameter, 6 cm) containing 7 mL of sterilized MEA medium were inoculated with each isolate and incubated for seven days at 30°C. Then, one plug of 6 mm was cut using a sterile cork borer from the colony periphery, transferred to the center of a culture medium containing citric pectin (Sigma), and incubated for seven days at 30°C (Uenojo and Pastore, 2006). The pectinolytic index was determined and expressed by the ratio of the diameter of the degradation halo and the diameter of the colony growth. The plates were flooded with 0.1% (w/v) Congo red solution for approximately 45 min. The plates were then washed with water. Pectin degradation was evidenced by a clear zone around the fungal growth.

Origin of yellow passion fruit peels

The yellow passion fruit peels used in this study were supplied by the Food Supply Center of Pernambuco, located in Recife, Pernambuco, Brazil.

Inoculum standardization

A 6-cm-diameter Petri dish containing 7 mL of sterilized MEA medium was inoculated with the selected isolate and incubated for seven days at 30°C. Then, five plugs of 6 mm were cut using the sterile cork borer from the colony periphery, and spores from these plugs were suspended in 30 mL of Tween 80/water (0.02%). The spore concentration was adjusted to 10^7 spores per gram and inoculated in the substrate used for SSF. The initial moisture content of the substrate was determined in accordance with the standards of Instituto Adolfo Lutz (Zenebon and Pascuet 2005).

Pectinase production by SSF

For pectinase production, the peels were submerged for 1 h in 2% (w/v) sodium hypochlorite solution and then washed in water. The peels were then crushed and incubated at 65°C until they were completely dehydrated. For fermentation, peels with a particle size between 3 and 8 mm were used as substrate in order to provide improved absorption and porosity to facilitate transport of oxygen as well as nutrients during SSF. The substrate was then placed in 250-mL Erlenmeyer flasks and irradiated with ultraviolet light for 2 h in a microbiological cabinet (Spier et al., 2008).

Experimental design and statistical analysis

The influence of substrate amount (Sa), initial moisture content (Im), and temperature (T) on pectinase production was evaluated from the results of the experiments performed according to a 2^3 factorial design (Bruns et al., 2006), plus four central points (Table 1). All statistical analyses were performed using Statistic 8.0 software (Statsoft, 2008).

Extraction of enzymes

Pectinase production was carried out for 96 h. The contents of the flasks were harvested at regular intervals (24 h). A mass of 5.0 g of the fermented mixture was mixed with 30 mL of 0.2 M acetic acid-sodium acetate buffer, pH 5.5 (1 g of the substrate per 2.5 mL of buffer) for 15 min. After maceration, extraction was performed using

Table 1. Variable levels of the 2^3 experimental design for the production of pectinases (Ug^{-1}) in SSF by *Aspergillus niger* URM4645 using yellow passion peels as substrate.

Variable	Level		
	Low (-1)	Central (0)	High (+1)
Sa (g)	5.0	10.0	15.0
Im (%)	30	50	40
T (ºC)	26	34	30

Sa, substrate amount; Im, initial moisture content; T, temperature.

a filter paper (Whatman no. 1) under vacuum. The extract was clarified by filtration and centrifugation at 5000 × *g* for 15 min (Spier et al., 2008). The supernatant was used as a crude enzyme extract and was subjected to enzymatic analysis.

Enzymatic assays

Endo-PG activity

Endo-PG activity was measured viscosimetrically (Tuttobello and Mill, 1961) using a reaction mixture containing 5.5 mL of 0.2% citric pectin in 0.025 M acetate buffer, pH 5.0, 1.0 mM ethylene diamine tetra acetic acid (EDTA), and 250 µL of the enzymatic extract. This mixture was incubated at 50°C for 10 min and then cooled in an ice bath. A viscosimetric unit (U) was defined as the amount of enzyme required to decrease the initial viscosity per min by 50% under the conditions described previously. Enzyme activity was expressed in units per gram (U g^{-1}).

