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Full Length Research Paper

Genetic diversity and demographic evolution of baobab (*Adansonia digitata* L., *Bombacoideae, Malvaceae*) populations in Senegalese Sahelian areas

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This study evaluated the spatial genetic structure of baobab (*Adansonia digitata* L.) populations from three agroecological sites located in sahelian zone of Senegal using *ITS1*, *5.8S rDNA* and *ITS2* gene sequences. To determine the extent of isolation, gene sequences were analyzed between and among three sahelian baobab populations. At least 25 haplotypes of baobab (*A. digitata* L.) were revealed in Senegal (6, 9 and 10, respectively in Dakar, Bandia and Widou Thiengoly). Private haplotypes found in each locality show that there is an adaptation of the plant to environmental conditions prevailing in each site. Indeed, nucleotide diversity was more important in Dakar (0.00527); it ranges from 0.00483 to 0.00060 for Bandia and Widou populations, respectively. Curves of mismatch distribution show that the population of Ferlo has undergone a recent demographic expansion. Although Bandia and Dakar populations were polyphyletic; each shows a balanced expansion. Fst values ranging from 0.62946 to 0.90712 correlates a strong genetic differentiation between sites. A correlation between geographic and genetic distances was not highlighted by the Mantel's test but phylogenetic trees of maximum likelihood and Bayesian inference have assigned two clades demonstrating that population of Ferlo (Widou) form a different ecotype from those of Bandia and Dakar.

Key words: *ITS1, 5.8S, ITS2*, *Adansonia digitata,* haplotype, genetic diversity, demographic evolution.

INTRODUCTION

It is important to understand the pattern of variation existing in populations of economically important trees, for use in domestication, conservation, management and tree breeding. Such a distribution should result in formation of distinct geographical races (Zobel and Talbert, 1984) that are adapted to various ecological conditions.

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Abbreviations: AFLP, Amplified fragment length polymorphism; **AIC,** Akaike information criterion; Hd**,** haplotype diversity; **ITS,** internal transcribed spacer; **Pi,** Nucleotide diversity.

This demand is to keep an appropriate level of genetic diversity to guarantee short-term viability and long-term evolutionary potential. In order to manage germplasm resources effectively in fruit tree domestication, one requires knowledge of the amount and distribution of genetic diversity present in natural populations (Mwase et al., 2006).

A. digitata L., the African baobab, is a stem-succulent tree native to the dry regions of tropical Africa (Wickens and Lowe, 2008). *A. digitata* is the oldest known tropical angiosperm species with reliable carbon dating results (Pâtrut, et al., 2007) and the best known of the eight species of *Adansonia*. The genus belongs to *Bombacoideae,* a subfamily of *Malvaceae* (Baum et al., 2004). The species is an autotetraploid species issued from a reduced aneuploid chromosomic type such as 4x = 176 (Baum and Oginuma, 1994). A phylogeographical analysis using polymerase chain reaction-restriction fragment length polymorphism (PCR-RLFP) of DNA chloroplast fragments designed to identify its centre of origin, after many decades of controversy, revealed that *A. digitata* probably originated from West Africa and migrated subsequently throughout the tropical parts of that continent and beyond, by natural and human-mediated terrestrial and overseas dispersal (Pock Tsy et al., 2009). This recent study on chloroplast DNA has shown that there are genetic differences between baobab populations from western and south-eastern Africa. In total, more than 300 uses have been reported for this species, with the most important ones being related to food, medicine and income generation for rural communities (Buchmann et al., 2010; Sarr et al., 2013). According to Sidibe and Williams (2002), anthropogenic pressures on the baobab in its natural habitat justify the absence of natural regeneration throughout its distribution area in Senegal.

Previously, molecular studies have been done to assess genetic diversity in baobabs (Assogbadjo et al., 2009; Kyndt et al., 2009; Pock Tsy et al., 2009; Larsen et al., 2009). Assogbadjo et al. (2009) showed that there was genetic structuring and low to high genetic diversity between baobab populations in different climatic regions of Benin (West Africa). Kyndt et al. (2009) found high levels of genetic structuring present in baobabs at a regional scale (Benin, Ghana, Burkina Faso and Senegal) and within populations, which was unexpected considering its dispersal by bats and human exchanges of seeds. However, Assogbadjo et al. (2009) using amplified fragment length polymorphism (AFLP) markers could not distinguish traditionally classified baobab morphotypes. Pock Tsy et al. (2009) established that the tetraploid *A. digitata*, or its diploid progenitor originated in West Africa and migrated subsequently throughout the continent, and beyond, through natural and human–mediated terrestrial and overseas dispersal. Larsen et al. (2009) developed and tested 18 microsatellite primers (SSR92 primers) for tetraploid *A. digitata* and its relatives showing different alleles per locus and different allele sizes. Most of the published results on baobab are from West Africa. However, there is limited published data from molecular studies in southern Africa. In spite of the paucity of genetic diversity information on baobab, domestication of some priority indigenous fruit species has been advanced in southern Africa (Akinnifesi et al. 2008). According to Larsen et al. (2009), it is pertinent to carry out gene flow studies in baobabs to provide insight into dispersal processes that shape the genetic structure. In addition, they indicated that estimates of seed dispersal and differentiation between populations are vital for monitoring impacts from human influence and for forecasting consequences of climate change.

Over time, baobab demography has been influenced substantially by anthropogenic factors (land-use patterns, trampling and browsing by domesticated livestock, clearing during cultivation), climate (prolonged drought), elephant damage (Wilson, 1988; Edkins et al., 2008), and fire (Chirwa et al., 2006) which have had adverse impact on genetic diversity. It is known that positive correlation exists among the levels of genetic diversity and fitness in plants (A´vila-di´az and Oyama, 2007). For baobab domestic-cation to succeed, it therefore requires understanding of the species' genetic diversity, since it is the fabric of evolution, the base material on which adaptation depends. High levels of genetic diversity confer a greater ability to respond to threats such as diseases, parasites, predators and environmental change (Amos and Harwood, 1998).

The only study on the population genetics of baobab, performed by a research group in Benin (Assogbadjo et al., 2006), indicated some degree of physical isolation of the populations collected in the three climatic zones of Benin, and inferred some impact of the environment and geographic distance on the level of genetic structuring among the analyzed populations. However, the study area was restricted in size. This study aimed at studying the levels of spatial structuring of baobab at different geographic scales. Specifically, we conducted a population genetic study of 11 baobab populations from four West African countries where the species is abundant and widely distributed in parkland agroforestry systems (Benin, Ghana, Burkina Faso, and Senegal). The goal of the research was to build and enhance a database for species conservation and domestication in the West African region (Kyndt et al., 2009).

Prior studies of chloroplast DNA markers (*psbA-trnH*, *trnL-trnF*) and the nuclear internal transcribed spacer (ITS) (*5.8S rRNA, ITS-1 and ITS-2*), combined or not with morphological traits, have been used to assess the genetic diversity and phylogenetic relationships within *Adansonia*. These data identified three lineages: one containing the Malagasy species, one containing the Australian species, and one containing the African species. A recent phylogenetic analysis (Pettigrew et al., 2012) demonstrated that *Adansonia kilima sp.* Nov. is a

Figure 1. Map of Senegal showing the geographical location of the studied baobab (*Adansonia digitata* L.) populations.

new diploid species from Africa, which co-exists with *A. digitata* in Africa. *A. digitata* and *A. kilima* were found to be genetically similar, suggesting that tetraploidy evolved relatively recently.

This current study was undertaken to assess genetic diversity and differentiation in subpopulations of baobab sampled from three sahelian zones in Senegal in order to choose genetically divergent individuals for *in vitro* cloning and other conservation measures. The specific objectives were to examine the genetic diversity within and among populations, the degree of genetic differentiation among populations and the mode of demographic expansion of different populations in these localities.

MATERIALS AND METHODS

Study site

In Senegal, phytogeographic regions are determined by rainfall. Basically, parallel isohyetes allow one to distinguish three regions from north to south: The sahelian (rainfall: 500 to 700 mm), the soudanian (rainfall: 700 to 900 mm) and the guinnean (rainfall: up to 1000 mm) regions. The three sites sampled are within the sahelian zone where the rainy season last from July to September (Figure 1). The choice of the three study sites was motivated by their contrasting floristic and ecological features although all in the Sahelian zone. Classified forest of Bandia (Latitude 14° 34' 60'' N, Longitude 16° 58' 0" E), 65 km away from Dakar, is located in the region of Thies and is therefore a protected natural site. It also includes the first private reserve of Senegal (1500 ha) and a natural stand of dense and ancient baobabs (Naegele, 1967). Dakar site is located on the peninsula of Cape Verde (Latitude 14° 40' 20 " N, Longitude 17° 25' 54" W), which is the most western point of the Sedimentary Basin of Senegal, as a spur bordered by the Atlantic Ocean. The formerly lush vegetation of this basaltic and rocky promontory, so contrasting with the arid hinterland, explains its name. Dakar is the capital city of Senegal and is increasingly urbanized. Natural wood forest stands have almost disappeared and individuals of emblematic baobabs are scattered throughout the city. Widou Thiengoly (Latitude 15° 20' N, Longitude 15° 26' W) is located in the Ferlo region, which is an agro-sylvopastoral and experimental site. It has been the subject of several reforestation programs and exclosure plots (19400 ha) subjected to controlled management (1975-1981). Ecological monitoring has been ongoing since 1981 (Hiernaux, 2006). Widou Thiengoly is also with the study area of OHM.i Tessekéré (Observatoire Homme Milieux. International), underpinned by an integrative socio-ecological systems in the Sahel. This area is typical of the African Sahel and is a bioclimatic transition zone between the Sahara area to the north and the savannas to the south. It is marked by ecological and human crises due to consecutive droughts (rainfall deficit, anthropic pressure on the environment, and changes in major ecological balances). This area is included in the Pan-African development and reforestation program, called the “Great Green Wall representing a fight against drought combined with promotion of rural development.

Sampling and records of vegetation

A. digitata leaves were collected, after the rainy season, from individuals in three populations located in Bandia forest (Thiès region), Dakar (Dakar region) and Widou Thiengoly (Ferlo, Louga region) sites, respectively. As recognized by Kyndt et al. (2009), a baobab population was defined as a group of baobab trees randomly and naturally distributed in a traditional agroforestry system within a 30 km maximum radius. Two different populations are isolated from each other by a distance of at least 50 km. For each population located in each different site, 15 to 20 individuals were sampled (Bandia: 15; Dakar: 15; Widou Thiengoly: 20) and encoded using the first letter of the locality. The 3 populations of baobab represented 50 individuals in total.

DNA extraction

For each sample, 25 mg of fresh leaves were ground with a 750 µl

of extraction buffer MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) preheated to 65°C as suggested by Gawal and Jarret (1991). The mixture was homogenized by vortexing and the tubes were incubated in a water bath at 65°C for 20 min, with a stir every 5 min to promote cell membrane degradation and the release of the DNA. The samples were then cooled at room temperature for 5 min. Cellular debris and proteins were eliminated by adding 750 µl of chloroform:isoamyl alcohol (CIAA solution; 24:1). The samples were centrifuged for 20 min at 13,000 rpm at 20°C. An aliquot of 600 µl of supernatant was collected for each sample, transferred to a new, sterile Eppendorf tube and an equal volume of isopropanol, cooled to -20°C, was added to precipitate nucleic acids. Microtubes containing DNA pellets were cooled at -20°C for 2 h and centrifuged again as previously. A series of centrifugation and washing of DNA was conducted using 70% ethanol. In order to degrade RNA, 6 µl RNase was added and the mixture was incubated at 37°C for 1 h. The extracted DNA was quantified and stored at -20°C.

Nuclear DNA amplification and sequencing

The nuclear ribosomal DNA region including *5.8S rDNA* and the internal transcribed spacers *ITS-1* and *ITS-2* were amplified using the primers designed by Sun et al. (1994). They were composed respectively, of AB101 (5' ACG AAT TCA TGG TCC CGT GAA GTG TTC G 3') and AB102 (5' TAG AAT TCC CCG GTT CGC TCG CCG TTA C 3'). The amplification was performed in a reaction volume of 25 µl containing 18.3 µl of water, 2.5 µl buffer (10x), 1 µl of additional $MgCl_2$, 0.5 additional of dNTPs, 0.25 µl of each primer, 0.2 µl of Taq polymerase and 2 µl of template DNA. Amplification conditions were done as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and elongation of the complementary DNA strand at 72°C for 1 min. A final extension at 72°C for 10 min completed the PCR. The visualization of the DNA fragments was done by gel electrophoresis in 1.5% agarose with a 0.5 x TAE buffer and stained with ethidium bromide. The size of the fragments was determined using a molecular weight marker (MW) composed of known size of several DNA fragments. For each individual, two PCR amplifications of 25 µl were carried out for sequencing. DNA sequencing step was performed by Macrogen (South Korea).

Genetic data analysis

The sequences were aligned by BioEdit software and TCS1.21 software (Clement et al., 2000) was used for the determination of haplotypes. This software also helped construct the network of haplotypes for estimating the plausibility of links between haplotypes in the network, using a 95% threshold. Nucleotide diversity (Pi) and haplotype diversity (Hd) were calculated by the DnaSp 5.10.01 software (Rozas et al., 2010). Tajima's D and Fu's F statistics (Tajima, 1989; Fu, 1997) were used to test for deviations from neutrality. Correlation between geographic distance and genetic was assessed with a Mantel Test (Mantel, 1967), implemented in XLSTAT software (Addinsoft, 2012). In all these analyzes, deletions were considered as a fifth state character (Felsenstein, 1985; Sanderson, 1989).

The phylogenetic tree was estimated using maximum likelihood in MEGA 5 software (Tamura et al., 2011). The Akaike information criterion (AIC) was used to estimate the best model of evolution. The GTR model was applied for reconstruction. The robustness of the nodes was assessed for 1000 bootstrap repetitions. The Bayesian approach was implemented by the Mrbayes 3.1.2 software (Huelsenbeck and Ronquist, 2001). The distribution of posterior probabilities in the tree reconstruction using the Bayesian approach was estimated by MC3 in which four chains were used simultaneously (three of which were "heated" gradually). One million (1,000,000) generations were performed for each chain by sampling parameters every 1000 generations. The convergence degree of the chains can be verified by examining the evolution of the likelihood function during the course of the "cold" chain to determine the burn-in period. Generations made during that period are removed from the analysis and the estimates become subsequent. Conservatively, the first 250,000 generations were discarded (25% of trees constructed) and inferences are then made on 750,000 generations, which corresponds to 75% of the trees constructed.

RESULTS

Polymorphism and genetic diversity

The results of the polymorphism analysis and genetic diversity are shown in Table 1. Genes targeted in this study were not amplified in F17 and F19 individuals. Of 715 bp of aligned sequences obtained from the 48 remaining accessions, 668 were conserved sites, 42 sites were variables, 13 sites were singletons and 28 were parsimony informative.

The number of variable sites was highest in the population of Dakar (17), followed by Bandia (14) and finally the Ferlo site in Widou Thiengoly (3). The number of parsimony informative sites is however higher at Bandia (12) than Dakar (11), whereas Ferlo has only one parsimony informative site (Table 2). Despite the relatively low nucleotide diversities in Dakar (0.00527 ± 0.00210) and Bandia (0.00483 ± 0.00210), haplotype diversities were high: 0.648 ± 0.134 and0.638 ± 0.129, respectively. By contrast, Ferlo, had low nucleotide and haplotype diversities (0.00060 ± 0.00024 and 0.363 ± 0.131, respectively).

Distribution and haplotypes network

On the set of sequences, 25 haplotypes were found. The haplotype found most frequently was H16, which was found in nine individuals, mainly from the Ferlo site (Widou Thiengoly). The haplotypes H1, H9 and H10 are common to both Dakar and Bandia. By constrast, Bandia has four private haplotypes (H3, H4, H5, and H6) and Dakar has six (H8, H11, H12, H13, H14 and H15). The site of Widou Thiengoly (Ferlo) presents only private haplotypes, meaning that no haplotypes were shared between Ferlo and the other two sites (Tables 3 and 4).

In the network (Figure 2), each ellipse represents a haplotype, and their size is proportional to the number of individuals corresponding to the haplotype. The lines between haplotypes represent mutational steps. The haplotype network shows three groups. Each group has a main haplotype and derived haplotypes. The first group consists of 13 individuals from Bandia and two individuals from Dakar for a total of 15 individuals. This group has a main haplotype (eight individuals) and five derived haplotypes. Dakar accessions prevail in the second

Table 1. Polymorphism of nuclear DNA (*ITS1*, *5.8S* and *ITS2*) of baobab populations in Senegal.

Parameter	Numbers of site
Total number of sequences	48
Conserved Sites (C)	668
Variable Sites (V)	42
Singleton Sites (S)	13
Informative Sites on parsimony (ISP)	28

Table 2. Polymorphism and genetic diversity in populations.

Parameter	Bandia	Dakar	Widou Thiengoly (Ferlo)
Number of Sequences (N)	15	15	18
Conserved sites (C)	692	689	650
Sites variables (V)	14	17	3
Singleton Sites (S)	2	6	2
Parsimony informative sites (ISP)	12	11	1
Nucleotide Diversity	0.00483 ± 0.00210	0.00527 ± 0.00210	0.00060 ± 0.00024
Haplotype Diversity	0.638 ± 0.129	0.648 ± 0.134	0.363 ± 0.131

Table 3. Distribution of individuals in the identified haplotypes

Haplotype (H)	Number of Individual	Individual	Haplotype (H)	Number of Individual	Individual
H1	8	B2, B5, B8, B9, B10, B13, D9, D15	H14	1	D11
H2	3	B7, B11, B14	H15	1	D5
H3	1	B6	H16	9	F1, F2, F8, F9, F11, F15, F16, F18, F20
H4	1	B15	H17	1	F14
H5	1	B3	H18	1	F3
H6	1	B1	H19	1	F6
H7	5	D1, D3, D4, D7, D12	H20	1	F7
H8	1	D8	H21	1	F10
H9	2	B4, D2	H22	3	F5, F13, F19
H10	2	B12, D10	H23	1	F4
H11	1	D6	H24	1	F17
H12	1	D14	H25	1	F12
H13	1	D13			

B, Bandia; D, Dakar; F, Widou Thiengoly (Ferlo).

Table 4. Haplotypes found in each site.

Site	Haplotypes	Individuals
Bandia	H1, H2, H3, H4, H5, H6, H9, H10	B2, B2, B5, B8, B9, B10, B13, B7 B11, B14, B6, B15, B3, B1, B4, B12
Dakar	H1, H7, H8, H9, H10, H11, H12, H13, H14, H15	D9, D15; D1, D3, D4, D7, D12; D8; D2; D10, D6, D14, D13, D11; D5
Widou Thiengoly (Ferlo)	H16, H17, H18, H19, H20, H21, H22, H23, H24, H25	F1, F2, F8, F9, F11, F15, F16, F18, F20, F14, F3, F6, F7, F10, F5, F13, F19, F4, F17, F12

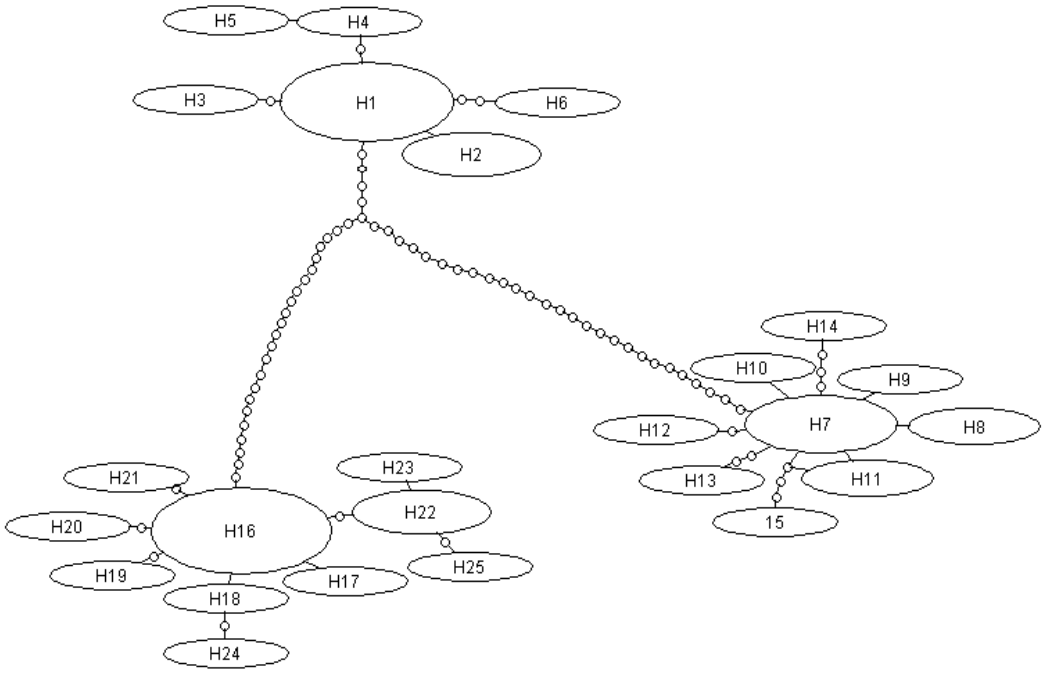

Figure 2. Relationships between haplotypes of baobab populations (*A. digitata* L.) from the three sites.