Exo-PG activity

Exo-PG activity was determined by measuring the release of reducing groups from citric pectin using the 3,5-dinitrosalicylic acid (DNS) reagent assay (Miller, 1959). The reaction mixture containing 0.5 mL of 0.5% citric pectin in 0.025 M acetate buffer, pH 5.0 and 0.5 mL of the enzyme extract was incubated at 50°C for 10 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 mmol of galacturonic acid per minute.

PL activity

PL activity was determined by measuring the increase in absorbance at 235 nm of the substrate solution (1.0 mL of 0.5% citric pectin in 0.2 M Tris-HCl buffer, pH 5.5) hydrolyzed by 1.0 mL of the enzyme extract at 40°C for 60 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 mmol of unsaturated uronide per minute, based on the molar extinction coefficient (ε_{235} = 5550 M^{-1} cm^{-1}) of the unsaturated products (Albershein, 1966). Enzyme activity was expressed in U g^{-1}.

PE activity

PE activity was determined by the pH decrease of the medium and by titration of carboxylic groups using a modified methodology (Siéssere et al., 1992). The reaction mixture contained 2.0 mL of 1% citric pectin in 0.025 M Tris-acetate buffer, pH 6.5 and 1.0 mL of the enzyme extract. The enzymatic reaction was carried out at 50°C

for 2 h and then quenched in a boiling water bath for 3 min. Then, the samples were cooled in an ice bath and the liberated carboxylic groups were titrated with a solution of NaOH (0.01 M), using three drops of the phenolphthalein with pH indicator whose turning point was detected by appearance of a pink color. The volume of NaOH solution spent in each titration was noted. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 microequivalent of a carboxylic group in 60 min of the reaction under the described conditions. The activity was expressed in U g^{-1}.

Enzyme characterization

Endo-PG, exo-PG, PE, and PL activities of the crude enzyme extract were measured at different pH and temperature values.

Optimum pH and temperature for enzyme activity

The effect of pH on pectinolytic activity was measured using the following buffers: sodium acetate buffer (pH 3.5-5.0), citrate-phosphate buffer (pH 5.0-7.0), Tris-HCl (pH 7.0-8.5), and glycine-NaOH buffer (pH 8.5-11.0). The optimum temperature in the range of 30-80°C was determined by incubating the reaction mixture at the optimum pH.

pH and temperature stability

The crude enzyme extract was diluted (1:1) in different buffers (pH 3.5-11.0, 0.2 M for endo-PG and PL and 0.025 M for exo-PG, for buffers as the cited above) and incubated at 25°C for 24 h. After incubation, endo-PG, exo-PG, PE, and PL activities were measured for the optimum pH and temperature values. The crude enzyme extract was incubated at temperatures ranging from 30°C to 80°C for 60 min to determine temperature stability. Subsequently, endo-PG, exo-PG, PE, and PL activities were determined.

RESULTS AND DISCUSSION

Screening for pectinase production

Fungi are important producers of enzymes, relatively easy to grow in controlled environments, and highly sensitive to genetic alterations, enabling enhanced strains to be obtained in terms of production and quality of enzymes (Santos, 2007). Screening is often the first step in selecting microorganisms with characteristics

Table 2. Pectinolytic Index for 25 isolates of *Aspergillus niger*.

Culture/Access number[a] URM	Øc	Øh	PI[b]
URM 2228	34	-	-
URM 2604	37	-	-
URM 3701	33	17	0.52
URM 3753	52	10	0.19
URM 3806	45	14	0.31
URM 3811	62	14	0.23
URM 3820	52	11	0.21
URM 3856	67	06	0.09
URM 3885	50	07	0.14
URM 4452	40	10	0.25
URM 4645	35	18	0.51
URM 5001	57	06	0.11
URM 5117	47	08	0.17
URM 5149	55	08	0.15
URM 5162	68	08	0.12
URM 5207	64	10	0.16
URM 5437	61	11	0.18
URM 5438	60	10	0.17
URM 5439	56	07	0.13
URM 5555	64	11	0.17
URM 5756	47	11	0.23
URM 5837	54	11	0.20
URM 5838	60	10	0.17
URM 5842	48	12	0.25
URM 5853	52	12	0.23

− = no halo. [a]Access number in the Micoteca URM culture collections (Recife, Pernambuco); [b](PI) pectinolytic index = ratio of the (Øh) ring diameter (mm) to the (Øc) colony diameter (mm).

intended for industrial applications. This allows for the characterization and selection of fungal strains with optimum enzyme production. In addition, the information obtained adds value to these microbial resources preserved in culture collections (Maciel et al., 2013).

Among the 25 *A. niger* isolates tested in the screening on solid medium, 23 (92%) showed potential pectinolytic enzyme production. Of these 23 isolates, 7 had a pectin degradation halo of less than 10 mm in diameter and 16 had halos that varied from 10 mm to 18 mm in diameter (Table 2). Of these 16 isolates, 11 were from plant-derived substrates and soil. Fungi isolated from soil are considered to exhibit high activity in biodegradation and biodeterioration processes through enzyme production, contributing to nutrient cycling and consequently to the maintenance of ecosystems (Allsopp and Seal, 1986). This may explain the reason for *A. niger* URM 4645 presenting a pectinolytic index of 0.51 (Table 2). Bezerra et al. (2012) reported that among 24 endophytic fungal isolates of *Opuntia ficus-indica* (forage cactus), only *A. japonicus* and *Penicillium glandicola* showed pectinolytic activity.

Production of pectinolytic enzymes

A. niger URM 4645 was the isolate selected for evaluating enzyme production at different substrate amounts, initial moisture contents, and temperatures by SSF using yellow passion fruit peels. High activities were obtained using this substrate, demonstrating that this agro-industrial residue is a good substrate for pectinase production by *A. niger* URM 4645. This substrate contains considerable amounts of valuable substances such as sugars, oils, fibers, polyphenols, and pectin (Arvanitoyannis and Varzakas, 2008), which contribute to pectinase production by filamentous fungi. Mrudulab and Anitharaj (2001) tested rice bran, wheat bran, sugarcane bagasse, orange peels, lemon peels, and banana peel for pectinase production. In their study, orange peel was the substrate that allowed highest pectinase production by *A. niger*. Moreover, Maller et al. (2011) concluded that agro-industrial residues, such as orange and lemon peels, induced production of high levels of PG by *A. niveus*. The use of orange peels can be highly economical at the industrial scale for PG production (Maciel et al., 2013).

Table 3. Results of the 2^3 design for endo-PG, exo-PG, PL, and PE production in SSF by *Aspergillus niger* URM 4645.

Run	Sa	Im	T	Endo-PG$_{96}$ (Ug^{-1})	Exo-PG$_{72}$ (Ug^{-1})	PL$_{96}$ (Ug^{-1})	PE$_{72}$ (Ug^{-1})
01	5.0	30	26	29.02	6.14	520,867.96	320.48
02	15.0	30	26	15.02	**7.98**	373,563.73	348.47
03	5.0	50	26	13.26	5.89	271,052.60	245.07
04	15.0	50	26	17.37	3.67	213,617.93	245.87
05	5.0	30	34	**31.25**	5.67	**551,299.39**	320.90
06	15.0	30	34	5.39	7.93	304,155.37	**447.93**
07	5.0	50	34	17.12	5.09	359,785.98	243.36
08	15.0	50	34	18.64	5.08	262,886.63	275.66
09(C)	10.0	40	30	16.87	5.26	284,650.96	301.29
10(C)	10.0	40	30	21.24	5.56	317,871.66	302.39
11(C)	10.0	40	30	22.22	6.20	315,238.53	300.22
12(C)	10.0	40	30	22.14	5.06	299,374.14	340.70

For endo-PG, exo-PG, PL, and PE, the subscript gives the cultivation time in hours. Sa, substrate amount; Im, initial moisture content; T, temperature.

A. niger URM 4645, when cultured using yellow passion fruit peels without the addition of nutrient solution, produced endo-PG, exo-PG, PL, and PE. Maximum endo-PG activity observed using 5.0 g of the substrate with an initial moisture content of 30% at 34°C was 31.35 U g^{-1} at 96 h of fermentation; maximum exo-PG activity observed using 15.0 g of the substrate with an initial moisture content of 30% at 26°C was 7.98 U g^{-1} at 72 h of fermentation; maximum PL activity observed using 5.0 g of the substrate with an initial moisture content of 30% at 26°C was 551, 299.39 U g^{-1} at 96 h of fermentation; and maximum PE activity observed using 15.0 g of the substrate with an initial moisture content of 30% at 34°C was 447.93 U g^{-1} at 72 h of fermentation (Table 3).