Table 5. Neutrality Indices of baobab populations (*A. digitata* L.).

Index	Bandia	Dakar	Widou Thiengoly
Tajima's D	- 0.98282	- 1.31710	- 1.44071
Fu ‘s Fs	1.506	0.704	- 2.135

group (nine haplotypes), with 15 individuals from this population and just two from Bandia. This second group presents a main haplotype (five individuals) and eight derived haplotypes. The third group consists of individuals exclusively originated from the Ferlo (Widou Thiengoly), with a main haplotype of nine individuals and nine derived haplotypes. The first group is separated from the second one by forty (40) mutational steps and from the third group by sixty seven (67) mutational steps. Between the second and the third group, there are ninety one (91) steps of mutation (Figure 2).

Demography of populations

Tajima's D and Fu’s Fs are negative in the Ferlo (Widou Thiengoly). Tajima's D is also negative in the Bandia site and at Dakar unlike Fu’s Fs which is positive in these two localities (Table 5). However, these values are not significant with p-values > 0.10, indicating that neutral evolution cannot be rejected. Mismatch distribution curves for the three populations taken altogether are multimodal (Figure 3a). Considering each site sampled, it appears that only the population of Ferlo presents a unimodal pattern, suggesting a recent expansion. Populations of Bandia and Dakar have multimodal curves revealing that they are in demographic equilibrium (Figure 3b, c and d).

Differentiation and genetic distances

All Fst values between the three populations (Dakar, Bandia and Ferlo) of baobab are high, with probability values highly significant (p less than 0.01). The Fst ranged from 0.62946 (between Dakar and Bandia) to 0.90712 (between Dakar and Ferlo (Widou Thiengoly) showing a partial isolation between these two populations (Table 6). Ferlo population is closed to the exchange of genes, contrary to what is observed between Dakar and Bandia where admixture is noted. The intra-population genetic distances (Kimura 2 parameter Model; Kimura, 1980) are low and vary from 0.001 to 0.006 (Table 7). Between populations, distances vary between 0.015 and

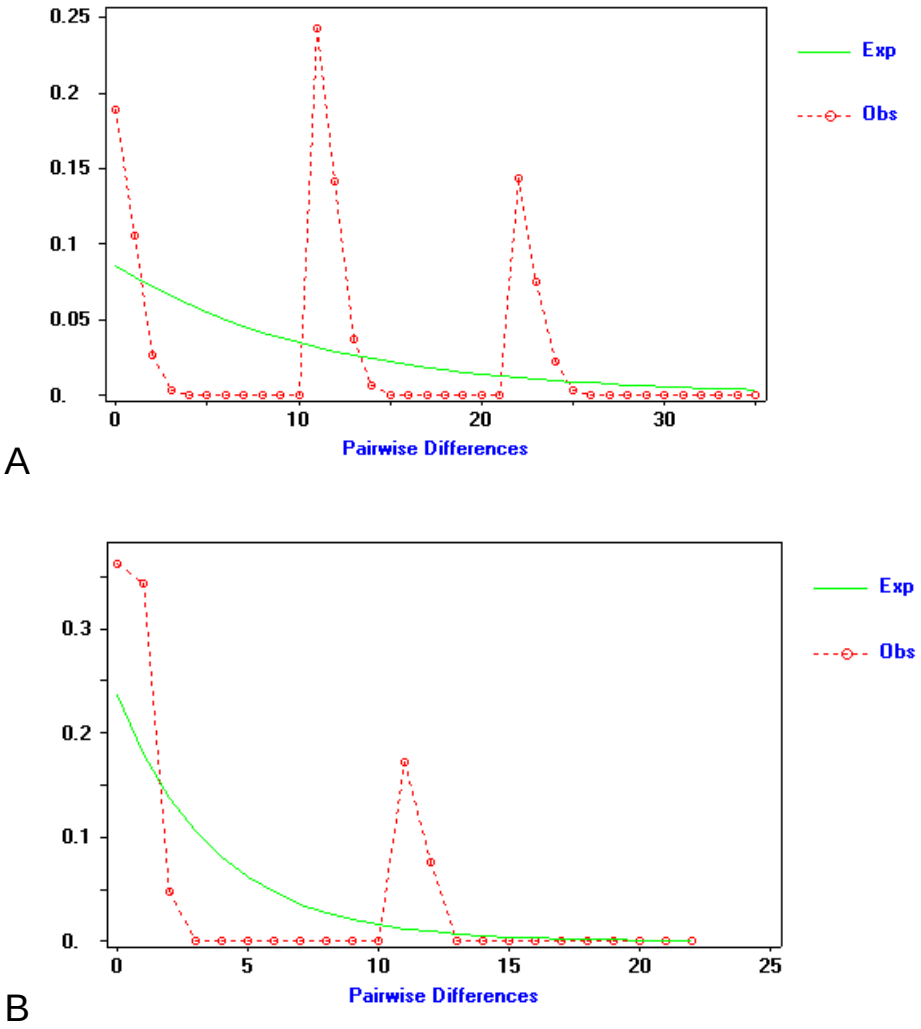

Figure 3. Distribution of the number of differences between haplotypes taken in pairs (mismatch distribution). **A,** All populations; **B,** population of Bandia; **C,** population of Dakar; **D,** population of Ferlo (Widou Thiengoly).

0.035. The highest is found between Dakar and Ferlo (0.035) and the lowest between Dakar and Bandia (0.021). The Mantel's test (Figure 4) revealed no correlation between the matrices of genetic differentiation, Fst and geographic distances (p = 0.500).

Phylogenetic trees

Phylogenetic relationships established with the Bayesian approach revealed the existence of two clades. One clade contained only individuals of the Ferlo (Widou Thiengoly) and is a monophyletic group and the second included those of Dakar and Bandia and is polyphyletic. These two clades were supported by high values of posterior probabilities (Figure 5). The subclades of these clades are not strong because posterior probability values are very low. In the second group, there is no clustering according to the geographic origin of individuals. The same groupings were obtained with the phylogenetic tree by the method of maximum likelihood. Clades are also supported by high values of bootstrap (100%) (Figure 6). This demonstrates that the groups are very strong confirming the very high values of posterior probabilities by the Bayesian approach, and consistent with the TCS analysis.

DISCUSSION

Studies in genetic diversity within a species are of paramount importance for understanding how a species will respond to environmental changes. Current patterns of genetic diversity can provide important clues to the

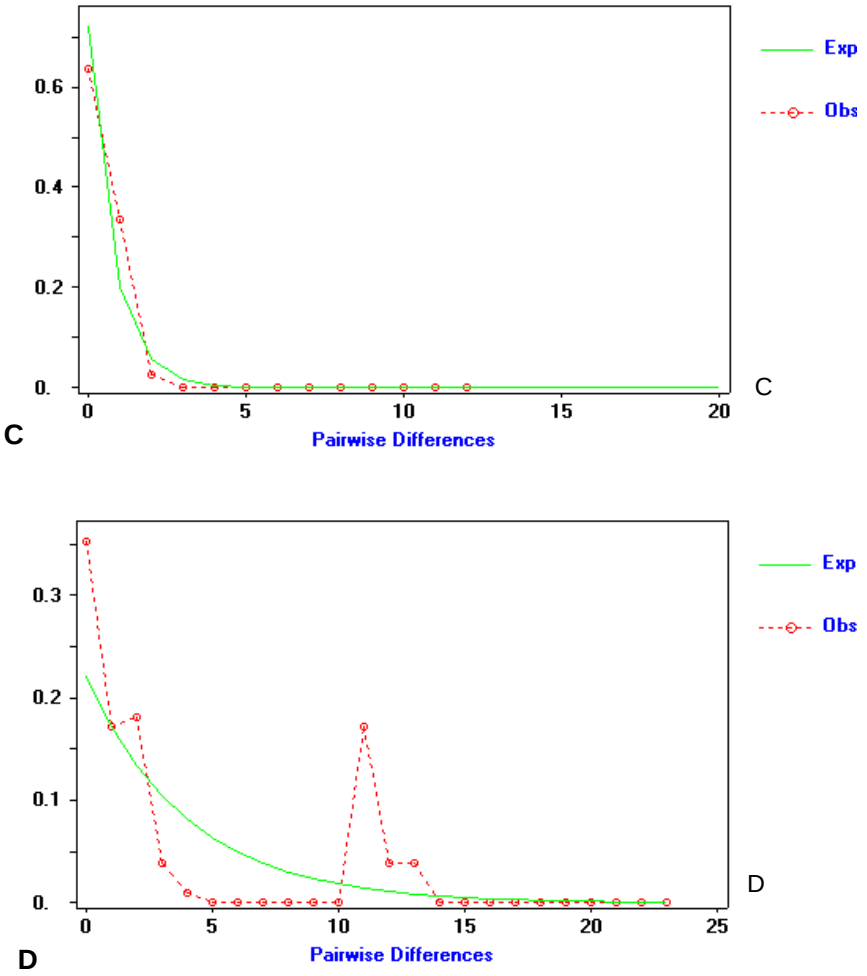

Figure 3. Contd.

history of the species and its current population structure (Heywood and Watson, 1995). In addition, knowledge about population genetics is fundamental for comprehending micro-environmental processes in plant populations that should be utilized in designing management, breeding and conservation strategies (Kyndt et al., 2009). Spatial genetic structuring in tree species is influenced by many biological forces such as gene flow through seed and pollen dispersal, tree density, fragmentation, colonization history, differential mortality, and micro-environmental selection (Kyndt et al., 2009). Genetic variation is the starting point for breeding programs and offers insurances against genetic erosion. Wild trees are genetically structured through natural processes such as mutation, genetic drift, selection, reproductive isolation, and migration (Buiteveld et al., 2007).

Our study reveals that Baobab (*A. digitata* L.) populations are quite diverse in Senegal, mainly in the sahelian zone. Indeed, 25 haplotypes were identified in three locations for 50 individuals sampled. This number of haplotypes is higher than that found by Pock Tsy et al. (2009) who identified five haplotypes in West Africa with a geographical distribution clearly structured. These authors had used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, which is less discriminative than sequencing, and chloroplast DNA as the genetic marker. Each site has a very large number of private haplotypes including the site of Ferlo (Widou Thiengoly), which contains only one haplotype cluster. The haplotype network shows three clusters separated by a large number of mutational steps. Some ellipses include individuals from Dakar and Bandia, which could be explained by the geographical proximity of these two sites that can promote the exchange of seed dispersal (most likely by humans) or pollen flow. More specifically, in agroforestry systems, all these factors may be influenced by human activity leading to many changes in ecosystem processes with various impacts (Allaye Kelly et al., 2004; Sanou et al., 2005). The results obtained by Assogbadjo et al. (2008) indicate a certain degree of physical isolation of popula-

Table 6. Genetic differentiation between populations of baobab (*A. digitata* L.) in Senegal.

Population	Bandia	Dakar	Widou
Bandia	-	-	-
Dakar	0.62946	-	-
Widou	0.85976	0.90712	-

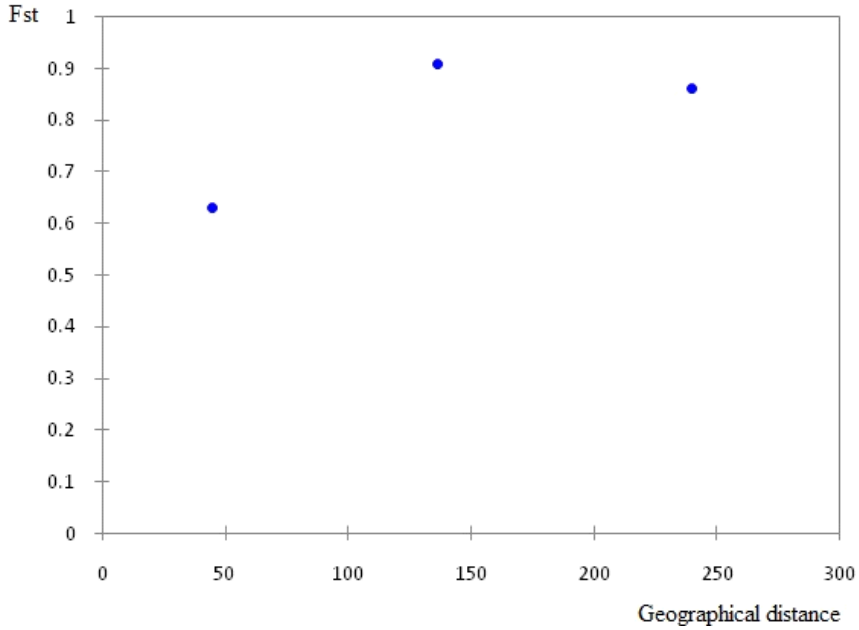

Figure 4. Correlation between geographical distance and genetic differentiation.

tions collected in different climatic zones. Indeed, the Ferlo region is in the forest grazing areas. This region is also the upper limit of baobab expansion in Senegal and has vulnerabilities and ecological characteristics different from those of Dakar, which in turn are also different from those of Bandia. Indeed, the existence of private and individual haplotypes and strong differentia- tion between Widou Thiengoly site and other sites can also be explained by the fact that the stands of baobab (*A. digitata* L.) in this area are considered as vestiges of wetter climatic periods, which cannot regenerate in the current rainfall conditions. Tests of exclosure plots in this area show that the dryer climate that high grazing pressure causes in a major factor preventing regeneration of this species (Miehe, 2002). Private haplotypes found in each locality could, thus, result from an adaptation of baobabs depending on the climate or agro-ecological zone. According to Kyndt et al. (2009), the distribution of seeds and tree improvement should recognize the presence of ecotypes and conservation measures should protect all populations due to the existence of alleles that are important for local adaptation. According to Buiteveld et al. (2007), forest ecosystem will only persist if genetic diversity of forest trees is dynamically maintained in view of environmental changes. The long-term viability of tree species within agroforestry systems depends upon a wide genetic base providing the capacity to adapt to environmental fluctuations or changing farmer requirements, such as changes in species use or planting niche (Lengkeek et al., 2006). Most forest species have evolved into distinct races (ecotypes, provenances), which should be recognized in tree breeding programs, as well as seed distribution for forest planting. Furthermore, it is known that although individuals within a race are similar from past heritage or selection pressures, they may also not necessarily be genetically identical (Zobel and Talbert, 1984).

Values of Tajima's D and Fu's Fs are negative in the Ferlo (Widou Thiengoly) but are not significant. According to Excoffier et al. (2005), a negative Tajima's D could correspond to a demographic expansion. Negative and non-significant values in the Ferlo site suggest a moderate demographic expansion. This last hypothesis is confirmed by the mismatch distribution curves that are unimodal in this locality. This contrasts those of Bandia

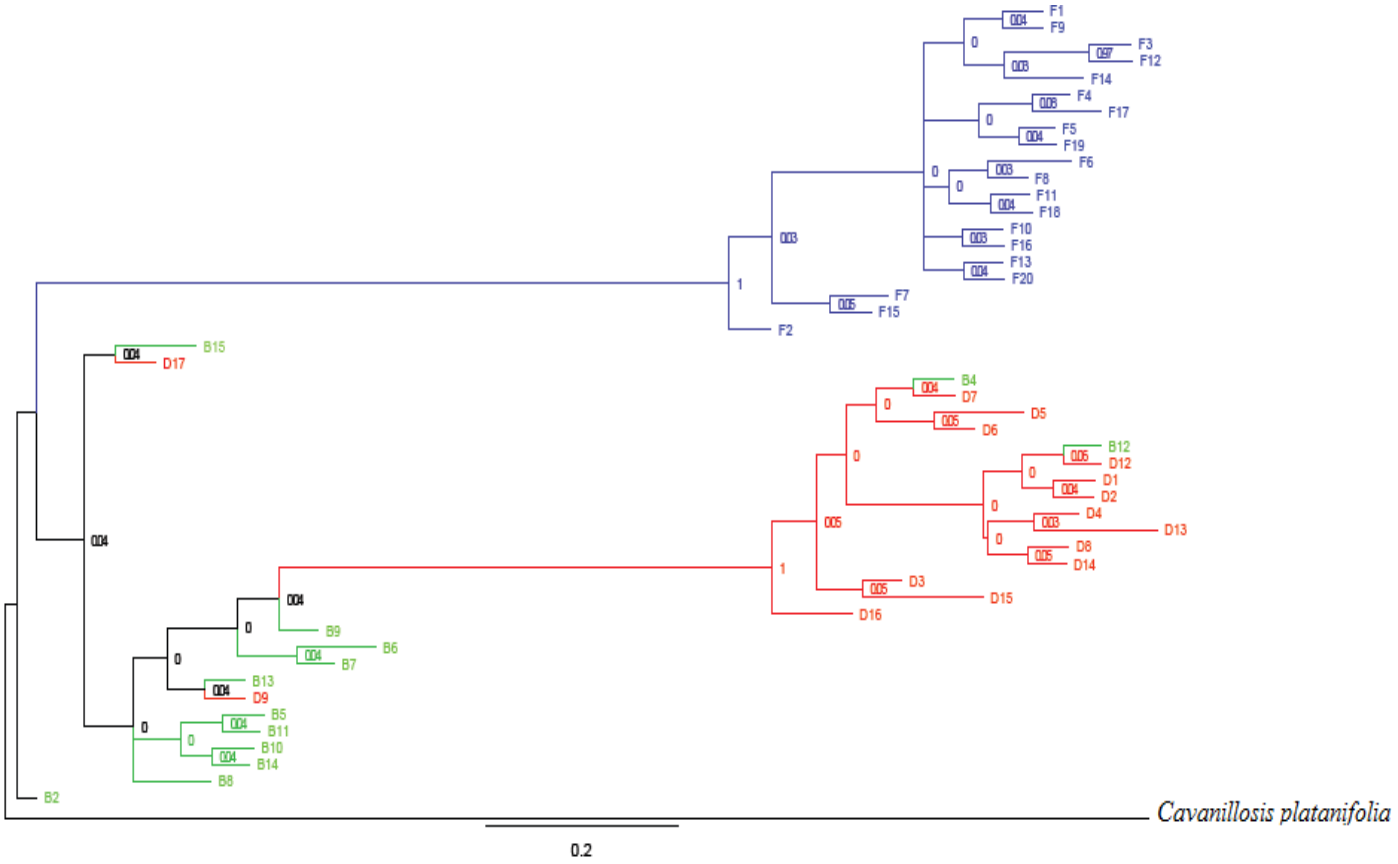

Figure 5. Phylogenetic relationships of baobab (A. *digitata* L.) populations with the method of Bayesian inference.

and Dakar, whose curves are multimodal and reveal that the populations of these two sites are in equilibrium. Fst values ranged from 0.62946 to 0.90712 and increased with the distance. These values were higher than those found by Kyndt et al. (2009) ranging from 0.02 to 0.28. Strong genetic differentiation is observed between Dakar and Bandia despite the potential for dispersal of seeds and pollen (by bats) within populations.

Genetic distances within populations are much lower than between populations. This indicates significant genetic differentiation between populations of different sites. The intra-population genetic distances (Kimura 2 parameter Model; Kimura, 1980) are low and vary from 0.001 to 0.006 (Table 7). Between populations, distances vary between 0.015 and 0.035. The highest is found between Dakar and Ferlo (0.035) and the lowest between Dakar and Bandia (0.021). The Mantel's test gives a value of P-value equal to 0.5 and does not reveal any correlation between geographic distance and genetic differentiation despite higher Fst values due to the distance. Distribution of seeds by humans could prevent isolation by distance. The active exchange of seeds in local markets allows improve gene flow between populations, the maintenance of genetic variation within populations and reduce genetic differentiation between populations (Chung et al., 2000). On the other hand, a weak positive and significant correlation (Z = 0.12, p = 0.64) between genetic distance and real geographic distance on the spot was reported by Munthali et al. (2013). According to these authors, gene flow is not directly influenced by the distance isolation. The organi-zation of genetic diversity appears to result essentially from spatially restricted gene flow with some influen-ces of seed exchange between humans (Kyndt et al., 2009).