Endo-PG activity obtained using yellow passion fruit peels was lower than that obtained by Fontana et al. (2005) and Maciel et al. (2011). Endo-PG activity obtained by Fontana et al. (2005) was 152 U g^{-1} using wheat bran and citrus pectin as substrates with 72 h of SSF. Using forage palm as substrates, Maciel et al. (2011) obtained endo-PG activity of 66.19 U g^{-1} after 96 h of SSF at 28°C.

Using the mixture of wheat bran and orange bagasse (1:1) as substrate, Silva et al. (2007) obtained exo-PG activity of 16.0 U g^{-1} with *P. viridicatum* RFC3 in SSF for 14 days. Patil and Dayanand (2006) obtained exo-PG activity of 17.1 U g^{-1} with *A. niger* after 96 h of SSF using sunflower head as substrate. Exo-PG activity obtained by these authors is higher than those obtained in the present study; however, in this study, exo-PG activity was approximately 8 U g^{-1} after 24 h of fermentation. Maciel et al. (2011) also obtained exo-PG activity of 3.59 U g^{-1} with *A. niger* URM 4645 after 24 h of SSF at 36°C using forage palm as substrate. Higher enzyme production during the first hours of fermentation allows a reduction in the cost of production compared to higher production

toward the end of the fermentation process (Maciel et al., 2013).

Martin et al. (2004) tested a culture medium containing orange peel, sugar cane bagasse, and wheat bran (1:1:1) as substrate and detected PL activities of 19.40 and 11.0 U g^{-1} at 144 and 96 h of SSF for *Moniliella* sp. and *Penicillium* sp., respectively. Using orange peels as substrate, Silva et al. (2002) observed PL production by *P. viridicatum*. The results show PL activity of 2.0 U g^{-1}, and when wheat bran was added to orange peels the PL activity rose to 3.54 U g^{-1}, showing the influence of medium composition on enzyme production. Maciel et al. (2011) detected PL activity of 40,615.62 U g^{-1} by *A. niger* URM 4645 at 72 h of SSF using only forage palm as substrate. Joshi et al. (2006) tested apple pulp for PE production and detected a maximum activity of 5.5 U g^{-1} by *A. niger* after 25 h of fermentation. These PL and PE activities were lower than those obtained in the present study, which found more satisfactory results without the addition of a carbon source or nutrient solution to increase pectinase production.

Comparing enzyme production by different microorganisms can be difficult because growing conditions and the methods of determination of enzyme activities are different. The present results show that SSF was suitable for pectinase production by *A. niger* URM 4645 using yellow passion fruit peels as substrate, facilitating and minimizing costs for the industry.

Results show that endo-PG, exo-PG, PL, and PE activities were obtained at different conditions and times of fermentation. Therefore, it was necessary to choose a common condition for pectinase production because the food industry uses enzyme complex in the preparation of the product. The substrate amount of 5.0 g and initial moisture content of 30% at 34°C with 96 h of fermentation was chosen using statistical analysis and is a common

Table 4. Effects calculated from the responses of the 2^3 design for endo-PG, exo-PG, PL, and PE production with 96 h of SSF by *Aspergillus niger* URM 4645.

Variable/Interaction	Endo-PG$_{96}$	Exo-PG$_{96}$	PL$_{96}$	PE$_{96}$
(1)	-4.94[a]	-0.46	-9.31[a]	1.03
(2)	-2.00	-3.96[a]	-14.72[a]	-4.50[a]
(3)	-0.32	1.16	2.27	0.46
1x2	6.34[a]	-1.36	5.50[a]	-0.99
1x3	-2.02	0.89	-3.19	0.10
2x3	1.75	0.93	4.06[a]	0.64
1x2x3	1.29	0.28	1.38	0.27

[a]Statistically significant values (at the 95% confidence level, $p < 0.05$). (1) Substrate amount, (2) Initial moisture content and (3) Temperature.

condition for the production of endo-PG, exo-PG, PL, and PE. This condition contributes to pectinase production on a large scale, facilitating and minimizing costs and production times for the industry.