Phylogenetic trees reveal the existence of two clades supported by very high bootstrap values (maximum likelihood tree) and posterior probability (Bayesian approach). One of the clades contained individuals from Dakar and Bandia and the other exclusively those of the Ferlo (Widou Thiengoly). It shows a structuring between populations of Ferlo which form a different ecotype from that one met in Bandia and Dakar. In addition to the distance between these regions, exchange of seeds between Ferlo and Bandia on the one hand, and between Dakar and Ferlo on the other hand would be low. Indeed, localities near Dakar as Bandia forest contain baobabs (*A. digitata* L.) and the exchange of seeds per trade is easier between these localities. It is also rare to have the bat-pollination between baobab populations separated by such distances. Knowledge about population genetics is,

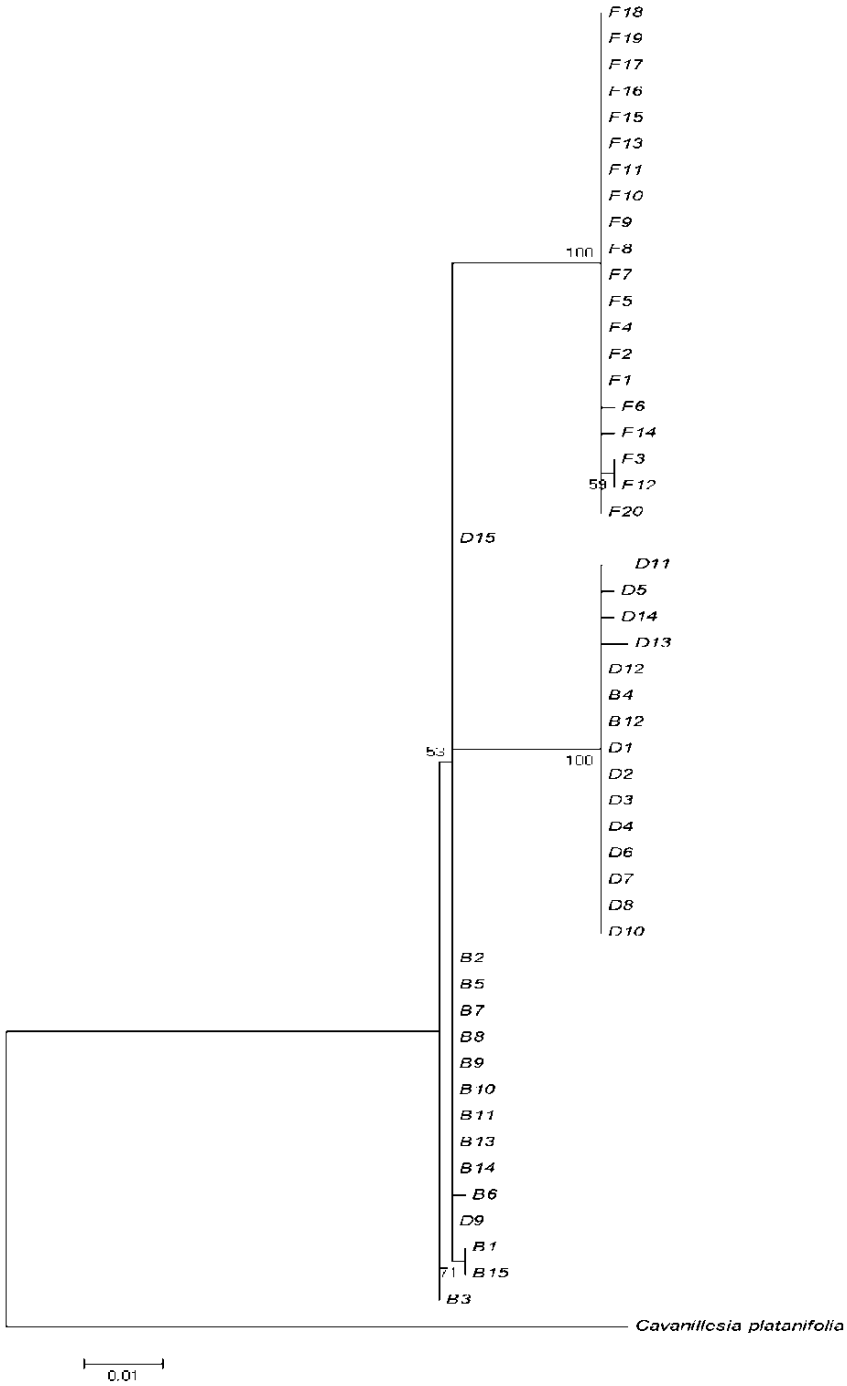

Figure 6. Phylogenetic relationships of baobab (*A. digitata* L.) populations with the method of maximum likelihood.

Table 7. Genetic distances of baobab (*A. digitata* L.) populations in Senegal.

Population	Within population	Population	Between population		
			Bandia	Dakar	Widou T.
Bandia	0.005	Bandia	-	-	-
Dakar	0.006	Dakar	0.015	-	-
Ferlo (Widou T.)	0.001	Ferlo (Widou T.)	0.021	0.035	-

however, of key importance for understanding micro-evolutionary processes in plant populations and supporting or developing appropriate use and conservation strategies (Lengkeek et al., 2006). Human trade promotes gene flow between remote populations of baobab (*A. digitata* L.) and pollination by bats between populations less distant from each other.

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African Journal of Biotechnology

Full Length Research Paper

Microtuberization, minitubers formation and *in vitro* shoot regeneration from bud sprout of potato *(Solanum tuberosum* L.) cultivar *K. badshah*

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Kufri badshah **is one of the important medium maturing, blight resistant potato varieties with round to oblong tubers; it has yellowish skin, shallow eye and white pulp. This variety is popular among farmers. The study on development of tissue culture protocol was carried out using sprout as an explant for initiation of culture in MS media supplemented with eleven different combinations of growth hormones: indole butyric acid (IBA), kinetin, naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D). The response for growth proliferation was observed. The treatment involving a combination of IBA, kinetin, NAA and 2,4-D gave good response for growth of shoot. The resultant shoots were sub-cultured further using nodal cutting as explants in same media for further multiplication. The developed plantlets were hardened in green house. Hardened plants were transplanted in the soil for further growth and development. The plants yielded 3-17 healthy minitubers. For microtuber production, high level of sucrose (8%) gave promising results than low level of sucrose (3%).**

Key words: *Kufri badshah*, explants, indole butyric acid (IBA), kinetin, naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and microtuber.

INTRODUCTION

Potato *(Solanum tuberosum* L.) is a popular and major vegetable crop in India. *S. tuberosum* L. belongs to the family, *Solanaceae* and is South America native. It is the most widely cultivated food crop after wheat, rice and maize, hence it is considered as the most important dicotyledonous and tuber crop (World book, Potato, 2000). Potato is normally propagated by planting the bud or 'eyes' present on the tubers. Micro propagation allows rapid multiplication of clones in a short duration under disease free, controlled environment on yearly basis. Potato can be easily micro propagated (Copeland, 1982; Espinoza et al., 1986). Micro propagated plants, when cultured under suitable conditions, produce *in vitro* micro-tubers (Wang and Hu, 1982). Micro-tubers are 2 to 10 mm diameter and originate as aerial structures from the micro-stems; although a few may also be formed in the medium. The use of 8% sucrose compared to 4 or 12% advanced the initiation of tuberization and gave more and larger micro-tuber (Garner and Jennet, 1989). Microtubers have become an important mode of rapid multiplication for pre basic stock in seed tuber multiplication as well as germplasm exchange (Zakaria et al. 2008).

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Abbreviations: GA_3, Gibberellic acid; **IBA,** indole butyric acid; **NAA,** naphthalene acetic acid; **2,4-D,** 2,4-dichlorophenoxy acetic acid.

Tuberization in potato highly complex development process, regulated by many factors amongst carbon source to nutrient media is the most important factor (Altindal and Karadogan, 2010). Micro-tubers, when grown in soil, produce mini-tubers of 5 to 25 mm diameter. Alternatively, micro propagated plants can be grown directly in soil to produce mini-tubers. The difference between micro- and mini-tubers is not only in their size but also in the way they are produced. Although some large sized micro-tubers may be of the same size or bigger than small minitubers, micro-tubers are produced in *vitro* from micro propagated plants; whereas mini-tubers are produced by growing micro propagated plants or micro-tubers in soil. Looking at the requirement of potato seed, alternative propagating material in potato can be tissue culture product. Therefore, the present experiment was planned.

MATERIALS AND METHODS

The research was conducted at Biotechnology laboratory Department of Genetics and Plant Breeding, C.P. College of Agriculture, S. D. Agricultural University, Sardarkrushinagar, North Gujarat in November 2009 to 2011. An indigenous elite potato (*Solanum tuberosum* L.) cultivar, *K. badshah* was collected from Main Potato Research Station, S. D. Agricultural University, Deesa (North Gujarat).

Culture media

To study the shoot-rooting and micro-tuber formation of potato in culture, Murashige and Skoog (1962) medium was used. This medium contained basal salts (macro and micro) and vitamins.

Tuber sprouting

The cultivars of mother potato were washed with water and treated with 0.3% gibberellic acid (GA_3). They were then packed in craft paper bags which were persevered in the dark at 21°C. Development of sprout took three to four weeks (Figure 1). 2 to 3 mm sprouts were excised from tubers and used as explants.

Shoot culture

The sprouts were cut into a 0.4 to 0.5 cm containing one bud in each explant. The explants were washed with tap water and then rinsed in 70% ethanol. They were treated with 0.1% $Hgcl_2$ (Mercury chloride) for 30 s and then washed with sterile distilled water. The explants were cultured in MS media (Murashige and Skoog, 1962), supplemented with different combination and concentration of growth hormones, indole butyric acid (IBA), kinetin, naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D). The cultures were incubated at 25 + 2°C under 16 h light period.

Sub-culturing nods

Cuts of shoots having nod were subcultured further for developed plantlets having shoots and roots placed in jar containing MS salts with different levels of kinetin, IBA, NAA and 2,4-D (Table 1).

Minituber production

The plantlets having 5-6 nodes with leaf, root mass were transferred to pot tray containing mixture of sand: vermin: compost: cocopit in ratio of 1:1:1 v/v and drenched with fungicide (Bavistin) under green house. Three to four mist irrigation was given to keep soil moist and to maintain the humidity for initial one week. (Figure 5). 8-10 days hardened plantlets were transplanted to normal fertile soil minitubers production.

Micro tuber culture

The nodal shoots were cut into 1 to 2 cm and inoculated in culture media containing half strength MS basal media supplemented with different levels of sucrose (Table 4). Subcultures were incubated at 18 to 20°C in dark room/condition.

RESULTS AND DISCUSSION

The present study was undertaken to establish the protocol for producing micro tuber in cultivar of potato using MS medium supplemented with different concentrations of IBA, kinetin, NAA and 2,4-D. Eleven combinations were tested for initiation and multiplication of shoots (Table 2). The shoots formations were started after one week of inoculation. Four kinds of results were observed among different combinations and concentrations of growth hormones viz.: single shoots, single shoot with branches, multiple shoots and both shoots as well as roots. The results differed according to the combinations of treatment: IBA 1.0 mg l^{-1} and NAA 1.0 mg l^{-1} + kinetin 2.0 mg l^{-1} were observed for single shoot. IBA 1.0 mg l^{-1} + NAA 2.0 mg l^{-1} + kinetin 1.0 mg l^{-1} were observed for single shoot as well as branches (Figure 2a). IBA 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + 2 4-D 1.0 mg l^{-1} and same combination except 2 4-D 1 mg l^{-1} were observed for combination multiple shoots. IBA 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + kinetin 2.0 mg l^{-1} and 2.0 mg l^{-1} IBA + 2.0 mg l^{-1} kinetin + 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} 2,4-D mg l^{-1} were observed for combination multiple shoots as well as roots (Figure 2b and Table 3). Shoot and root formation were found to be better in combined treatment of IBA and kinetin than in single treatment of IBA or kinetin (Khuri and Moorby, 1996).

Observation of shoot regeneration was recorded from 36 explants. Results in Table 3 indicate that treatment T_6 was superior as it gave three to five shoots per explants in 4 weeks and number of nodes per shoot was three to four. These shoots were sub-cultured (Figure 3) for further multiplication in same media using nodal cuttings. The frequency of regeneration of shoots was recorded (78%) in three weeks in treatment T_6 (Figure 4). These shoots may be used for further nodal cutting or may be allowed to root. The shoots having 5-6 nodes with leaf and sufficient amount of root mass were shifted in green house for hardening (Figure 5). After seven days of hardening, these were transplanted into soil for further growth and development (Figures 6 and 7). The plantlets

Figure 1. 3-4 weeks old etiolated sprouts emerging from tubers cultivar *K. badshah* after treated of 0.3% GA_3 and persevered in dark at 21°C.

Table 1. Concentration and combination of different growth hormones.

Treatment	Growth hormone			
	IBA (mg l^{-1})	Kinetin (mg l^{-1})	NAA (mg l^{-1})	2 4-D (mg l^{-1})
T_1	1.0	1.0	1.0	0.0
T_2	1.0	1.0	2.0	0.0
T_3	1.0	2.0	1.0	1.0
T_4	1.0	1.0	2.0	0.0
T_5	1.0	1.0	1.0	1.0
T_6	2.0	2.0	2.0	1.0
T_7	2.0	2.0	1.0	1.0
T_8	2.0	1.0	1.0	1.0
T_9	2.0	2.0	0.0	0.0
T_{10}	2.0	2.0	1.0	0.0
T_{11}	2.0	2.0	2.0	1.0

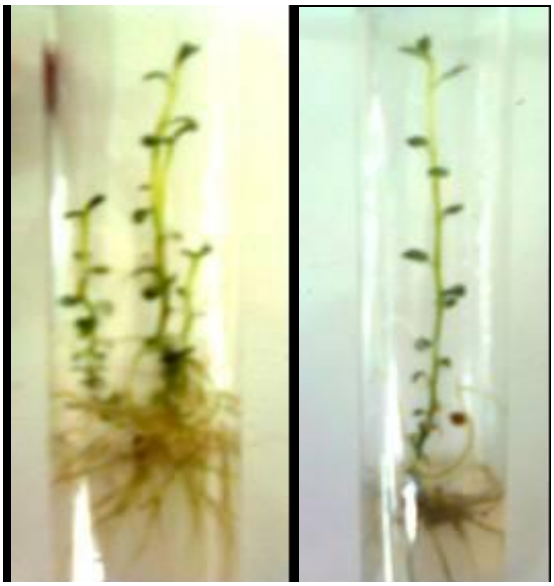
Table 2. Percentage of shooting from sprouts of potato (*S. tuberosum*) in different concentration and combinations (2009-2010).

Treatment	No. of explants kept	No. of explants shooting	Shooting (%)
T_1	06	05	83.33
T_2	06	04	66.66
T_3	06	04	66.66
T_4	08	06	75.00
T_5	06	05	83.33
T_6	09	07	88.88
T_7	09	08	77.77

yielded 3 to 17 healthy minitubers (Figure 8).

The microtubers were developed from the one month old shoots which were cuts in small pieces (1-2 cm including nodes). It was subcultured in combination with half strength MS media supplemented with different levels of sucrose viz.: 8 gl^{-1} (8% sucrose), 7 g l^{-1} (7% sucrose), 6 g l^{-1} (6% sucrose), 5 g l^{-1} (5% sucrose), 4 g l^{-1} (4% sucrose) and 3 g l^{-1} (3% sucrose) (Table 4). Microtuber appeared after three to four weeks of inoculation. The morphology of the microtuber appeared after 6th days of culture (Figure 9). Similarly, Desire (1995a, b) reported that from the 12th day sessile microtuber becomes round in shape with diameter of 2 to 3 mm (Figure 9); thereafter with the growth of cornical cells and high accumulation of starch and protein, the final size becomes 4-5 mm. In the present study, results

a



b

Figure 2. a, Shoot development with branching (IBA 1.0 mg l^{-1} + NAA 2.0 mg l^{-1} + kinetin 1.0 mg l^{-1}); b, multishoots and root development (IBA 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + kinetin 2.0 mg l^{-1} and 2.0 mg l^{-1} IBA + 2.0 mg l^{-1} kinetin + 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} 2,4-D mg l^{-1}).

Table 3. Treatments used for shoot and root proliferation (2010-11).

Treatment	No. of explants kept	No. of plantlets/ explants (range)	Number of nodes/ shoot	Plantlets survived for hardening (%)	Minituber/ plant (range)
T4	36	2-3	2-3	62	3-17
T6	36	2-3	2-3	78	3-17

Table 4. Percentage of microtuber development from shoots of potato (*S. tuberosum*) in different sucrose level (2009-2010).

S/N	Sucrose level (g/l)	No. of explants kept	Tuber developed	Percentage tuber developed
1	8	06	04	80
2	7	06	02	40
3	6	06	02	40
4	5	06	01	20
5	4	06	01	20
6	3	06	00	00

Figure 3. Nodal sub culturing from shoot.

Figure 4. Nodal cutting developed into plantlets.

Figure 5. The shoots having 5-6 nodes with leaf and sufficient amount of root mass shifted to green house for hardening.

Figure 6. Minituber transplanted to soil for further growth and development after seven days of hardening.

Figure 7. Normal development and growth of plantlets in field condition.

Figure 8. 3-17 healthy minitubers yielded by the plantlets under normal field condition.

Figure 9. Microtuber formation.

show that half strength MS supplemented with 8% sucrose media developed tuber, whereas 3% sucrose media did not develop any tuber (Table 4). These results are supported by the findings of Uddin (2006), which showed that the presence of high level sucrose (8%) was beneficial and led to the production of slightly larger microtuber and higher yield. Similarly, number and weight of microtuber, formation of shoots, shoots length were found superior at sugar concentration of 8%, which was also reported by Fatima et al., (2005). And significantly, slower microtuber growth rates were observed when sugar concentration was 4% instead of 8%; this is in line with Yu et al. (2000). From the present investigation it can be concluded that low level (3%) of sucrose was not found suitable for the microtuber production under invitro conditions. Similar results were also supported by El-sawy et al. 2007 and Hoque, 2010.

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Full Length Research Paper

Influence of vesicular arbuscular mycorrhiza (VAM) and phosphate solubilizing bacteria (PSB) on growth and biochemical constituents of *Marsdenia volubilis*

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A field experiment was carried out to find out the effect of biofertilizers, vesicular arbuscular mycorrhiza (VAM), and phosphate solubilising bacteria (PSB) individually and in combination on growth and physiological attributing properties of *Marsdenia volubilis* plant under nursery conditions. The plant seedlings were harvested at various intervals: 30, 60 and 120 days after transplantation. The inoculation of microbial cultures VAM and PSB resulted in enhancement of growth parameters like plant height, root length, fresh weight and dry weight of shoot and root, leaves/plant, leaf area/plant , chlorophyll content, reducing and non-reducing sugars, starch, lipid and protein contents in root and shoot samples. These parameters were maximum with dual inoculation than individually. The results emphasize the importance of microbial biofertilizers inoculations for rapid growth of seedlings of plant (*M. volubilis*) in nurseries and illustrate the advantage of inoculating soils of low microbial population with indigenous microbes.

Key words: Biofertilizers, vesicular arbuscular mycorrhiza (VAM), phosphate solubilising bacteria (PSB), *Marsdenia volubilis,* growth and biochemical parameters.

INTRODUCTION

Soil fertility is diminishing gradually due to soil erosions, loss of nutrients, accumulation of salts and toxic elements, water logging and unbalanced nutrient compensation. Organic wastes and biofertilizers are alternate sources to meet the nutrient requirement of crops. In recent years, biofertilizers have emerged as a promising component of integrating nutrient supply system in agriculture. Thus, biofertilizers are organic products containing specific microorganisms in concentrated forms, derived from the soil root zone (rhizosphere) (Mishra and Dadhich, 2010). Consequently, microbial fertilizers are considered as an important part of environment friendly sustainable agricultural practices, with low cost inputs; mainly including nitrogen fixing, phosphate solubilizing, potash mobilizing and plant promoting microorganisms. Vesicular arbuscular mycorrhizal (VAM) fungi improve plant growth through phosphorous nutrition. In addition to phosphorous, they also help in the uptake of other nutrient elements. Nutrient absorption by fungal symbionts is due to external hyphae of the fungus proliferating beyond the nutrient depletion zone and reaching the source of nutrients. Mycorrhizal fungi appear to be extremely advantageous to crops grown in soils with low fertility. The improved plant growth is also attributed to the production

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Abbreviations: VAM, Vesicular arbuscular mycorrhizal; **PSB,** phosphate solubilizing.bacteria.

of growth promoting substances, tolerance to drought, salinity and transplantation shock, resistance to soil-borne plant pathogens and synergetic interactions with other beneficial rhizosphere microorganisms. Phosphorous is one of the major plant nutrients limiting plant growth. Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular application of chemical fertilizers. However, a large proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to plants. Phosphorus and other major nutrients are involved in cell division and development, photosynthesis, breakdown of sugar, energy transfer, nutrient transfer within the plant and cell signal transduction (Sharma and Namdeo, 1999). There are several microorganisms which can solubilize the unavailable phosphorous. Bacteria like *Bacillus megaterium, Bacillus polymyxa* and *Pseudomonas straita* are important phosphate solubilizing microorganisms. Many fungi, *Aspergillus* and *Pencillium* species are potential solubilzers of bound phosphates. They solubilize the bound phosphorous through secretion of organic acids and make it available to the plant, resulting in the improved plant growth and yield. Therefore, phosphate dissolving microorganisms play some part in correcting phosphorous deficiency in plantation soils. They may also release soluble inorganic phosphate into soil through decomposition of phosphate rich organic compounds. These microbial inoculants can substitute almost 20-25% of the phosphorous requirement of plants. In view of this, the supply of these elements to plant is essential for achieving optimum growth and crop yield.