The results of the statistical analysis, including the effects of each variable studied in the experimental design in order to produce endo-PG, exo-PG, PL, and PE at 96 h of SSF, are shown in Table 4. The initial moisture content and temperature were not significant variables at a 95% confidence level for endo-PG activity. Substrate amount and temperature were not significant variables at a 95% confidence level for exo-PG and PE activities. Temperature was not a significant variable at a 95% confidence level for PL activity (Table 4). The variable substrate amount had a negative effect on endo-PG and PL activities, indicating that smaller substrate amounts increase the enzyme activities. However, our results were different from those obtained by Maciel et al., (2011). These authors studied pectinase production by *A. niger* URM 4645, and highest enzyme activities were obtained in that study when a higher substrate amount was used (10.0 g).

The initial moisture content also showed a negative effect during 96 h of fermentation for exo-PG, PL, and PE activities, which indicated that an increase in these enzyme activities was obtained by reducing the initial moisture content (Table 4). This means that pectinase production was enhanced when the fermentation conditions were as follows: 5.0 g of substrate and 30% initial moisture content. This could be explained by the fact that under such fermentation conditions, the fungus showed good growth and was capable of producing pectinases to hydrolyze the pectin in the carbon sources. Substrate amount and temperature play an important role in influencing the process parameters and thus the yield of enzymes in SSF (Ustok et al., 2007). However, in the present study, the variable temperature was not shown to play an important role in pectinase production.

Some interaction effects were significant (Table 4), demonstrating a dependent relationship between them. For endo-PG and PL activities, the substrate amount and initial moisture content (1 × 2) interacted positively, demonstrating that an increase in the values of these variables results in an increase in these activities. For PL activity, the initial moisture content and temperature (2 × 3) interacted positively, such that an increase in the values of these variables resulted in an increase in the activity. The cultivation of microbial cells in an excess of water can lead to sticking of particles, limited gas exchange, and higher vulnerability to bacterial contamination, whereas that in low levels of water is correlated with reduced microbial growth, reduced enzyme stability, substrate swelling, and diffusion of nutrients (Silva et al., 2007).