In the present study, *Marsdenia volubilis* plant was selected due to its high medicinal value. *M. volubilis* is an important medicinal plant belonging to the family *Aselepiadaceae.* It is a tall woody climber, grows 11 m height and 95 cm in girth with dense lenticillate and pustular branches. This plant is widely used in ayurvedic medicine in India. The leaves are used for snake bites and to cure boils and abscesses as it has potent antimicrobial activity against a wide range of fungal and bacterial species which causes the diseases in human beings. The plant bark is widely used in the case of anorexia and nervous dyspepsia and roots and tender stalks are considered emetic and expectorant. The flowers and unripe fruits are eaten as vegetable. In view of medicinal importance of *M. volubilis*, there is a need to develop efficient, low cost cultivation methods for this plant which are suitable to various climatic conditions to obtain higher yield, hence there is a need to improve plantation of this tree, with implementation of organic farming and application of biofertilifers.

MATERIALS AND METHODS

Location of the study

The plants of *M. volubilis* were maintained under glass house conditions in the medicinal plant garden of Botany Department, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. The climate was warm and humid at the time of starting the experiment. There was monsoon rain for few days which gave the favorable climate for the seed germination. The weekly average maximum and minimum temperatures ranged between 27.1 to 36.2°C and 14.6 to 23.7°C, respectively, during the experimental period.

Collection of biofertilizers

Glomus mosseae and *Bacillus megaterium* were obtained from Regional Biofertilizers Development Centre, Bangalore Division, India.

Experimental design

The pot culture experiment was carried out under greenhouse conditions to know the response of *M. volubilis* plant to *G. mosseae* and *B. megaterium* inoculation. The *M. volubilis* plants were grown in plastic pots containing a sterilized mixture of soil and sand (1/1 w/w). The pots were placed according to a completely randomized design. Seeds of *M. volubilis* were surface sterilized with 0.05% sodium hypo chloride for 45 min before sowing them into a 5 cm depth of growth media. Five to six seeds were sown in each pot and after a week of germination time, they were thinned to one plant per pot. The plants were grown in a greenhouse under natural photoperiods (23.5/18°C day/night, 6000/4000 lux light intensity) for three months. Inoculum of *G. mosseae* (20 g/kg soil), and 20 ml of *B. megaterium* was laid around the seed.

The following treatments were established to know the response of *M. volubilis* to the inoculation with VAM fungi and phosphate solubilising bacteria (PSB): T_1, Control (without inoculation of microorganisms); T_2, inoculated with VAM (*G. mosseae*); T3, inoculated with PSB (*B. megaterium*); T4, inoculated with both *G. mosseae and B .megaterium*.

Growth parameters

The growth parameters of *M. volubilis,* shoot length, root length, number of leaves, leaf area, fresh and dry biomass of shoot and root were measured on every 30^{th}, 60^{th} and 90^{th} day of the plant growth in all the treatments with or without biofertilizers.

Physiological parameters

The physiological characteristics such as chlorophyll content, reducing and non- reducing sugars, starch, lipid and protein contents in root and shoot samples on 30^{th} , 60^{th} and 90^{th} day were studied with and without inoculum treated plants. The biochemical properties, chlorophyll content (Arnon, 1949), starch (Mc Cready et al., 1950) carbohydrates (Highkin and Frankel, 1962) total lipids (Bligh and Dyer, 1959), and total proteins (Lowry et al., 1951) were estimated.

Statistical analysis

Two-way analysis of variance (ANOVA) was carried out at a 0.05 level of significance on the data and SPSS version 13.0 was used. The values corresponded to each table in the results.

RESULTS AND DISCUSSION

Influence of biofertilizers both VAM and PSB showed

Table 1. Effect of VAM fungi and PSB on shoot length (cm) of *M. volubilis*.

Treatment	Incubation days (after treatment)		
	30	60	90
T_1	3.15 (0.40)	5.40 (0.36)	10.80 (0.60)
T_2	5.67 (0.25)	10.40 (0.40)	21.10 (0.56)
T_3	4.63 (0.45)	9.87 (0.50)	18.77(0.45)
T_4	6.23 (0.65)	11.60 (0.60)	24.37 (0.61)
LSD	0.87	0.90	1.05
SE	0.37	0.72	1.52

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

Table 2. Effect of VAM fungi and PSB on root length (cm) of *M. volubilis*.

Treatment	Incubation days (after treatment)		
	30	60	90
T_1	5.13 (0.40)	9.57 (0.31)	17.37 (0.45)
T_2	14.10 (0.46)	20.53 (0.55)	27.83 (0.55)
T_3	12.60 (0.50)	17.53 (0.65)	22.00 (2.00)
T_4	14.77 (0.65)	22.87 (0.35)	30.50 (0.40)
LSD	0.96	0.92	2.04
SE	1.17	1.28	1.41

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

Table 3. Effect of VAM fungi and PSB on leaf number of *M. volubilis*.

Treatment	Incubation days (after treatment)		
	30	60	90
T_1	3.87 (0.31)	5.33 (0.31)	8.47 (0.31)
T_2	5.43 (0.45)	8.43 (0.31)	12.0 (0.26)
T_3	4.83 (0.25)	7.40 (0.30)	11.60 (0.20)
T_4	6.0 (0.20)	9.63 (0.40)	13.60 (0.40)
LSD	0.60	0.63	0.57
SE	0.13	0.38	0.52

Values with in the brackets indicate standard deviation. Each value represents mean of six replications.

significant effect on growth and physiological characteristics of *M. volubilis*. The data presented in Tables 1, 2, 3, 4, 5 and 6 indicate that biofertilizers had significant effect on shoot length, root length, fresh weight of shoot and root, dry weight of shoot and root, leaves / plant, leaf area of plant. All characteristics under study were significantly higher in combined inoculation of VAM and PSB (T_4), than other inoculations and control. The biofertilizers treated plants exhibited increased shoot length compared to un-inoculated plants. The maximum shoot length was recorded in T_4 plants (24.37 cm) at 90 days of plant whereas the co-inoculation of biofertilzers (T_4) exhibited maximum root length (30.50 cm) and the root length was found minimum (5.13 cm) in T_1 treatment after 90 days. The maximum number of leaves were observed in T_4 treatment (13.60) followed by T_2 (12.00) and T_3 (11.60). In contrast, least leaves were counted in the control (T_1). The leaf area differed significantly in treated plants compared to the control. On the 30^{th} day, the maximum leaf area was found in T_4 plants (34.1 cm^2) and the minimum in T_1 (22.42 cm^2). On the 60^{th} and 90^{th} day, inoculated individually PSB or VAM or in combination performed better compared to control (T_1). The maximum leaf area was recorded in T_4 plants whereas the least leaf area was observed with control. The plant biomass was improved along with increasing the incubation periods.

Table 4. Effect of VAM fungi and PSB on leaf area (cm^2) of *M. volubili*.

Treatment	Incubation days (after treatment)		
	30	60	90
T_1	22.42 (0.00)	29.05 (0.56)	36.52 (1.14)
T_2	30.66 (0.00)	42.57 (0.99)	57.40 (1.50)
T_3	26.54 (0.00)	35.37 (0.66)	48.56 (0.97)
T_4	34.10 (0.00)	45.99 (1.52)	59.43 (1.90)
LSD	0.00	1.90	2.68
S E	0.00	1.99	2.75

Values with in the brackets indicate standard deviation. Each value represents mean of six replications.

Table 5. Effect of VAM fungi and PSB on fresh biomass of *M. volubilis*.

Treatment	Incubation days (after treatment)								
	Shoot fresh biomass (g)			Root fresh biomass (g)			Total fresh biomass (g)		
	30	60	90	30	60	90	30	60	90
T_1	0.64 (0.08)	1.01 (0.03)	1.88 (0.04)	0.34 (0.07)	0.64 (0.10)	1.14 (0.08)	0.96 (0.14)	1.32 (0.12)	3.15 (0.06)
T_2	1.06 (0.10)	2.63 (0.06)	3.96 (0.13)	0.51 (0.02)	1.09 (0.15)	1.60 (0.09)	1.60 (0.17)	3.63 (0.14)	5.64 (0.19)
T_3	1.00 (0.11)	2.58 (0.23)	3.92 (0.10)	0.49 (0.01)	0.93 (0.07)	1.44 (0.05)	1.48 (0.12)	3.21 (0.23)	5.24 (0.20)
T_4	1.37 (0.17)	3.19 (0.29)	4.33 (0.14)	0.60 (0.02)	1.23 (0.11)	1.73 (0.07)	1.93 (0.13)	4.15 (0.27)	6.07 (0.21)
LSD	0.23	0.36	0.21	0.08	0.21	0.14	0.27	0.37	0.33
SE	0.08	0.25	0.29	0.03	0.07	0.05	0.11	0.33	0.34

Values with in the brackets indicate standard deviation. Each value represents mean of six replications.

Table 6. Effect of VAM fungi and PSB on dry biomass of *M. volubilis*.

Treatment	Shoot dry biomass (g)			Root dry biomass (g)			Total dry biomass (g)		
	Incubation days (after treatment)								
	30	60	90	30	60	90	30	60	90
T_1	0.13 (0.04)	0.25 (0.05)	0.74 (0.09)	0.07 (0.01)	0.11 (0.03)	0.20 (0.01)	0.18 (0.02)	0.39(0.11)	0.89 (0.06)
T_2	0.45 (0.09)	0.83 (0.05)	1.46 (0.11)	0.14 (0.01)	0.25 (0.06)	0.57 (0.08)	0.55 (0.07)	1.03 (0.16)	1.97 (0.13)
T_3	0.37 (0.09)	0.65 (0.05)	1.25 (0.11)	0.11 (0.01)	0.24 (0.06)	0.55 (0.13)	0.51 (0.13)	0.99 (0.22)	1.77 (0.11)
T_4	0.50 (0.11)	0.92 (0.06)	1.65 (0.24)	0.15 (0.01)	0.35 (0.10)	0.65 (0.09)	0.63 (0.10)	1.23 (0.22)	2.15 (0.17)
LSD	0.16	0.10	0.28	0.02	0.12	0.17	0.17	0.35	0.23
SE	0.05	0.07	0.11	0.01	0.03	0.06	0.06	0.11	0.15

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

Table 7. Effect of VAM and PSB on chlorophyll content of *M. volubilis*.

Treatment	Chorophyll 'a' (mg/g)			Chlorophyll 'b' (mg/g)			Total Chlorophyll (mg/g)		
	Incubation days (after treatment)								
	30	60	90	30	60	90	30	60	90
$T_{1(Control)}$	0.58 (0.04)	0.69 (0.04)	0.85 (0.02)	0.95 (0.02)	1.05 (0.03)	1.15 (0.03)	1.53 (0.21)	1.95 (0.01)	2.15 (0.03)
$T_{2(VAM)}$	0.77 (0.03)	0.96 (0.02)	1.23 (0.03)	1.26 (0.04)	1.39 (0.03)	1.63 (0.03)	2.11 (0.03)	2.38 (0.02)	2.45 (0.03)
$T_{3(PSB)}$	0.68 (0.02)	0.86 (0.03)	1.10 (0.04)	1.19 (0.04)	1.34 (0.03)	1.44 (0.04)	2.09 (0.03)	2.28 (0.02)	2.41 (0.02)
$T_{4(VAM +PSB)}$	0.86 (0.03)	1.0 (0.04)	1.34 (0.08)	1.31 (0.03)	1.45 (0.02)	1.77 (0.03)	2.39 (0.03)	2.48 (0.02)	2.91 (0.03)
LSD	0.05	0.06	0.09	0.06	0.05	0.06	0.20	0.03	0.05
S E	0.02	0.02	0.03	0.03	0.03	0.06	0.06	0.03	0.07

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

The improvement of growth parameters in the present study may be due to functions of biofertilizers, availability of nitrogen, phosphorous, and certain growth harmones like auxins, gibberlins, vitamins and organic acid secreted by bioinoculants which increase the surface area per unit root length and were responsible for root hair. Similarly, reports were made by Gupta et al. (1999), Ahmad et al., (2004), Nandre et al. (2005), Chadrasekar et al. (2005), Nabila et al. (2009), Zaki et al. (2010) and Abou El-Yazeid and Abou–Aly (2011).

The influence of biofertilizers on biochemical properties was studied. Content of Chlorophyll a, b and total chlorophyll were estimated and shown in Table 7. Maximum chlorophyll a, b were observed in T_4 (1.34, 1.77) and least in T_1 (0.85, 1.15). There was a significant difference in chlorophyll-a, chlorophyll-b and total chlorophyll content among the treatments and different days. This may be due to the increase in stomatal conductance and carbon assimilation (Levy and Krikun, 1980). Krishna and Bagyaraj (1981) observed that bundle sheath chloroplasts were larger and numerous in mycorrhizal plant. Increased chlorophyll 'a', chlorophyll 'b' and total chlorophyll content were also reported by Mathur and Vyas, (2000) Bhoopander Giri et al. (2003), Kate et al. (2005) and Senthilkumar and Sivagurunathan (2012).

Maximum amount of carbohydrates were observed in plants treated with biofertilizers individually and combined form. With increasing plant incubation days, the reducing sugar content also improved ranging from 1153.42 to 1317.07 µg/g in T_4 plants whereas the least in T_1 in all treatments except control as shown in Tables 8 and 9. The polysaccharide starch content was also maximum in T_4 in all incubation days than the control. Improvement in carbohydrate content in all biofertilizers treated plants may be due to Increased carbon fixation, activation of enzymes and increased photosynthetic rate increased reducing and non reducing sugar contents in different mycorrhizal plants was observed by Krishna and Bagyaraj (1981), Mathur and Vyas (2000) and Nelson and Achar (2001).

The influence of biofertilizers on lipid content in shoot and root parts of *M. volubilis* is shown in Table 10. The maximum lipid content was recorded in shoot samples of T_4 (9.61, 17.39 and 26.77 mg/g) and minimum in the control plants. Similarly, the lipid contents in roots of *M. volubilis* studied in selected plants results are shown in Table 10. The increments in lipid content of biofertilizer inoculated plants were due to the formation of lipid bodies in arbuscular trunks and intercellular hyphae. In this mutualistic symbiosis, the fungus acquires carbon as hexose within the root and stores predominantly as triacylglycerol. Stimulation of mycorrhizal activity in presence of PSB may attributes for more lipid content in dual inoculated plants.

The total protein content was estimated in plants parts treated with biofertilizers individually or in combination of both. The protein content in shoot and roots of T_2, T_3 and T_4 plants on 30^{th}, 60^{th} and 90^{th} days were significantly higher when compared to protein content of Control (T_1) plants. Maximum shoot protein content was recorded in T_4 plants and minimum in control (Table 11). Significant increase in the protein content of both shoot and root tissue of inoculated plants over control plants attributes to the accumulation of more Nitrogen and phosphorous in treated plants. Maximum protein content in dual inoculated plants is due to the increase of plant membrane proteins and/or to the presence of proteins from the fungal partner. Similar reports were made by Mathur and Vyas (2000), Nelson and Achar (2001) and Shehata and Khawas (2003), Senthilkumar and Sivagurunathan (2012).

Table 8. Effect of VAM fungi and PSB on carbohydrate content in shoot of *M. volubilis*.

Treatment	Reducing sugar (µg/g)			Non-reducing sugar (µg/g)			Starch (mg/g)		
	Interval days (after treatment)								
	30	60	90	30	60	90	30	60	90
$T_{1(Control)}$	709.24 (10.27)	850.24 (15.20)	926.58 (28.65)	433.22 (11.90)	474.39 (21.12)	554.08 (6.79)	14.35 (0.39)	19.33 (0.38)	24.94 (0.70)
$T_{2(VAM)}$	888.10 (10.95)	1099.21 (10.00)	1112.91 (1.52)	524.92 (8.59)	571.23 (9.45)	595.89 (4.45)	20.29 (0.12)	28.14 (0.19)	31.42 (0.66)
$T_{3(PSB)}$	762.43 (9.05)	1040.03 (10.92)	1105.44 (10.53)	521.87 (6.86)	542.70 (10.07)	582.62 (7.23)	19.84 (0.14)	26.30 (0.39)	30.53 (0.14)
$T_{4(VAM +PSB)}$	1153.42 (7.40)	1184.80 (6.19)	1317.07 (15.09)	542.00 (6.48)	574.70 (8.09)	602.37 (7.78)	25.46 (0.49)	31.71 (0.16)	37.63 (0.17)
LSD	17.94	20.84	32.14	16.46	24.99	12.61	0.61	0.56	0.92
SE	51.79	37.16	41.89	12.97	12.57	5.83	1.19	1.36	1.36

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

Table 9. Effect of VAM fungi and PSB on carbohydrate content in roots of *M. volubilis*.

Treatment	Reducing sugar (µg/g)			Non-reducing sugar µg/g)			Starch (mg/g)		
	Interval days (after treatment)								
	30	60	90	30	60	90	30	60	90
$T_{1(Control)}$	298.94 (2.51)	496.09 (5.99)	668.03 (5.41)	315.69 (2.91)	351.03 (4.33)	446.84 (5.59)	4.88 (0.31)	5.72 (0.12)	6.07 (0.15)
$T_{2(VAM)}$	464.00 (4.37)	764.08 (4.56)	825.84 (3.81)	550.97 (3.84)	566.66 (3.34)	618.00 (3.00)	6.17 (0.07)	11.26 (0.04)	13.40 (0.13)
$T_{3(PSB)}$	397.87 (2.69)	714.69 (5.89)	831.00 (3.61)	534.61 (3.21)	558.14 (4.72)	606.27 (0.49)	5.98 (0.06)	8.56 (0.05)	9.35 (0.12)
$T_{4(VAM +PSB)}$	555.03 (2.38)	775.49 (6.17)	851.55 (2.74)	584.45 (6.70)	619.39 (5.16)	653.81 (4.44)	6.65 (0.10)	12.68 (0.05)	14.84 (0.09)
LSD	5.84	10.73	7.56	8.35	8.37	7.31	0.32	0.13	0.23
SE	28.21	34.07	22.16	31.94	30.92	24.00	0.20	0.80	1.04

Values within the brackets indicate standard deviation.Each value represents mean of six replications.

Table 10. Effect of VAM fungi and PSB on total lipid content of *M. volubilis*.

Treatment	Shoot lipid content (mg/g)			Root lipid content (mg/g)		
	Interval days (after treatment)					
	30	60	90	30	60	90
$T_{1(Control)}$	5.72 (0.04)	9.44 (0.08)	15.63 (0.09)	4.07 (0.08)	6.53 (0.10)	10.52 (0.09)
$T_{2(VAM)}$	8.44 (0.07)	15.46 (0.05)	25.22 (0.41)	7.13 (0.11)	10.18 (0.06)	13.98 (0.15)
$T_{3(PSB)}$	8.33 (0.07)	15.49 (0.06)	23.44 (0.08)	5.15 (0.05)	9.65 (0.07)	13.17 (0.07)
$T_{4(VAM +PSB)}$	9.61 (0.06)	17.39 (0.06)	26.77 (0.07)	8.18	11.54 (0.12)	15.66 (0.10)
LSD	0.11	0.12	0.41	0.15	0.17	0.20
SE	0.21	0.42	0.69	0.40	0.44	0.45

Values within the brackets indicate standard deviation. Each value represents mean of six replications.

Table 11. Effect of VAM fungi and PSB on total protein content of *M. volubilis*.