Enzyme characterization

For the characterization of endo-PG, exo-PG, PL and PE produced by *A. niger* URM 4645, the crude enzyme extract obtained under the following conditions was used: 5.0 g of the substrate with an initial moisture content of 30% at 34°C (Figure 1 and Table 3, Run 05).
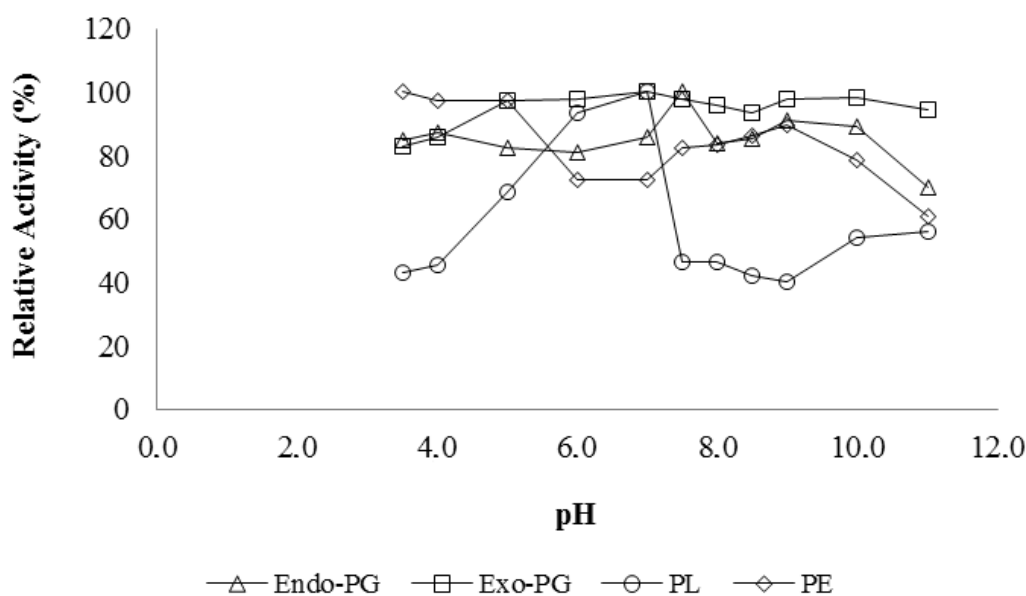
The effect of pH on endo-PG, exo-PG, PL, and PE activities is shown in Figure 2. Optimum endo- and exo-PG activities were observed at pH 7.5 and 7.0, respectively. Endo- and exo-PG showed a second peak of activity at pH 9.0 and 10.0 with 91 and 98.5% of maximum activity, respectively. The optimum pH for endo- and exo-PG was higher than that for most fungal PG already described, which show higher activities at an acidic pH (Favela-Torres et al., 2006). Maciel et al. (2011) obtained the maximum endo- and exo-PG activities of *A. niger* URM 4645 at pH 5.0 and 7.0, respectively. The maximum activity of exo-PG from *P. viridicatum* RFC3 was observed at pH 6.0 (Silva et al., 2007), *Moniliella* sp. SB9 at pH 4.5, and *Penicillium* sp. EGC5 at pH 4.5-5.0 (Martin et al., 2004). Freitas et al. (2005) obtained the maximum exo-PG activity of *Monascus* sp. and *Aspergillus* sp. at pH 5.5, and Phutela et al. (2005) obtained the maximum PG activity of *A. fumigatus* at pH 5.0.

Optimum PL activity was obtained at pH 7.0. PL showed a second peak of activity at pH 6.0 with 93% of
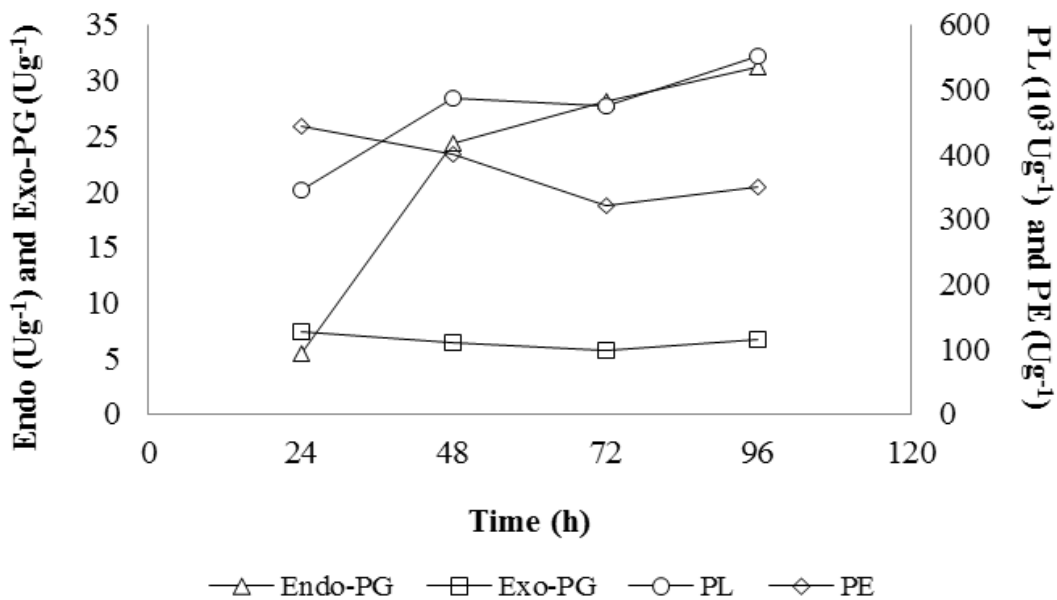
Figure 1. Endopolygalacturonase (Δ- endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊ - PE) activities under the optimum conditions used to produce these four enzymes (5.0 g substrate and 30% moisture at 24°C with 96 h of solid state fermentation (SSF) - Run 05).
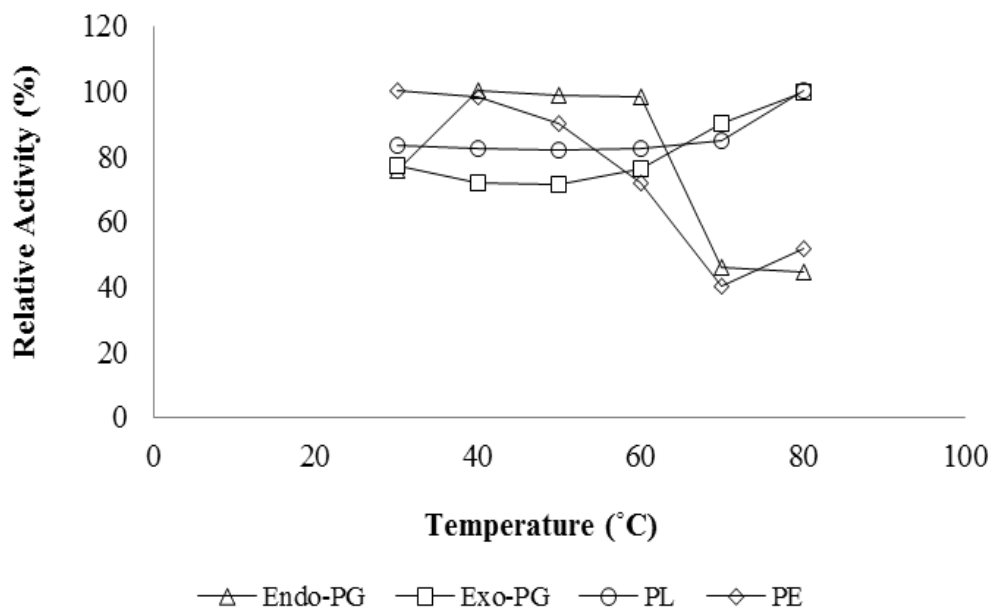

Figure 2. Effect of pH on the activities of endopolygalacturonase (Δ - endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊ - PE) produced by *Aspergillus niger* URM 4645.

maximum activity. Optimum pH reported for PL was acidic (5.0) for *A. niger* (Maciel et al., 2011), neutral (7.0) for *P. expansum* (Silva et al., 1993), and basic (8.0) for *A. flavus* (Yadav et al., 2008) and *A. terricola* (Yadav et al., 2009). Piccoli-Valle et al. (2001) obtained the maximum

PL activity of *P. griseoroseum* at a pH close to neutral (pH 5.0-7.0).

Optimum PE activity was obtained at pH 3.5, but PE showed a second peak of activity with 97% of maximum activity at pH 4.0 and 5.0. Dinu et al. (2007) obtained the

Figure 3. Effect of temperature on the activities of endopolygalacturonase (Δ - endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊- PE) produced by *Aspergillus niger* URM 4645.

maximum PE activity of *A. niger* MIUG 16 at pH 4.2-4.4. Optimum PE activity of *A. niger* and *P. griseoroseum* was observed at pH 4.0 and pH 4.5 to 5.0, respectively (Joshi et al., 2006; Piccoli-Valle et al., 2001). Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi show reasonably good growth over a broad range of pH (2.0 to 9.0), with an optimum range of pH 3.8 to 6.0 (Gowthaman et al., 2001).

Endo-PG was stable at pH 7.0 to 8.0, exo-PG at pH 6.0 to 8.0, PL at pH 6.0-7.5, and PE at pH 3.5-5.0. After incubation for 24 h at pH 3.5-11.0, more than 40% of the pectinase activity was maintained. The results are in agreement with those obtained by Silva et al. (2002) with PG from *P. viridicatum* RFC3 that was stable at pH 5.0 to 8.0, retaining 80% of its activity at pH 9.0. Furthermore, Silva et al. (2007) reported the stability of exo-PG from *P. viridicatum* RFC3 at pH 7.0 to 10.0. Maciel et al. (2011) observed that endo-PG and exo-PG from *A. niger* URM 4645 were stable at pH 3.5 to 11.0, and more than 40 and 70% of the endo- and exo-PG activities were maintained, respectively. Freitas et al. (2005) found that exo-PG from *Monascus* sp. was stable at pH 4.5-6.0, while that from *Aspergillus* sp. was stable at pH 4.0.