Treatment	Shoot protein content (mg/g)			Root protein content (mg/g)			Total protein content (mg/g)		
	Interval days (after treatment)								
	30	60	90	30	60	90	30	60	90
$T_{1(Control)}$	3.52 (0.05)	4.57 (0.06)	5.20 (0.05)	0.82 (0.07)	1.12 (0.05)	1.34 (0.03)	4.33 (0.11)	5.88 (0.02)	6.84 (0.02)
$T_{2(VAM)}$	6.28 (0.06)	6.55 (0.07)	6.81 (0.06)	1.56 (0.08)	1.77 (0.04)	1.99 (0.06)	7.84 (0.14)	8.32 (0.11)	8.80 (0.12)
$T_{3(PSB)}$	5.28 (0.09)	5.89 (0.05)	6.21 (0.07)	1.44 (0.05)	1.65 (0.03)	1.78 (0.04)	6.72 (0.05)	7.54 (0.08)	7.99 (0.11)
$T_{4(VAM +PSB)}$	6.53 (0.06)	7.20 (0.05)	8.10 (0.09)	1.69 (0.05)	1.98 (0.05)	2.32 (0.07)	8.22 (0.10)	8.85 (0.05)	10.42 (0.10)
LSD	0.12	0.11	0.13	0.11	0.08	0.09	0.20	0.14	0.17
S E	0.24	0.16	0.23	0.10	0.07	0.08	0.34	0.23	0.31

Values within the brackets indicate standard deviation.Each value represents mean of six replications.

Conclusion

In this study, treatment of biofertilizers in combination with *G. mosseae* and *B.megaterium* significantly enhanced the growth parameters which included, shoot length, root length, leaves, leaf area, biomass of root and shoot and biochemical constituents, total chlorophyll, carbohydrate lipid and protein content in *M. volubilis* when compared to the control.

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Nutrients, phytochemicals, fungal flora and aflatoxin in fresh and salted *Vernonia amygdalina* leaves

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In this study, the effect of salting on the pH, phytochemicals, fungal flora and nutrient composition of *Vernonia amygdalina* leaves was investigated. There was a decrease in pH from 5.88 for the fresh, to 5.80, 5.73, 5.24, and 5.02 for the light brined, light salted, heavy salted and light brine + vinegar treated leaves, respectively. Leaves treated with water alone had a pH of 6.63. Compared with the fresh leaves, there was a decrease in all the nutrients investigated, except for sodium and calcium which increased significantly. The fresh sample showed the highest concentration of total phenolic compounds (TPC). Compared with the fresh, the highest loss of TPC was for the light brine + vinegar preserved leaves (51.69%) and the lowest loss was for the water treated (27.28%). The heavy salted and light salted leaves lost about a third (36.05 and 33.42%, respectively) and the light brined 45.93% of their TPC. In terms of organoleptic properties, reduction in fungal count and genera, and loss of moisture, heavy salting appears to be the most effective preservation treatment. These, coupled with only moderate loss of nutrients and phytochemicals is recommended as an effective technique for the preservation of *V. amygdalina* leaves. Screening of the fresh and treated produce indicated the presence of aflatoxin.

Key words: *Vernonia amygdalina* leaves, salting, nutrients, phytochemicals, fungal flora, aflatoxin.

INTRODUCTION

The salting or brining of vegetables offers tremendous possibilities both for their commercial and home preservation. In the process, the salt exerts a selective action on the naturally occurring organisms to promote a desirable fermentation. Salt tolerant microorganisms use as their nutritive material the soluble constituents that diffuse out of the vegetable as a result of the action of the salt on vegetable tissue. These fermentative organisms bring about the production of various compounds, principally lactic acid but also acetic acid (both of which result in a reduction of pH), alcohols and considerable amounts of gas. The production of sufficient amount of acid makes the medium unsuitable for the growth of food spoilage bacteria. In addition, the acid and other microbial metabolites alter the flavour of the food. Substances and organisms in fermented foods can cause changes in the composition and/or activity of the gastrointestinal microbiota resulting in several health benefits (Perdigon et al., 1987; FAO/WHO, 2001; FAO, 2007; Farnworth, 2004; Farnworth et al., 2007; Granato et al., 2010).

Vernonia amygdalina, variously known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo), is a tropical shrub, 1-3 m in height with petiole and leaf of about 6 mm in diameter, and elliptic in shape (Igile et al., 1995). The leaves are dark green in colour, with a characteristic odour and a bitter taste. The species is indigenous to tropical Africa where it is found wild or cultivated (Bosch et al., 2005). The leaves are eaten, after crushing and washing thoroughly to remove the bitterness (Mayhew and Penny, 1998). As with other high yielding leafy vegetables, post-harvest losses may

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occur due to inadequate preservation.

Previous authors have reported nutrient composition and antimicrobial activity (Oboh and Masodje, 2009) and blood lipid control activity of the methanolic and aqueous extracts of *V. amygdalina* leaves (Adaramoye et al., 2008; Oboh and Enobhayisobo, 2009), and the effects of blanching and drying, and salting on their organoleptic characteristics and nutrient composition (Osunde and Makama, 2007; Aliero and Abdullahi, 2009; Oboh and Madojemu, 2010), In this study, the effect of salting on the phytochemicals, fungal flora and nutrient composition of *V. amygdalina* leaves was investigated. The fresh and salted leaves were also screened for the presence of aflatoxin.

MATERIALS AND METHODS

To investigate the effect of salting on the nutrient composition, phytochemicals, mycological flora and organoleptic properties of *V. amygdalina* leaves, a study was conducted using the following treatments:

Heavy salting

V. amygdalina leaves were gently washed to remove dirt, and the water drained. Salt (37.5 g) and leaves (150 g) were mixed well in a plastic bucket. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. Brine made of salt (37.5 g) and water (150 ml) was added until the pressure plate was slightly submerged. The buckets were stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Light salting

Washed and drained *V. amygdalina* leaves (150 g) were mixed well with dry salt (3.75 g) in a plastic bucket, packing tightly. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Light brining

To washed and drained *V. amygdalina* leaves (150 g) light brine was added (3.75 g salt dissolved in 150 ml water). The brine was added to the vegetable in layers (that is, put a layer of vegetables, add brine, put another layer of vegetables add more, and so on) in a plastic bucket, and packed tightly. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to27.0°C.

Light brine + vinegar

A solution of salt (7.5 g), white vinegar (7.5 ml) and water (150 ml) was added to rinsed and drained *V. amygdalina* leaves (150 g) in layers (that is, put a layer of vegetables, add brine and vinegar, put another layer of vegetables add more, and so on) in a plastic bucket and packed tightly. The mixture was covered with 2 layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Analytical procedure

Determination of nutrients

Vitamin C, β-carotene, carbohydrates, protein and moisture: Ascorbic acid was determined titrimetrically using the 2,6-dichlorophenolindophenol method according to Osunde and Musa Makama (2007). β-Carotene was determined spectrophotometrically according to the method of Nagata and Yamashita (1992). The sample (100 mg) was vigorously shaken in 10 ml of an acetone-hexane mixture (4:6) for 1 min and filtered through Whatman no. 4 filter paper. The absorbance was measured at 453, 505, and 663 nm. The content of β-carotene was calculated according to the following equations:

$$\beta\text{-carotene (mg/100 ml)} = 0.216\ (A_{663}) - 0.304\ (A_{505}) + 0.452\ (A_{453})$$

Where, A is absorbance. Total carbohydrate was determined spectrophotometrically at 620 nm using the anthrone method (Hedge and Hofreiter, 1962). Nitrogen was determined using the Kjedahl method and protein calculated as total nitrogen x 6.25. Moisture content was determined by drying 5 g wet sample to constant weight in a ventilated oven at 60°C (AOAC, 1984). Moisture content was calculated as follows:

$$\%\ \text{Moisture} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100\%$$

Ether extract was determined by Soxhlet extraction with petroleum ether (40-60°C) and calculated as:

$$\%\ \text{Ether extract} = \frac{\text{Weight of sample before extraction} - \text{Weight of sample after extraction}}{\text{Weight of sample before extraction}} \times 100\%$$

Minerals: Minerals were determined according to Novosamsky (1983) and Okalebo (1985). Iron content was determined by atomic absorption spectrophotometry, after wet digestion with nitric acid-perchloric acid. Calcium was determined by ethylene diamine tetra acetic acid (EDTA) titration. Potassium and sodium were determined by flame photometry.

Phytochemical studies

Qualitative analysis was carried out according to Malec and Pomilio (2003) and Evans (1996).

Determination of total phenolic compounds (TPC)

TPC was determined spectrophotometrically according to Azizah et al. (2007). The standard curve was prepared as follows: To each gallic acid standard (50, 100, 150 upto 500 mg/l) and sample, 0.5 ml of 0.2 N Folin-Ciocalteau reagent was added. After 8 min, 1.5 ml

sodium carbonate (7.5% w/v) was added. The mixture was kept in the dark for 1 h and absorbance was measured at 765 nm. TPC content was read off the standard curve and expressed as mg of gallic acid equivalent (GAE) per litre of sample.

Microbiological analysis

Sterilization

The solid and liquid media were sterilized by autoclaving at 121°C for 15 min. Glassware was sterilized in the oven at 100°C for 1 h and allowed to cool down before use while the media were left to cool to 45°C before pouring into the Petri dishes

Enumeration and isolation of fungal isolates

Extract of fresh or preserved *V. amygdalina* leaves (1 ml) was aseptically transferred into 9 ml sterile distilled water in McCartney bottles and mixed thoroughly. Serial dilutions up to 10^{-2} and 10^{-3} were then carried out and 1 ml from dilutions 10^{-2} and 10^{-3} were transferred into the Petri dishes. Potato dextrose agar was poured into the Petri dishes and allowed to solidify. The plates were then incubated at ambient temperature (30±2°C) for three to five days and observed daily for growth of fungi. Discreet colonies were counted using a colony counter (Digital Colony Counter-Labtech, UK). Sub-culturing of the occurring fungi was made on sterile PDA plates to obtain pure isolates. The pure isolates were stored in PDA slants at 4°C for further laboratory studies.

Identification of Isolates

Pure cultures of the isolates obtained were used for identification. The identification of the isolates were based mainly on the structural features as observed from the growing colonies in plants (Sutton et al., 1998) and slide mounts seen under the microscope. The plate identification involved colour, presence of mycelia, spores and production of fruiting bodies. In the microscopic examination, a wet mount of each isolate was prepared on a microscopic slide, covered with a cover slip, stained with lactophenol cotton blue dye and viewed under the low and high power magnification to ascertain its features. These features were compared with those described in standard fungi manuals (Barnett and Hunter, 1998; Raper and Fennel, 1973).

Determination of percentage of fungal occurrence

The percentage frequency of occurrence of the fungal isolates was determined by dividing the occurrence of individual fungal isolates with the total occurrence. This was expressed as a percentage as follows:

$$\frac{X}{N} \times \frac{100}{1}$$

Where, X is the total number of each organism in the samples and N is the total number of all organisms in the samples.

Screening for aflatoxin

Screening for aflatoxin was done by the Contaminants Bureau FDA method (Heinrich, 1990; Richard et al., 1993) with modification, as follows:

Sample preparation

Fresh or salted *V. amygdalina* leaves (5 g) were ground to pass a no. 20 sieve, mixed thoroughly and placed in a 50 ml, glass-stoppered Erlenmeyer flask with 2.5 ml of H_20, 2.5 g of diatomaceous earth, and 25 ml of $CHCl_3$. The mixture was shaken for 30 min and filtered. The first 10 ml of the extract to emerge from the filter was collected and placed in the column.

Column preparation

Column chromatography was carried our using a 22 x 300-mm chromatographic tube packed with silica gel and anhydrous Na_2SO_4 in $CHCl_3$. The extract was added on top of the silica gel. The column was washed with 150 ml of hexane, followed by 150 ml of anhydrous ether, which were discarded. Aflatoxins were then eluted with 150 ml of solvent mixture MeOH-$CHCl_3$ (3:97v/v) and the entire fraction was collected and evaporated to dryness.

Thin layer chromatography (TLC)

Residue was dissolved in chloroform-acetonitrile (4:1v/v) and applied on a TLC plate, pre-coated with silica gel 60. Plates were developed for about 1 h using a chloroform-acetone-isopropanol (8:1:1v/v/v) solvent mixture in an equilibrated tank. Plates were removed from the tank, dried and examined under UV light.

RESULTS AND DISCUSSION

Table 1 shows the result of an organoleptic evaluation of the fresh and treated *V. amygdalina* leaves. The fresh leaves were a bright dark green in colour, had a distinctive green leaf smell and were firm, with slightly rough surface. Light brined leaves had a dull dark green colour with a slightly offensive smell. They were deformed and their surface was rougher than that of the fresh leaves. Light salted leaves were similar to the light brined in structure but were closer to the fresh leaves in texture.

Light brine + vinegar treated leaves had an almost smooth texture and their structure was better maintained than that of the light brined and light salted leaves. Leaves subjected to heavy salt treatment had properties similar to those of the original leaves but had a salty taste. Leaves treated with water alone had a dull dark green colour and a very offensive smell. They had a deformed structure and a rough texture. Based on these observations, heavy-salted leaves were the closest to the fresh leaves in organoleptic properties.

The pH values of fresh and fermented *V. amygdalina* leaves are given in Table 2. There was a decrease in pH from 5.88 for the fresh, to 5.80, 5.73, 5.24, and 5.02 for the light brined, light salted, heavy salted and light brine + vinegar treated leaves, respectively. These values indicate that salting resulted in fermentation, with production of acid and decrease in pH, the better preserved heavy-salted and light brine vinegar treated leaves having the lowest values. Leaves treated with water alone had a pH of 6.63.

Table 1. Organoleptic characteristics of fresh and fermented (*Vernonia amygdalina*).

Property	Fresh	Light brine	Light salt	Light brine+ vinegar	Heavy salt	No salt
Colour	Bright dark green	Dull dark green	Dull dark green	Dull light green	Bright dark green	Dull dark green
Odour	Fresh leafy smell	A slight but not offensive smell	A slight but not offensive smell	A slight but not offensive smell	Fresh leafy smell retained	Very offensive smell
Appearance and texture	Firm with slightly rough surface	Slightly deformed structure; surface slightly rougher than that of the fresh leaves.	Slightly deformed structure; similar to the fresh leaves in texture.	Retained more of the original structure than the light salted and light brined. Leaves had an almost smooth texture	Similar to that of the fresh leaves.	Deformed structure rough surface

Table 2. pH values for fresh and fermented *Vernonia amygdalina* leaves.

Treatment	pH
Fresh	5.88
Light brine	5.80
Light salt	5.73
Light brine + vinegar	5.02
Heavy salting	5.24
No salt	6.63

Table 3 shows the nutrient content of fresh and fermented leaves. Compared with the fresh leaves, there was a decrease in the content of all the nutrients investigated, except for sodium and calcium which increased significantly. This is in agreement with previous findings (Oboh and Madojemu, 2010). The water treated leaves had less of each nutrient than the fresh or fermented leaves, except for their protein content, which was not significantly different from that of the heavy-salted and light brine and vinegar treated leaves. Loss of nutrients during the salting of vegetables has been observed previously (Jones and Etchells, 1944) and might be due to leaching into the aqueous medium in which the leaves were fermented. The sodium content was much higher in the salted samples than in the fresh sample because of the addition of salt to the fermentation medium. The higher calcium levels in the salted leaves (relative to the fresh) were due, probably to the presence of calcium impurity in the salt (Jones and Etchells, 1944) and/or the presence of this mineral in the water used in the experiments.

Compared with fresh sample, the moisture content of the fermented leaves showed a significant decrease due to the osmotic effect of the salt. The heavy-salted leaves had the lowest moisture content (less than half of that of the fresh leaves). This, coupled with their lower pH gave an environment less favourable than the fresh leaves, for the proliferation of spoilage microorganisms, resulting in excellent preservation as indicated by the organoleptic properties of the leaves subjected to this treatment.

The results of tests for phytochemicals and the total phenolic compounds content of the fresh and preserved leaves are presented in Table 4 and 5. These compounds when present in the diet could provide biological and pharmacological benefits. Saponins and glycosides were found in all the leaves (fresh, salted and unsalted) and tannins were present in all except the unsalted. Steroids were detected in the fresh, light salted and water treated (unsalted) leaves. Flavonoids and alkaloids were not detected in any of the samples. The fresh sample gave the highest value of total phenolic compounds (TPC). The highest loss of TPC was for the light brine + vinegar preserved leaves (51.69%). The lowest loss was for the badly preserved water treated leaves (27.28%). Of the salted leaves, the heavy-salted and light-salted lost about a third of their TPC (36.05 and 33.42% respectively). Compared with the fresh, light brining resulted in the loss of 45.93% of the total phenolic compounds. Thus, the best preserved leaves in terms of organoleptic properties (that is, the heavy-salted) lost only about a third of their TPC.

Table 6 shows the fungal counts of fresh and fermented leaves. In all cases, salting resulted in a decrease in fungal count. The fresh leaves had a higher fungal count (120×10^2 cfu/ml) than the salted samples (49, 47, 43 and 8×10^2cfu/ml respectively for light brined, light salted, light brine + vinegar treated and heavy salted leaves, respectively). Unsalted leaves had the highest count (292×10^2cfu/ml, about a two and half-fold increase, compared with the fresh leaves). Heavy salting was the most effective

Table 3. Nutrients of fresh and fermented *Vernonia amygdalina* leaves.

Bitter leaf	Fresh	Light brine	Light salt (dry)	Light brine ± vinegar	Heavy salt	No salt
Fe (mg/100 g)	16.50±0.71^{b}	11.80±0.14^{b}	9.80±0.00^{a}	11.20±0.00^{c}	10.15±0.07^{c}	9.65±0.07^{a,c}
Ca (mg/100 g)	235.26±0.01^{c}	257.82±0.01^{c}	245.29±0.01^{c}	242.79±0.01^{c}	240.28±0.01^{c}	220.25±0.07^{a,b}
K (mg/100 g)	23.57±0.01^{c}	24.48±0.01^{c}	27.15±0.07^{c}	25.39±0.01^{c}	20.82±0.01^{a,c}	9.03±0.01^{a,c}
Na (mg/100 g)	1.56±0.01^{c}	165.48±0.01^{a}	161.39±0.01^{a}	204.80±0.14^{a,b}	289.77±0.01^{a}	1.48±0.01^{a}
Protein (%)	4.59±0.01^{b}	3.13±0.00^{a}	2.63±0.01^{a,c}	1.98±0.01^{a,c}	1.65±0.01^{a,c}	2.20±0.01^{a,c}
Carbohydrate (mg/100 g)	0.054±0.039^{b}	0.001±0.001^{a,c}	0.001±0.001^{a,c}	0.022±0.002^{a,c}	0.029±0.002^{a,c}	-
Lipid (g/100 g)	0.85±0.07^{c}	0.55±0.07^{a,b}	0.35±0.07^{a,b}	0.51±0.01^{a,c}	0.62±0.03^{a,c}	0.31±0.01^{a,c}
Moisture (%)	74.60±4.26^{c}	46.00±4.37^{a,c}	48.40±3.50^{a,b}	43.80±7.45^{a,b}	36.90±1.94^{a,b}	48.70±3.52^{a,b}
β-carotene (mg/100 g)	0.44±0.67^{b}	0.35±0.59^{a,c}	0.37±0.58^{a,c}	0.31±0.52^{a,b}	0.28±0.46^{a,b}	0.02±0.02^{a,b}
Vit. C (mg/100 g)	1049.03±2.87^{b}	821.29±0.01^{a,c}	625.52±0.01^{a,c}	680.84±0.03^{a,c}	582.94±0.06^{a,b}	285.12±0.01^{a,c}

Values are recorded as mean ± standard deviation of three independent samples. t-Test: ^{a}, Values differ significantly compared with the fresh sample mean $P < 0.05$; ANOVA: ^{b}, mean values differ significantly from other means within the same group $P<0.05$; ^{c}, values are not significantly different $P < 0.05$ within the same group.

Table 4. Phytochemicals of fresh and salted *Vernonia amygdalina* leaves.

Sample	Saponin	Tannin	Flavonoid	Alkaloid	Steroid	Glycoside
Fresh	+	+	-	-	+	+
Light brine	+	+	-	-	-	+
Light salt	+	+	-	-	+	+
Light brine + vinegar	+	+	-	-	-	+
Heavy salt	+	+	-	-	-	+
No salt	+	-	-	-	+	+

+, Present; -, absent. Values are recorded as mean ± standard deviation of two independent samples. t-Test: ^{a}, values differ significantly compared with the fresh sample mean $P < 0.05$; ANOVA: ^{b}, mean values differ significantly from other means within the same group $P < 0.05$; ^{c}, values are not significantly different $P < 0.05$ within the same group.

for the reduction of fungal load (93.3% reduction) and was therefore the most effective preservation treatment.

Table 7 shows the occurrence of fungal species and genera in fresh and salted *V. amygdalina* leaves. *A. nige*r occurred in the fresh, light brined and the water treated (no salt) leaves. *A. flavus* was associated with all the samples (fresh and treated) and *Penicillium* with the light salted and water treated. *Fusarium* was associated with only the light salt and vinegar treated leaves.

Mycotoxins are small (MW ~ 700), toxic chemical products formed as secondary metabolites by a few fungal species that readily colonise crops and contaminate them with toxins in the field, between harvest and drying, and during storage. The major fungal genera producing mycotoxins are *Aspergillus, Fusarium* and *Penicillium.* The most common mycotoxins are aflatoxins, ochatoxin A, fumonisins, deoxyni-valenol, T-2 toxin and zearalenone (Turner et al., 2009 and Zheng et al., 2006).

A green colour characteristic of aflatoxins B_1 and B_2 (Surekha et al., 2011) was observed when

Table 5. Total phenolic compounds (TPC) of fresh and salted leaves.

Sample	Total phenolic compounds (mg/100g)	Loss relative to the fresh (%)*
Fresh	0.799 ± 0.001^{b}	-
Light brine	$0.432 \pm 0.001^{a,c}$	45.93
Light salt	$0.532 \pm 0.001^{a,c}$	33.42
Light brine + vinegar	$0.386 \pm 0.002^{a,c}$	51.69
Heavy salt	$0.511 \pm 0.001^{a,c}$	36.05
No salt	$0.581 \pm 0.001^{a,c}$	27.28

*Percentage loss relative to the fresh = 0.799 - TPC/ 0.799 x 100.

Table 6. Fungal counts of fresh and fermented *V. amygdalina* leaves.

Sample	10^2 CFU/Ml	loss relative to the fresh (%)
Fresh	120	-
Light brine	49	59.16
Light salt	47	60.83
Light brine + vinegar	43	64.17
Heavy salt	8	93.33
No salt	292	-

Table 7. Occurrence of fungal species and genera, and aflatoxin in fresh and preserved leaves.

Leaves	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium* spp.	*Fusarium* spp.	Aflatoxin
Fresh	+	+	-	-	+
Light brine treated	+	+	-	-	+
Light salt treated	-	+	+	-	+
Light brine + vinegar treated	-	+	-	+	+
Heavy salt treated	-	+	-	-	+
Water (no salt) treated	+	+	+	-	+

+, Present; -, absent.

Table 8. Frequency of occurrence of fungi isolates.

Fungal isolate	Percentage frequency of occurrence
Aspergillus flavus	79.12
Aspergillus niger	74.21
Penicillium sp.	48.26
Fusarium sp.	19.36

leaf extracts were cleaned up, separated by thin layer chromatography (TLC), and viewed under ultra violet light. Aflatoxins are highly toxic and carcinogenic secondary metabolites of *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius. A. flavius* produces only aflatoxin B_1 and B_2, while the other two species produce both aflatoxins B and G (Baydar, 2007). In this study, only *A. flavus* was found to be associated with the leaves. Some species of *Fusarium* produce the mycotoxins zearalenone and fumonisin B_1, which are possibly carcinogenic in humans. One species of *Penicillium, Penicillium verrucosum* produces ocratoxin, which is suspected to be a human carcinogen (GASGA, 1997).

Results (Table 8) show that the percentage frequency of occurrence of fungi associated with fresh, salted and unsalted leaves was highest for *A. flavus* followed by *A.*

niger, and Penicillium. Fusarium had the lowest occurrence. Unlike the other treatments which gave leaves with two or more organisms, only *A. flavus* was found in the heavy salted leaves, which in addition, had the lowest fungal count.

Conclusion

In terms of organoleptic properties, reduction in fungal count and genera, and moisture reduction, heavy salting appears to be the most effective preservation treatment. These, coupled with the only moderate loss of nutrients and phytochemicals recommend it as an effective technique for the preservation of *V. amygdalina* leaves. A rigorous study is required to identify and quantify the mycotoxins present in the preparation.

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Full Length Research Paper

Use of *Lactococci* isolated from Moroccan traditional dairy product: Development of a new starter culture

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Ninety (90) strains were isolated from Moroccan traditional dairy product and identified using biochemical and molecular tests. Among the 20 identified lactic acid cocci, two strains were selected for their important acidifying activity: *Lactococcus lactis* subsp*. lactis* (AML8) and *Lactococcus lactis* subsp*. cremoris* (BML2). Both revealed remarkable acidifying activity, especially when they were mixed in cultures, even in greater values than in the traditional Lben (a Moroccan dairy product). Sensory analysis showed that the so fermented milk has been more appreciated than the Lben. These results show the potential of the two strains as possible culture starter for fermented dairy product.

Key words: *Lactococci*, acidifying activity, fermented milk, Lben.

INTRODUCTION

The isolation of microorganisms from natural habitats is, for the food industry, an inexhaustible source of new and useful strains. Lactic acid bacteria (LAB) are important for the food and dairy industries, because lactic acid, as well as other organic acids produced by these bacteria is a natural preservative (Mohd Adnan and Tan, 2007). Among LAB, *Lactococcus lactis* is the most extensively studied organism with many works dealing with the physiology of growth, carbon and nitrogen metabolism, or regulatory networks. *Lactococcus lactis* metabolism is described as homo-fermentative and produces only L (+) lactic acid (2-hydroxypropanoic acid) with high yield (Kaloyan et al., 2008).

In Morocco, the Lben is one of a large variety of traditional fermented dairy products highly appreciated by consumers. Despite their usual non-conformity to the official regulatory standard they contribute undeniably to urban-rural income (Benkeroum and Tamime, 2004). Reduced costs and low energy requirements of such industries helps stimulate rural and semi rural development. The biodiversity of the microorganisms involved in this process is a fundamental factor for the conservation of the specificity and original characteristics of dairy products. Lactic acid bacteria, belonging especially to the *Lactococcus* and *Leuconostoc* genus, are the dominant microorganisms in traditional Lben (Tantaoui et al., 1983). Lben contains also a considerable amount of yeasts (*Candida lusitaniae, Candida tropicalis* and *Candida albicans*) (Samet-Bali et al., 2012). *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus* have also been detected (Benkerroum and Tamine, 2004; Feresu and Nyathi, 1990). This indicates that, despite real efforts in this field, the technology remains often applied without a global understanding of the principles regulating the fermentation process and the conditions required to ensure quality and safety of the product. Such approach

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presents a significant risk for consumer health and leads to marketing damaging (Motarjemi, 2002). In this context, many traditional Moroccan dairy products failed their transfer to the industrial scale which requires a good knowledge of the performances and the technological potentialities of the wild-type lactic bacteria implied in their fermentation.

The objective of the present study was to identify, phenotypically and genotypically, *Lactococci* isolated from traditional Moroccan dairy product samples and to screen these isolates for desirable properties such as acidity. The sensorial characteristics of fermented milk obtained by using these strains will also be compared with those of the traditional Lben.

MATERIALS AND METHODS

Traditional Moroccan dairy product samples

A total of 50 samples of traditional dairy product were collected and aseptically transported in iceboxes (4°C) from different areas of Morocco (northern, north-central and east regions), mainly from small farms located around the cities. The samples were collected in various seasons, over a period of two years.

Isolation of LAB

10 ml of traditional dairy product sample were homogenized with 90 mL of normal saline (9 g/ L) then 10 fold serial dilutions were prepared using autoclaved normal saline and 1 ml of each serially diluted sample was poor-plated in duplicate on MRS and M17 agar media (Biokar, Diagnostics, France) and incubated at the optimal temperature at 35°C for 24 h.

After incubation, the isolated colonies were purified by streaking twice onto appropriate agar media plates. For immediate use, they were maintained at 4°C and streaked every 4 weeks on agar plates. For long-term storage, purified isolates were kept at -20°C in skim milk plus glycerol (85/15 (v/v).

Identification of LAB

Morphological and biochemical identification

The isolates obtained were identified by morphological and biochemical techniques according to the criteria given by Teixeira (2000). All isolates were initially characterized for their morphology by microscopy and Gram staining, and then tested for oxidase and catalase production. Gram positive and Catalase negative cocci were used for further identification. Growth at 10, 35 and 45°C was examined on M17 broth. Carbohydrate fermentations were determined twice with the aid of the API 50 CH medium according to the manufacturer's instructions (BioMerieux, Marcy-l'Etoile, France). Salt tolerance was tested by incorporating 2, 4 and 6.5% of NaCl on M17 broth. Production of CO_2 was also evaluated in M17 broth containing inverted Durham's tube.

Molecular identification

Genetic identification of presumptive *Lactococcus* sp. was carried out by molecular techniques based on the amplification and sequencing of the 16S rRNA gene. This methodology is currently the most used for bacterial phylogeny. It allowed the establishment of large databases. Bacterial DNA extraction from bacteria was carried out according to standard methods (Marmur, 1961). For polymerase chain reaction amplification, universal primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-TACGGCTACCTTGTTACGACTT- 3') were used to amplify the 16S rRNA gene (Weisberg et al., 1991). Direct sequencing of the PCR products was performed on an ABI PRISM sequencing apparatus (ABI Prism 3130 Genetic Analyser, Applied Biosystem) and data analysis was done by sequence analysis software (Sequence analysis 5.2.0, Collection software: foundation data collection version 3.0).

Sample preparation and physico-chemical characterization

Culture conditions

Pure and mixed cultures: Individual strains were pre-grown for 24 h at 35°C in Ultra High Temperature treated milk (UHT). Subsequently, 2% (v/v) of each culture was added to 100 mL UHT milk to obtain approximately 10^7 CFU/ mL and incubated at 35°C. Mixed cultures of two strains were performed at 35°C by inoculating UHT milk with 1% of each strain (pre-grown for 24 h).

Traditional way of manufacturing Lben: The traditional Lben was prepared in the laboratory by spontaneous fermentation of raw milk at the temperature of 35°C until coagulation which may take up to 16 h. For gelation, the product, known as Raib, is churned during approximately 45 min for obtaining the Lben and some amount of raw butter called zebda beldia (Tantaoui-Elaraki et al., 1983; Benkerroum et Tamine, 2004).

Physico-chemical characterization

The dry matter, fat content, pH, titrable acidity (equivalent lactic acid), lactose, enumeration of cells and biomass were followed. Each measurement was performed in quadruplicate.

Dry matter: Dry matter was determined according to standard methods (Afnor, 1993).

Fat content: Fat content in milk was determined by Gerber method as described by FAO (1997). Into butyrometer, 10.94 mL of sample was slowly added to 10 mL of sulphuric acid, then 1 mL of Amyl alcohol. The mixture was thoroughly stirred and centrifuged at 1100 rpm for 5 min. Then, the butyrometer was removed and placed in water bath at 65°C from 3 to 4 min. The fat content (in percent) was established according to the butyrometer scale.

pH: The pH values of inoculated milk were continuously measured by a glass electrode pH-meter (Inolab pH 730) and recorded automatically at 3-min intervals.

Titrable acidity: Titrable acidity, expressed in Dornic Degree (°D), was determined by titration with a standard solution of NaOH 0.1N using phenolphthalein as the indicator.

Lactose: Samples preparation and lactose analysis was performed according to the method of Fraysse et al. (2003) using an Agilent HP3DCE capillary electrophoresis instrument (Waldbronn, Allemagne) equipped with a CE LIF/UV cassette. The laser induced fluorescence detector was a Zetalif Discovery (Picometrics, France) coupled with a 488 nm argon laser (26 mW) (Spectra Physics, USA). Capillary (Polymicro Technologies, USA) dimensions were 14 cm effective length and 50 µm internal diameter. Running buffer was aminocaproic acid 40 mM with 0.02% hydroxypropylméthyl-

cellulose at pH 4.5 and the electrophoretic separation was conducted at 20 kV. Calibration was performed by labeling standard lactose solution prepared in M17 medium.

Enumeration of cells: Viable cell counts (CFU/mL) were determined by plating diluted samples in sterile normal saline on M17 agar, and incubating for 48 h at 35°C.

Biomass: Biomass concentration was determined by optical density (OD) measurement using the method of clarification described by Raynaud et al. (2005). One volume of milk culture was mixed with two volumes of a solution of clarification (EDTA 15 mm, NaOH 0.15 mm). The cellular pellets were washed 3 times with normal saline (NaCl 0.9 %) and suspended in the same solution again. The absorbance at 580 nm of the sample can then be measured directly or after dilution, in order to remain the spectrophotometer linearity range.

Sensory analysis

Sensory analyses have been conducted by a panel of five persons, previously trained for the sensory evaluation of manufactured dairy products. They were invited to compare the dairy product obtained by mixed culture to traditional Lben. The evaluation was scored in a 9-point negative to positive scale according to the method of Zamora et al. (2011).

RESULTS AND DISCUSSION

Identification of isolates

A total of 90 strains were obtained from 50 traditional dairy product samples harvested in different regions of Morocco. Twenty (20) isolates was identified as Gram-positive cocci. The colonies observed on M17 plates were small (1 mm diameter), compact and regular. Cells were revealed to be catalase and oxidase negative, non-spore-forming and homofermentative (absence of CO_2 production during glucose and galactose fermentations).

On API 50 CH trays, the 20 isolated cocci produced lactic acid from lactose, glucose, galactose and fructose. They were subdivided into two groups: (1) strains ADH (+) (arginine dihydrolase), acetoin (-) growing at 45°C and with 4% of NaCl, (2) strains ADH (-), acetoin (-) unable to grow at 45°C, sensitive to 4% salt concentrations. These results suggest that the group 1 isolates belong to the *Lactococcus lactis* subsp *lactis* and group 2 to the *Lactococcus lactis* subsp *cremoris*. In fact, according to the study of Badis et al. (2004) these tests distinguished lactococci from other related microorganisms and differentiated between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris.* Subsequently, the 20 strains were genetically identified. 16S rRNA genes were amplified by PCR. The sequences obtained with fd1 and rp2 primers were 571 bp and 505 bp, respectively. It has been reported that the initial 500-bp sequence provides adequate differentiation for bacterial identification (Clarridge, 2004). Some reports indicate that less sequence was required: 400 bp or even less than 200 bp (Wilck et al., 2001). The sequences were compared to the EMBL, Gen Bank, DDJB and PDB databases, using BLAST NR 2.2.11 software through the National Center for Biotechnology Information (NCBI). The alignment of these sequences with nucleotide databases revealed a very high similarity (99%), with the nucleotide sequences of the genes coding for 16S rRNA bacterial strains of genus *Lactococcus* only. For twelve strains of *Lactococcus* (AML), the percentage of the highest similarity (99%) was obtained with strain of *Lactococcus lactis* subsp. *Lactis* (I 11403). For eight other strains of *Lactococcus* (BML), the percentage of the highest similarity (99%) was obtained with strain *L. lactis* subsp. *cremoris* (MG1363).

Acidifying activity

The lactic acid plays a particularly important role in fermented dairy industry. It acts as a natural preservative and for sensory characteristics of the product (acidity, flavor). Therefore, acidification activity is an important parameter for the selection of lactic starter culture strains. The time required to acidify milk from pH 6.7 to 5.5 (tpH 5.5) was determined at 35°C for 12 *L. lactis* subsp. *lactis* strains (AML) and the eight *L. lactis* subsp. *cremoris* strains (BML). Each strain was inoculated in milk in quadruplicate. As shown in Table 1, the time to reach pH 5.5 (tpH 5.5) by *L. lactis* subsp. *lactis* strains was significantly lower than by *L. lactis* subsp. *cremoris* ($P < 0.05$). The observed differences between both *Lactococci* (BML2 and AML8) may be due the fact that acidifying activity of each strain is linked to its specific proteolytic activity and nutrient transport system (Albenzio et al., 2001). Moreover, following the pH in milk during growth of the *L. lactis*, two strains characterized by low tpH 5.5 values could be highlighted out of the 20 strains; *L. lactis* subsp. *lactis* (AML8; tpH 5.5: 198 ± 12 min) and *L. lactis* subsp. *cremoris* (BML2; tpH 5.5: 237±13 min). These tpH 5.5 are inferior to that obtained in the study of Casalta et al. (1995) by *L. lactis* (416 min) in the same type of milk (cow's milk). Low values for tpH 5.5 reflect a high acidification activity for the considered starter culture (Chammas et al., 2006). A rapid decline of pH during the initial step of dairy fermentation is essential for the milk coagulation and the prevention or reduction of undesired microflora development. Two strains (AML8 and BML2) were retained for further experiments.

The optical density (OD) of the cultures was determined at the beginning of fermentation. They were 0.31 for *L. lactis* subsp. *lactis* and 0.29 for *L. lactis* subsp. *cremoris* in milk. This indicates that the number of cells used in the preparations was very similar, indicating well controlled procedures**.**

Pure culture of L. lactis subsp. lactis (AML8)

The titrable acidity was 56.7°D after 6 h and 64.4°D after

Table 1. Time (min) required to acidify milk from pH 6.7 to 5.5 at 35°C.

Lc. lactis		*Lc. cremoris*	
Strains [a]	**tpH 5.5 (min)** [b]	**Strains** [a]	**tpH 5.5 (min)** [b]
AML1	202± 12	BML1	241±17
AML2	295± 12	BML2	237±13
AML3	216±13	BML3	303±14
AML4	264±12	BML4	266±11
AML5	245±11	BML5	298±12
AML6	226±9	BML6	317±11
AML7	235±12	BML7	323±15
AML8	198±12	BML8	292±11
AML9	236± 17		
AML10	218±11		
AML11	229±13		
AML12	237±18		

[a]AML, *L. lactis* subsp. *lactis* strains ; BML, *L. lactis* subsp. c*remoris* strains. [b]Values ±SD. Inoculation percentages were 2%.

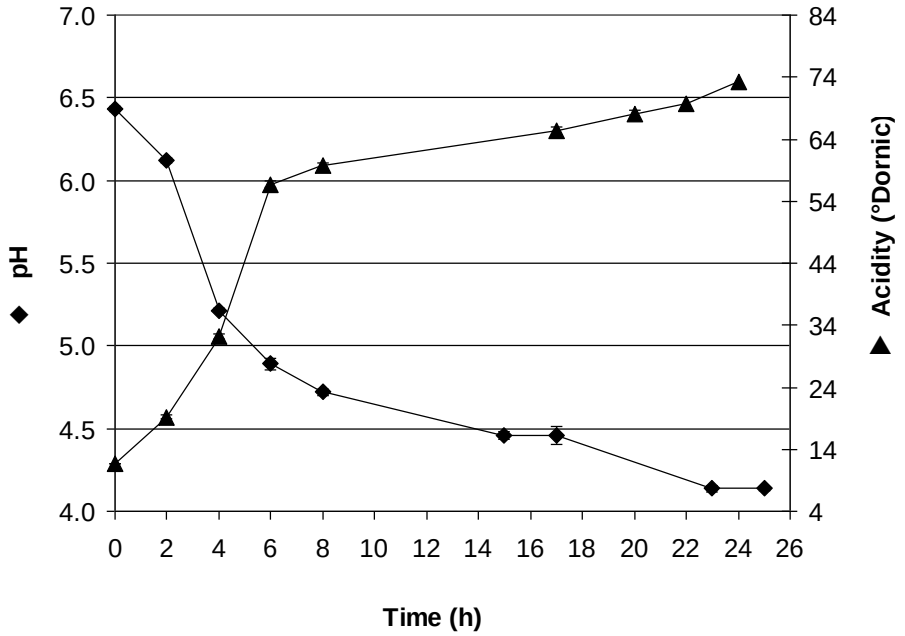

Figure 1. : Mean pH and acidity values of the cultures of *L. lactis* subsp. *lactis* AML8 in milk during 24h. ◆ pH; ▲ acidity.

16 h (Figure 1). These values are similar to those obtained by Alonso-Calleja (2002) who reported approximately 54°D after 6 h for milk inoculated with *L. lactis* subsp. *lactis*.

The pH of *L. lactis* subsp. *lactis* (AML8) cultures correlate well with the titrable acidity (Figure 1). Indeed, his progressive increase is accompanied with a decrease of pH values which reaches the value of 4.46 after about 16 h (Figure 1). The highest value of titrable acidity (73.2°D) was reached after 24 h. At the same time, the pH reached the value of 4.15.