In the present study, PL was stable at acidic to neutral pH (4.0-7.0). However, Yadav et al. (2008, 2009) reported the stability of PL from *A. flavus* and *A. terricola* at pH 4.0-10.0 and 4.0-9.0, respectively. PE from *A. japonicus* was stable at pH 3.5 to 5.5 (Semenova et al., 2003) and that from *Aureobasidium pullulans* was stable at pH 4.0 to 6.5 (Manachini et al., 1988).

Regarding the optimum temperature, maximum endo-PG and PE activities were obtained at 40 and 30°C, respectively. The maximum for exo-PG and PL activities were obtained at 80°C (Figure 3). Phutela et al. (2005), studying pectinases from *A. fumigatus* TF3 and Yadav et al. (2009) working with *A. terricola* MTCC 7588, obtained maximum PL activity at 50°C. Silva et al., (2002) observed that maximum PG and PL activities were obtained at 55 and 50°C, respectively. *Monascus* sp. and *Aspergillus* sp. exhibited maximum exo-PG activity at 60 and 50°C, respectively (Freitas et al., 2005). Dinu et al. (2007) and Silva et al. (2007) showed an optimum activity of PG produced by *A. niger* MIUG 16 at 40°C and that of exo-PG produced by *P. viridicatum* RFC3 at 60°C.

Regarding temperature stability, endo-PG was stable at 40°C after 60 min of incubation, but the activity decreased with an increase in temperature, and 64% of its activity was maintained after incubation at 40°C. Exo-PG and PL were stable at 60 to 80°C, maintaining 99 and 128% of their activities after 60 min at 80°C, respectively. PE was stable at 30 to 60°C, but the activity decreased with an increase in temperature. Maciel et al. (2011) showed that endo-PG and PL were stable at 50 and 80°C, respectively, and maintained 60% of their original activities. Silva et al. (2002) showed that at 40°C for 60 min, PG and PL activities were maintained at 100 and 80% of their original activities, and at 50°C, they were maintained at 55 and 60% of their original activities, respectively. Yadav et al. (2008) showed that 98% of stability was maintained at 50°C for PL produced by *A. flavus*, and the stability decreased at temperatures above

50°C. Exo-PG produced by *Monascus* sp. and *Aspergillus* sp. was stable at temperatures up to 50°C (Fontana et al., 2005). PL and PE produced by *A. japonicus* showed stability between 40 and 50°C (Semenova et al., 2003). The most important factor among all the physical variables affecting the production of enzymes and metabolites is probably incubation temperature because enzymatic activities are sensitive to temperature (Krishna, 2005). The high stability at a certain temperature suggested that the pectinases (endo-PG, exo-PG, PL and PE) are sufficiently acceptable for commercial application.

Conclusions

The results presented demonstrate the feasibility to produce pectinases using *A. niger* URM 4645 and yellow passion fruit peels as substrate in SSF. Using 5.0 g of substrate and an initial moisture content of 30% at 34°C with 96 h of incubation was determined to be the best condition for the pectinases production at the same time. Variable substrate amounts and initial moisture contents showed significant effects on pectinase production. Optimum endo-PG activity was obtained at pH 7.5 at an optimum temperature of 40°C. Optimum exo-PG and PL activities were obtained at pH 7.0 at an optimum temperature of 80°C. Optimum PE activity was obtained at pH 3.5 at an optimum temperature of 30°C. Endo-PG was stable at pH 7.0 to 8.0 at 40°C; exo-PG and PL at pH 6.0-8.0 and 6.0-7.5, respectively, and temperature 60 to 80°C; and PE at pH 3.5 to 5.0 and temperature 30-60°C. Enzyme production optimization clearly demonstrated the impact of process parameters on the yield of pectinolytic enzymes for use in commercial applications.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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