Martinez-Moreno (1976), Nunez and Medina (1979), Mas and Gonzalez-Crespo (1992) and Alonso-Calleja (2001) divided the strains of *L. lactis* subsp. *lactis* into two groups, depending on the acid production rate: fast acid producer (F) strains and slow acid producer (S) strains. They have shown that the difference between fast (F) and slow (S) acid producer strains is the titrable acidity at 6 h: higher than 30°D in F strains. Moreover, they indicate

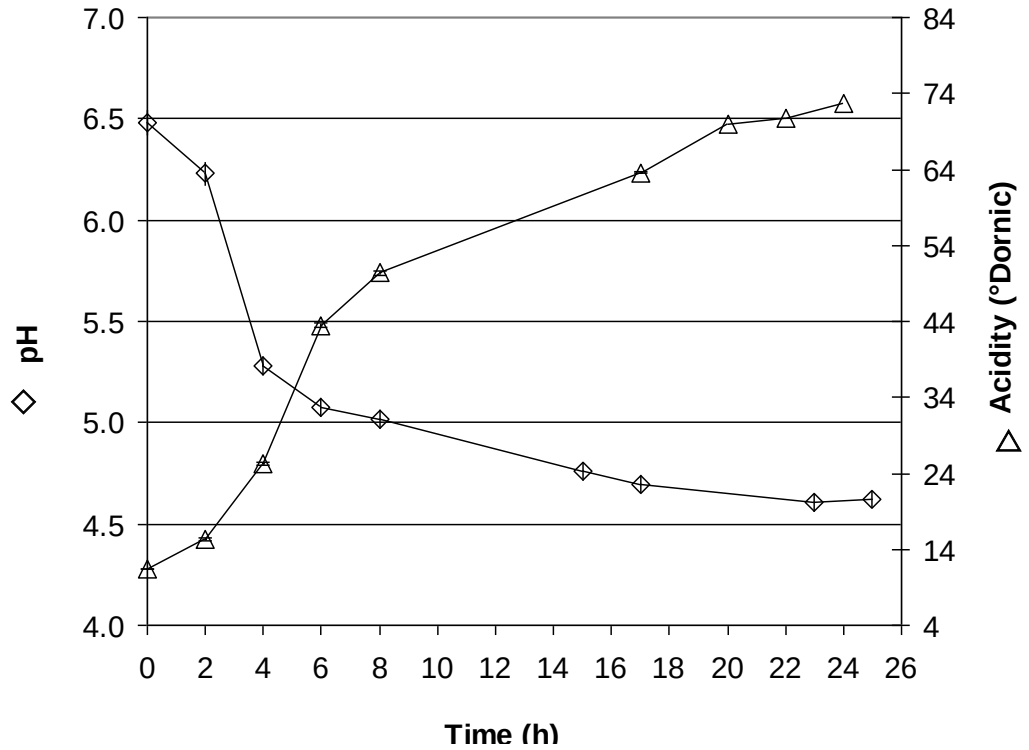

Figure 2. Mean pH and acidity values of the cultures of *L. lactis* subsp. *cremoris* BML2 in milk during 24h. ◇ pH; △ acidity

that the rapid acid producer *L. lactis subsp. lactis* strains have the potential as a starter culture in industry.

These data are very interesting as they indicate that *L. lactis* subsp. *lactis* AML8 strain, whose titrable acidity widely exceeds 30°D at 6 h, is a good candidate for use in fermented dairy product.

Pure culture of L. lactis subsp. cremoris (BML2)

The pH and titrable acidity in pure culture of *L. lactis* subsp. *cremoris* (BML2) behavior were similar to those of *L. lactis* subsp. *lactis* (AML8). However, the titrable acidity obtained was inferior, in particular after 6 h of incubation. The value obtained was 43.5°D (Figure 2). It is nevertheless higher than 30°D; therefore we can also consider the *L lactis* subsp. c*remoris* strain as fast acidifying strain.

Mixed culture of L. lactis subsp. cremoris (BML2) and L. lactis subsp. Lactis (AML8)

In mixed culture, the titrable acidity was compared with those obtained in pure culture. The titrable acidity after 16 h incubation was significantly higher in mixed culture (78.6°D) (Figure 3) than pure culture (64.4°D) ($P < 0.05$). Similarly, after 24 h of incubation, the value of titrable acidity reached in the mixed culture was 91.5°D, whereas it was only 73.2° D for pure cultures.

These results suggest a synergy relationship between the two subspecies involved. Our finding are in agreement with those obtained by Kimoto-Nira et al. (2012), indicating that a mixed culture of two different species, *L. lactis ssp. lactis* strain 54 and *Lactococcus raffinolactis* strain 37; stimulated greater acid production during fermen-tation in milk than occurred with pure culture fermentation.

The pH of milk fermented with mixed cultures and the Lben presented similar behaviors (Figures 3 and 4). Both of them decline from an initial value of 6.8 and 6.7 respectively to reach, after 24 h, the value of 4.5. In the milk fermented with mixed culture, the value of titrabe acidity is correlated with that of pH. The progressive decline of the latter was accompanied by the increase in the titrable acidity which reached 91.5 °D after 24 h. In contrast, in traditional Lben, the value of titrable acidity remained low (around 15°D) in the early hours. It reaches the value of 39°D only after 10 h; even sometimes after 14 h of incubation. Therefore, the decrease in pH observed within the first hours of incubation of traditional Lben (Figure 4), is not correlated to the titrable acidity. This could be explained by the fact that the traditional Lben is produced by an undefined mixture of microorganisms found in the raw milk able to produce a variety of other acids resulting in a pH decrease (Benkerroum and Tamine, 2004; Feresu and Nyathi, 1990).

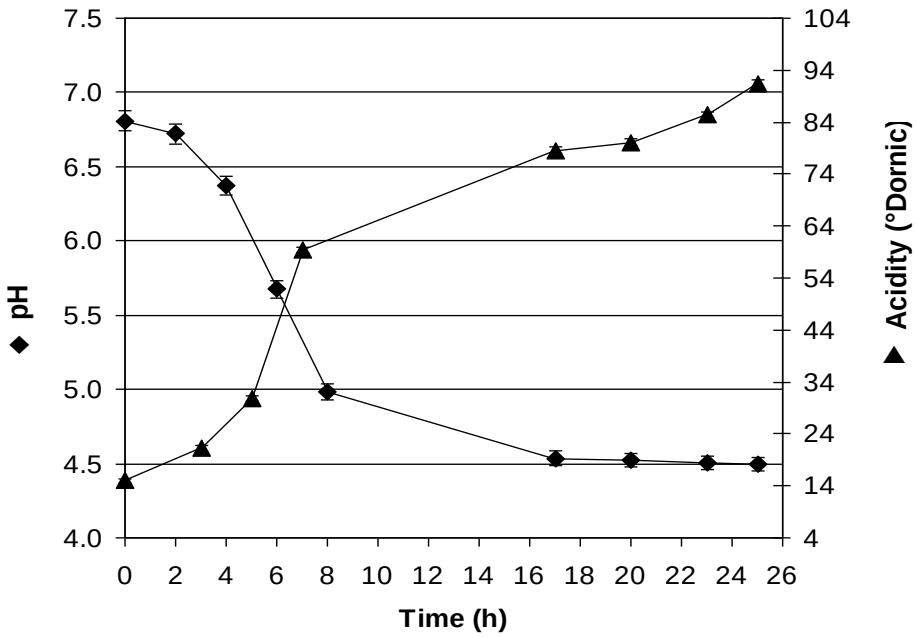

Figure 3. Mean pH and acidity values of the mixed culture in milk during 24h. ◆ pH; ▲ acidity.

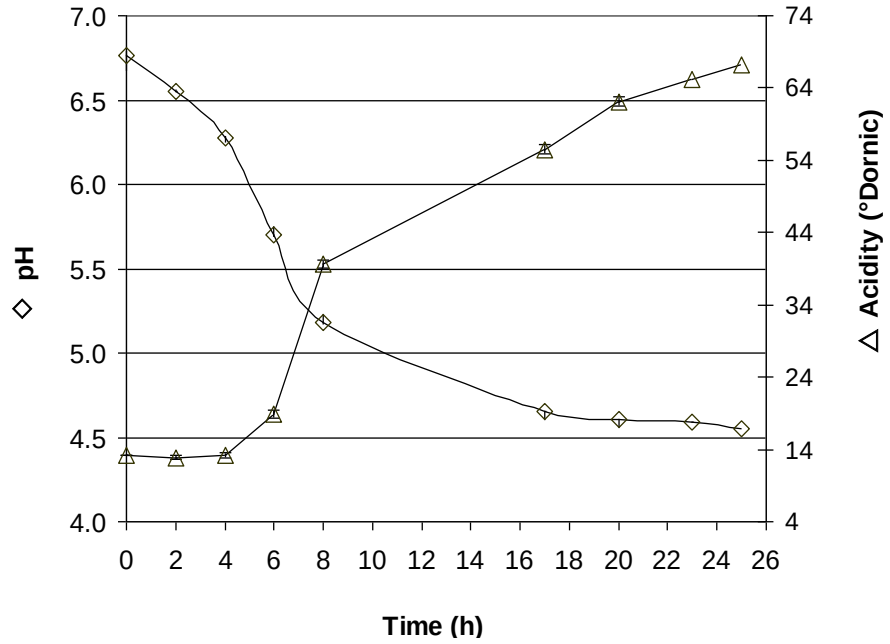

Figure 4. Mean pH and acidity values of the Lben during 24h. ◇ pH; △ acidity.

The progressive acidification of the medium promotes the lactic acid bacteria activity and thus the titrable acidity, but only after 16 h.

Generally, the manufacturers cannot control the natural acidification demonstrating irregular kinetics and therefore results in the heterogeneity of the products obtained. Moreover, bad hygiene results in the presence of hazardous microorganisms in the final product. Therefore, the acidification managed by using well controlled starter cultures is a less risky alternative than natural sowing.

Table 2. Physicochemical analyses of sterilized whole milk, fermented milk, raw milk and traditional Lben.

Sample	pH[a]	Titrable Acidity (°Dornic) [a]	Lactose (g/L) [a]	Fat content (g/L) [a]	Dry matter (g/L) [a]
Sterilized whole milk	6.08±0.01[b]	15.00±0.14[b]	50.00±0.18[b]	30.54±0.15[b]	104.00±0.67[b]
Fermented milk	4.49±0.01[c]	91.50±0.26[c]	30.00±0.38[c]	28.40±0.42[c]	96.20±0.58[c]
Raw milk	6.76±0.02[b]	13.20±0.25[d]	40.00±0.17[d]	32.50±0.54[d]	110.00±0.45[d]
Traditional Lben	4.55±0.02[c]	67.20±0.32[e]	29.90±0.22[c]	5.20±0.36[e]	87.90±0.81[e]

[a] Mean value±S.D. Means are average from four samples.[b, c, d] and [e] The letters indicate significant differences (P<0.05) between samples.

Table 3. Sensorial parameters.

Paramter	Sensorial parameter	Mean [a]
Odor and flavour	Lacteous	-0.93 ± 0.55
	Yeast	-0.40 ± 0.67
	Fermented	2.00 ± 0.31
Texture and taste	Viscosity	3.00 ± 0.44
	Color	-0.20 ± 0.37
	Acid	1.20 ± 0.37

[a] Mean value±S.E. Mixed culture is compared to Lben. Odor, flavor, texture and taste were scored in a 9-point negative to positive scale (0=no differences with Lben ; ±1=minimal differences; ±2=noticeable differences; ±3=considerable differences; ±4: very considerable differences).

Physico-chemical characterization

During fermentation, the lactose content decreased in fermented milk and in traditional Lben below 30.00±0.38 g/L (40%) and 29.90±0.22 g/L (33%) respectively. Thus, the breakdown of lactose increases the titrable acidity to more than 90°D in fermented milk and 67.20°D in traditional Lben. These results were similar to those reported by Tantaoui-Elaraki et al. (1983) for Moroccan Lben and Samet-Bali et al. (2012) for Tunisian Lben.

The observed difference in fat and dry matter in fermented milk and traditional Lben could be due to different processing conditions. Thus, the use of whole milk in the former case and the elimination of butter granules after churning operation for making traditional Lben significantly reduce its fat content (5.20 ± 0.36 g/L).

Sensory analysis

During growth in milk, the bacteria exert an influence on the microstructure and the sensorial properties of coagulated milk. *L. lactis* subsp. *lactis* strains are resistant to stress and can grow well to provide adequate acidification required for curd formation. *L. lactis* subsp. *cremoris* strains, more sensitive to stress, may contribute to the development of sensory properties (Kim et al., 1999).

For the obtained products, the panel detected a difference in all the sensory attributes (Table 3). The milk fermented with mixed culture has been classified as less yeasty and less lacteous. Generally, the best perception of odor in the Lben could be due to ethanol, volatile compound found in the Lben (Benkerroum and Tamine, 2004).

On the other hand, the values attributed to the viscosity and the acid taste indicated a better perception of the so fermented milk than the Lben. It is very likely that the higher fat content of fermented milk has contributed to its better taste. The correct texture of the fermented milk could be related to the presence of exopolysaccharides (EPS). Gruter et al. (1992) previously reported their production by *Lactococcus lactis* species and we have also detected their synthesis by the species used in this work (results not shown).

Conclusion

This study describes the isolation, identification and acidifying activity of wild strains of lactic acid bacteria from traditional dairy products. It has allowed us to distinguish two interesting strains.The collected data, especially their acidifying capacity, revealed they both have a high potential for future use in the production of fermented dairy products. The mixture culture in milk of these two strains

provided a safe fermented dairy product with the desired sensory features. Additional studies are underway to better determine others technological properties of these strains, especially the production of EPS and aroma. This could lead to the selection of these bacteria to produce a new mixed starter.

On the other hand, they may be used in the artisanal dairies which are very common in Morocco. This would allow a better control of fermentation conditions, and therefore better products quality.

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Full Length Research Paper

Effects of natural plant tenderizers on proteolysis and texture of dry sausages produced with wild boar meat addition

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This study was conducted to develop a method for improving tenderness and overall qualities of tough wild boar meat used to dry sausage production with direct addition of raw pineapple (*Ananas comosus*), mango (*Mangifera indica*), kiwifruit - fuzzy kiwi (*Actinidia deliciosa*), or ginger (*Zingiber officinale roscoe* - ginger rhizome) juices contained a plant proteolytic enzyme. Dry-sausages were subjected to various chemical, mechanical and sensory evaluations. An increase in proteolysis was observed in all enzyme-treated samples compared to the control and as a consequence an improvement in juiciness, tenderness and overall acceptability scores were observed. Ginger or kiwifruit juice-treated sausages received better scores for texture, flavor, and overall acceptability. From these results, it is shown that those enzymes as a raw plant juices could be used as tenderizers in dry sausage production.

Key words: Dry sausages, wild boar meat, plant enzymes, proteolysis, texture, sensory properties.

INTRODUCTION

The technology of dry-cured sausages allows many variations as long as the basic concepts (reduction of pH and water activity) are kept in mind (Roca and Incze, 1990). Consequently, these products vary greatly across all of the producer countries, although their manufacture always involves a combination of fermentation and dehydration processes. During the ripening of fermented sausages, the proteins and lipids experience great changes. Proteolysis influences both texture and flavor development due to the formation of several low-molecular-mass compounds, including peptides, amino acids, aldehydes, organic acids, and amines. All of them are important flavor compounds or precursors of flavor compounds (Demeyer et al., 1995; Fadda et al., 2001). Lipolysis plays an essential role in the development of dry-sausage flavor. Lipids are hydrolyzed by enzymes, generating free fatty acids, which are substrates for the oxidative changes that are responsible for flavor compounds (Samelis et al., 1993; Stahnke, 1995; Verplaetse, 1994).

Fermented sausages are usually prepared from seasoned raw meat stuffed in casings and allowed to ferment and mature (Moretti et al., 2004; Živković et al. 2012). The meat of different species of adult, well-fed animals is preferred in raw sausage production: Pork, beef, poultry but also goat, lamb and venison, or combinations thereof. The use of game in meat technology is worth emphasizing, especially since in recent

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years consumer interest in game meat as an alternative for pork and beef is now increasing. The reason for the increase in consumption of venison is its high nutritional value. Thus cured, fermented, and dried products from different game species have appeared on the market (Paleari et al., 2000; Soriano et al., 2006). Despite the increased popularity of game meat, there is a lack of research comparing the nutritional and sensory qualities of meat and meat products, especially fermented sausages, from different game species (Vioque et al., 2003; Soriano et al., 2006; Van Schalkwyk et al., 2011).

It is well known that venison's lack of rich fat, a little thicker connective tissue and/or higher amount of red fibres, compared to for example pork meat can cause it to become tough (Lachowicz et al., 2004; Żochowska-Kujawska et al., 2009, 2010). In the last years different tenderization techniques of beef and sheep meat as well as venison were applied. These techniques include mechanical tenderization, elevated-temperature storage, calcium chloride injection, electrical stimulation, muscle stretching, shock-wave pressure, and enzymatic tenderization (Koohmaraie, 1992; Cheftel and Culioli, 1997; Żochowska-Kujawska et al., 2012).

The enzymes of vegetable origin, such as papain from papaya, bromelin obtained from raw pineapple, ficin derived from figs and zingabaine from ginger as well as bacterial collagenase (Foegeding and Larick, 1986; Stanton and Light, 1987; Dransfield and Etherington, 1981; Naveena et al., 2004; Weiss et al., 2010) were often used for postmortem meat tenderization. These enzymes have regulatory approval (U.S.D.A) for meat tenderization and have been used in various forms as marinades, injection in brine, pre-slaughter injection into the animal's vascular system, and incorporation into various spices as meat tenderizers (Dransfield and Etherington, 1981). These exogenous enzymes have very broad specificities and, therefore, generally indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), sometimes resulting in over- tenderization and a mushy-textured product (Miller et al., 1989).

The proteolytic activity of these enzymes is still a matter of discussion. The same enzyme can show differing results in activity levels and optimal pH and temperature ranges depending upon the substrate (Sullivan and Calkins, 2010). For example Sullivan et al. (2010) studied the tenderization effects of seven enzyme randomized treatments (papain, ficin, bromelain, homogenized fresh ginger, *Bacillus subtilis* protease, and two *Aspergillus oryzae* proteases) in *Triceps brachii* and *Supraspinatus* and they found that except for ginger treatment, all samples treated with enzymes showed improvement in both sensory and instrumental tenderness analysis, however, papain was the enzyme that caused the greatest tenderness in meat. Whereas Ionescu et al. (2008) found a better tenderization for samples treated with bromelin compared to those treated with papain. Kiwifruit has also been studied as a source of actinidin and Han et al. (2009) investigated the ability of prerigor infusion of kiwifruit juice to improve the tenderness of lamb. It was confirmed by Wada et al. (2002) who found that a purified form of actinidain increased the solubility of collagen and thus making it attractive for improving the tenderness of semimembranous muscle.

Addition of enzymes such as lipases (Fernandez and Rodriguez, 1991) and aspartyl-proteinases (Díaz et al., 1993) to dry fermented sausages to enhance flavor has been already considered but with conflicting results. However according to the study of Melendo et al. (1996) plant enzymes such as bromelain have a significant effects on texture of dry sausages, especially with shorten drying period. Despite the increased popularity of natural techniques of improved game meat (Żochowska-Kujawska et al., 2012), there is a lack of research establishing exactly, which enzyme is more suitable to texture and sensory properties of game dry sausages.

The study presented here aimed at evaluating the effects of selected plant enzymes used in our experiment as raw plant juices on the texture and sensory properties of dry sausages produced using traditional process with wild boar meat addition.

MATERIALS AND METHODS

Raw materials

Investigations have been done on dry-sausages produced from pork and wild boar lean meat. Pork was obtain from commercial pigs (about 6 months of age) whereas venison was from a total of 9 male of wild boars hunted during spring in the forest of the Western Pomeranian district in Poland, kept at 4°C for 48 h after shot. The carcass weights of the wild boars were 40±3 kg while their ages were about 3 years. Carcasses were used to obtain hams of pH 5.6 - 5.8. Each ham was deboned, and cleaned of external fat. The lean meat from trimming of biceps femoris semimembranusus and *Quadriceps femoris* muscles was used for sausage production.

Dry-sausages were prepared using a traditional production process as follows: 40% lean pork, 50% lean wild boar meat, 10% pork belly, 3% curing salt, 1% sugar, and spices (red pepper, garlic). Microflora of those traditionally produced fermented sausages originated from the raw material or from the environment in which the sausages were made.

Lean meat and pork belly were minced individually using a 14 mm plate. Sausage mixture was divided into five batches and raw pineapple, mango, kiwifruit, and ginger juices contained enzymes such as bromelain, magneferin, actinidin, and zingabain, respectively, were added to four of them at 5%. Each raw juice was obtained from fresh fruits, purchased at the local market, after peeled, diced and homogenized for 5 min and then centrifuged at 1000 × g for 5 min. The obtained supernatant was used for further study.

The last batch produced without any plant enzymes addition was the control. All batches were stuffed into 45 mm diameter natural casings (15 sausages were made from each batch) and the sausages were held for 48 h at 22-26°C and 80-85% relative humidity (RH) to allow fermentation. Afterwards, sausages were transferred to a drying chamber at 14-18°C and 70-80% RH and ripened for 14 days. Then sausages were divided into two parts and to counteract a substantial reduction of the sausage weight due to the evaporation, one of them was stuffed in additional fibrous

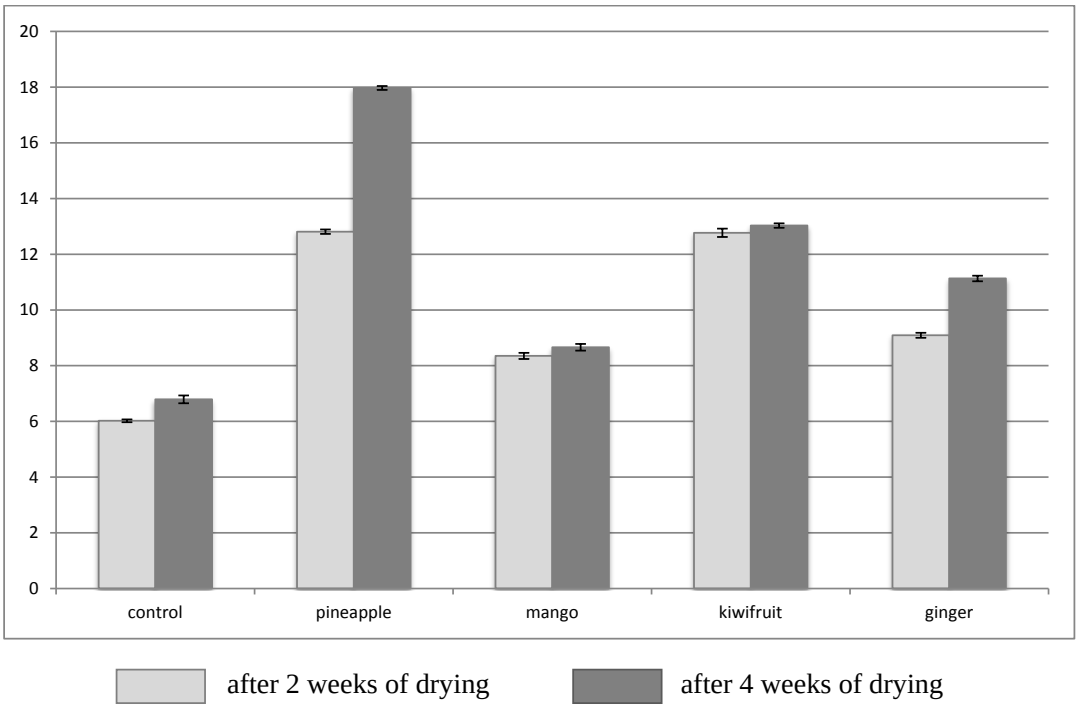

Figure 1. Proteolysis index of dry cured sausages produced with natural plant enzymes addition after two and four weeks of drying.

collagen casings and subjected ripened for another 14 days at 12-14°C and 70-75% RH. Sausages were evaluated at 14 day of ripening (first batch) and at the end of ripening process (28 days, second batch).

Methods

Proteolysis index

The sausages were ground separately in a meat grinder after removing the casing. Proteolysis index were determined as total nitrogen in ca. 2 g of minced sausage by using the Kjeldahl method and calculated as N x 6.25 following the AOAC (2012) method and expressed as percent ratio between the nitrogen soluble in 5% trichloroacetic acid and the total nitrogen (Careri et al., 1993). The proteolysis index was measured 5 times on each batch.

Texture measurement

Sausage texture were measured following the texture profile analysis (TPA) procedures (Bourne, 1982), with an Instron 1140. The test involved driving a 0.61 cm diameter shaft twice, into a sample down to 80% of their original height (16 mm), using a crosshead speed of 50 mm min^{-1} and a load cell of 50 N. The force-deformation curve obtained during the TPA test served to calculate meat hardness, cohesiveness, springiness, and chewiness (Bourne, 1982). The TPA test was repeated 7-9 times on each sausages and three sausages were examined in each batch.

Sensory parameters evaluation

The sensory evaluation of the sausage samples was assessed by a trained expert panel of 7 members with, in general, a minimum of four years experience in texture analysis of meat and meat products. The sausage springiness, tenderness, cohesiveness, chewiness, juiciness, palatability, taste: sweet, bitter, sour as well as off-flavor and overall attractiveness were assessed using a 6-points scale as follows: 1 point: The least spring, tender, cohesive and samples assessed as not sweet, bitter, sour, without off-flavor, with the low juiciness, palatability, chewiness and overall attractiveness; 6 points: the most spring, tender, cohesive, juicy, sweet, bitter, sour, with off-flavor and with the highest palatability, chewiness, and overall attractiveness. The data presented in this study was a mean calculated from a set of scores obtained from each member.

Statistical analyses

Statistical analyses of the data involved the calculation of the mean values and standard deviations (SD) for each sample of sausages. The differences in texture, proteolysis index, and sensory properties between the samples were studied using the analysis of covariance. Treatment differences were tested for significance at the 5% level. All the calculations were performed with Statistica® v.7.0 PL software.

RESULTS AND DISCUSSION

Proteolysis

As shown in Figure 1, the addition of plant enzymes regardless of drying time, enhanced proteolysis index (Pi) by about 27-165% compared to the control dry fermented

sausages. Of all the sausages tested, the highest values of Pi as an effect of protein degradation were recorded in samples produced with pineapple and kiwifruit juices, lower value of this parameter was typical for ginger addition and the lowest were found in sausages with mango addition. Thus, it can be said that these enzymes can “digest” muscle proteins when they are mixed with meat. As shown in the study of Wada et al. (2002) some fruit proteases affect the structure of myosin and actin filaments. It is widely known that most of the exogenous plant enzymes used to tenderize meat have a good activity in temperature above and close to the environment during the fermentation or drying process. For example, Zhao et al. (2012) reported that almost all of the myofibrillar proteins (MHC and AC) were degraded into fragments with molecular weights lower than 20 kDa when meat was treated with bromelain or papain at 37°C. Thus, a higher proteolysis index recorded in our work can be attributed to dry sausages produced with exogenous enzymes addition compared to the controls. Those enzymes have a strong activity towards all the myofibrillar proteins and in favourable conditions may result in extensive degradation of myofibrillar proteins and meat structure (Zhao et al., 2012).

Another reason for the high proteolysis index could be that some enzymes can also hydrolyze the connective tissue. According to the study of Wada et al. (2002) some plant enzymes can also hydrolyze collagen and elastin, which helps to tenderize meat especially rich in connective tissue like beef or venison meat. Ketnawa et al. (2010) also confirmed that bromelain from pineapple peels can extensively degrade the collagen from beef and giant catfish skin. Also Ketnawa and Rawdkuen (2011) showed that the high TCA-soluble peptides content in bromelain treated samples was due to greater muscle protein hydrolysis. Fragmentation of both myofibrillar proteins and collagen tissue when treated with ammonium hydroxide resulted in the tenderization of buffalo meat (Naveena et al., 2011). The prolongation of enzymatic ageing of dry sausages for 4 weeks, led to the higher changes in proteolysis compared to the samples tested after 2 weeks of drying (Figure 1). For example, due to the 28 days of drying Pi of sausages with pineapple and ginger juices addition had increased about 40.2 and 22.4%, respectively, relative to the sausages dried for 2 weeks. At the same time, corresponding changes in Pi of other sausages were about 2-8%.

Textural properties

Differences in intensity of proteolysis during ripening of dry fermented sausages could be connected with the differences in the textural parameters of these products observed in this study (Figure 2). As suggested in this work, addition of exogenous proteolytic enzymes, by breaking of the protein chains of muscle and collagen fibers and by their structural damage resulted generally in a reduction of muscle hardness, springiness and chewiness compared to dry sausages produced with any enzymes addition.

There were few differences in texture changes among the enzymatic treatments (Figure 2). The sausages with the pineapple or kiwifruit juices were 70-75% or 35-39% less hard than control, respectively, regardless of drying time, while sample with mango juice had the highest numerical value and was tougher than all treatments. As shown in this work, enzymatic activity of enzymes coming from ginger was lower than enzymes from pineapple and kiwi fruit and no significant effect of mango juices on hardness changes was found. Probable reason for small changes in the hardness of the dry sausages produced from the mango juice addition, compared to control, was the fact that in these studies the juice derived directly from the fruit which contains a complex of enzymes as magneferin, catechol oxidase and lactase (Jha et al., 2010) was used, not a proteolytic enzyme in pure form.

The degradation of muscle protein plays a major role in determining the tenderness of meat during post-mortem storage (Koohmaraie et al., 2002). Our results show more pronounced effect of enzymes coming from pineapple and kiwifruit on protein proteolysis and thus texture changes compared with other exogenous proteolytic enzymes. According to the study of Wada et al. (2002), plant thiol proteases have very broad specificities and therefore, they indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderization to a mushy-textured product.

The level of hydrolysis was more accentuated when longer enzyme action times was used. Regardless of enzymes addition, longer sausage drying was found to induce in general the additional decrease in the hardness, cohesiveness, springiness and chewiness (Figure 2). However changes in textural parameters between sausages after 2 and 4 weeks of drying were dependent on plant enzymes addition. For example, 4 weeks of drying without any enzymes addition induced a 9% and 24% decrease in sausage hardness and chewiness, respectively, and about 8% changes in other textural parameters, compared to sausages after 2 weeks of drying. Whereas the prolongation of enzymatic drying of sausages produced with pineapple, ginger and kiwifruit juices, similar to changes observed in this study in Pi value, led to the higher changes in textural parameters compared to the samples with other exogenous enzymes. For example, due to the 4 weeks of drying, hardness of sausages with those juices addition had decreased about 24, 17 and 13%, relative to the samples dried for 2 weeks. And lower texture changes compared to those observed in control were found in sausages manufactured with mango and vegetables juice addition (by about 5% for hardness and 25% for chewiness).

As shown in this work, enzymatic activity of pineapple

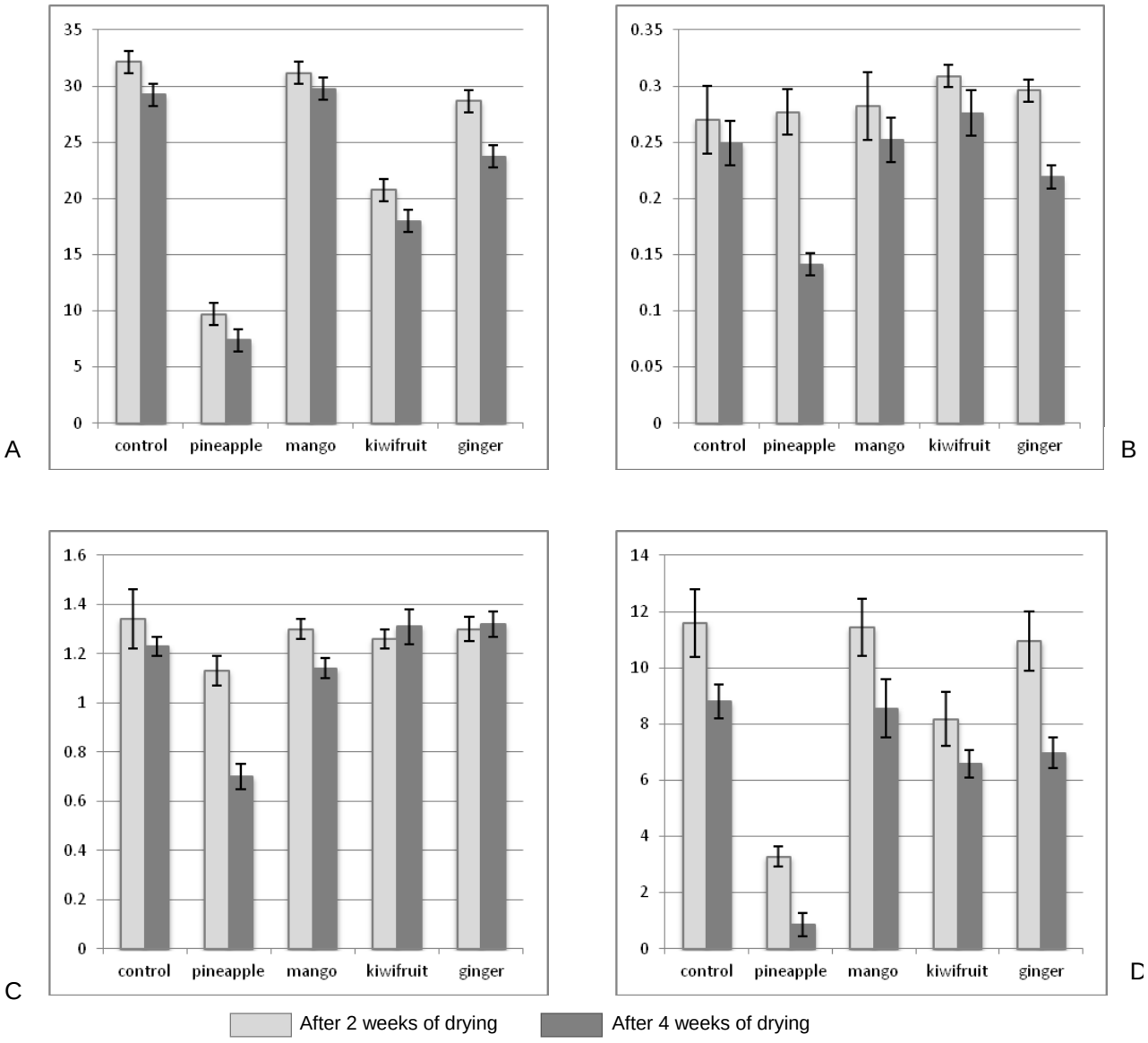

Figure 2. Texture of dry cured sausages produced with natural plant enzymes addition after two and four weeks of drying. A, Hardness (N); B, cohesiveness (-); C, springiness (cm); D, chewiness (Nxcm).

and kiwifruit juices were higher than other fruit enzymes activity. According to the study of Ionescu et al. (2008), the bromelain exhibits a more accentuated hydrolytic action on collagen than on myofibrillar proteins. As can be seen in other research, the activity of these enzymes strongly depends on pH. Kim and Taub (1991) found that pH 5.0 which is slightly similar for environment occurred in fermented sausages, is the optimal pH for bromelain activity. Thus probably, the conditions occurred in our work during dry sausages production were optimal for bromelaine activity and for this reason the highest texture changes were observed when the enzyme from pineapple juice affected on connective tissue from wild boar meat.

Also McKeith et al. (1994) found a significant increase in tenderness when a bromelaine solution was injected into muscle versus dipping or tumbling in brine.

When the effect of kiwi juice addition was considered, it was found that similar results obtained in this study were also achieved by Samejima et al. (1991) who demonstrated that actinidin could degrade the insoluble collagen under unheated conditions, and could also digest elastin into peptide fragments (Wada et al., 2004). They also suggested that kiwifruit tenderizing effects were also partially due to the degradation of the connective tissue in muscle.

In our research the tenderizing effects of ginger juicewas shown but was lower than those occurred when pineapple

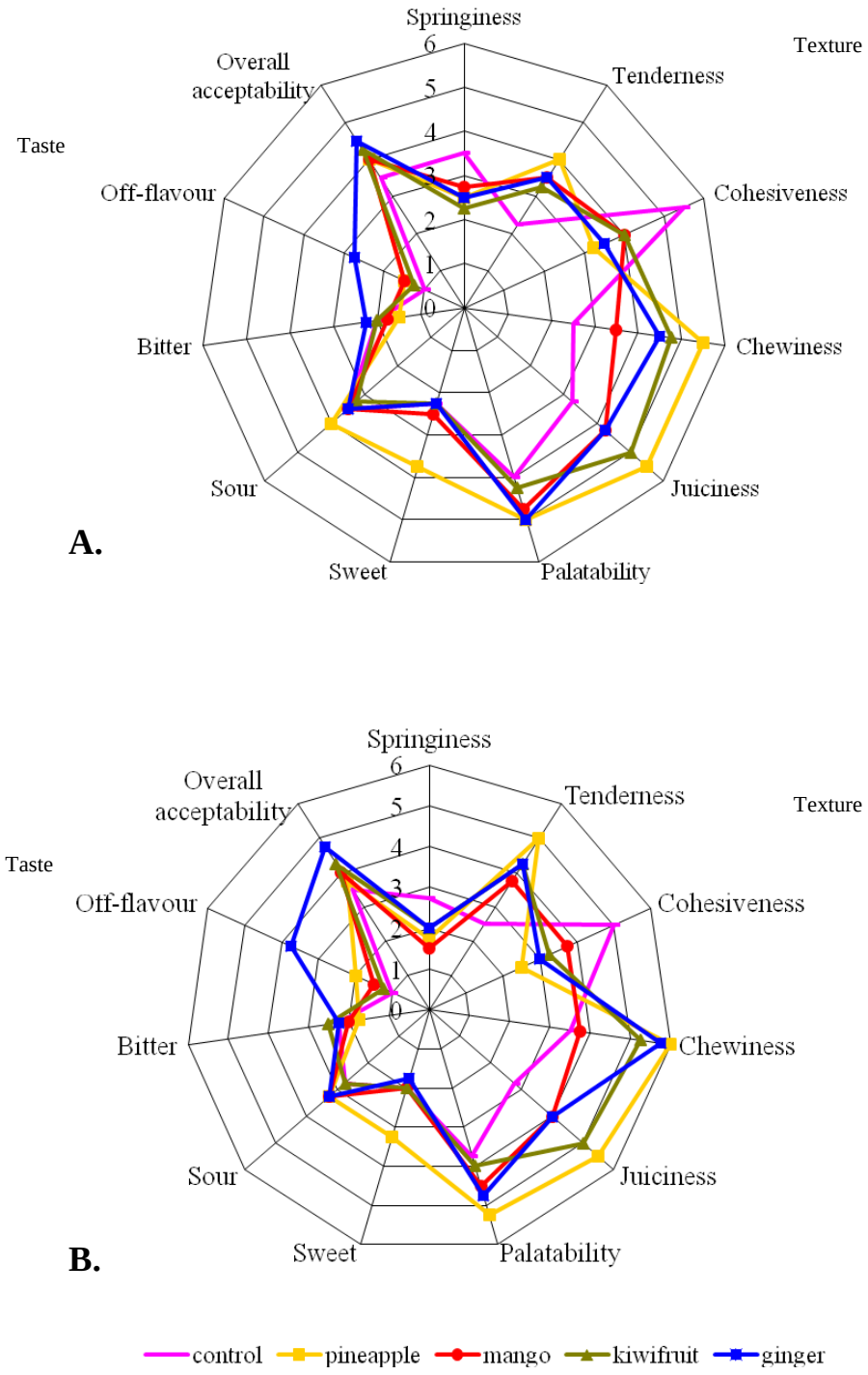

Figure 3. Sensory properties of dry cured sausages produced with natural plant enzymes addition after two (A.) and four (B.) weeks of drying.

and kiwifruit juices were used in dry sausage production. As reported by Naveena et al. (2004), zingabaine could effectively be used for tenderization of tough meat. Also Lee et al. (1986) explained that higher concentration of ginger extract extensively degraded the myofibrils and the degradation appeared to begin at I band of each sarcomere and progressed towards the M line

According to the study of Naveena and Mendiratta (2001), this enzyme has an advantage over other tenderizing agent as a greater proteolytic activity in heated condition, which is desirable. According to the study of Mansour and Khalil (2000) ginger has been shown to

have a powerful proteolytic enzyme, which can be used as tenderizing agent for tough meat. Thus, decrease in hardness of sausages produced with pineapple, kiwifruit and also ginger juices observed in this study could be connected with combined proteolysis of two main muscle proteins such as collagen and actomyosin.

Sensory evaluation

Sensory evaluation confirmed that all plant enzymes produced an improvement in tenderness, chewiness juiciness and palatability of dry sausages compared with untreated controls (Figure 3). The pineapple-treated samples received higher scores for those parameters and these sausages were rated as the sweetest, sour and as the least bitter, regardless of the time of drying. Thesensory panel also detected improvements in tenderness and juiciness when the ginger, kiwifruit and mango juices were added. Off-flavours were detected in the ginger-treated samples but those sausages were rated superior and most preferred by the panelists. Also samples produced with pineapple or mango juice addition were characterized by slightly perceptible off-flavours, however, in this case the finished products have been evaluated positively by a panelists. The least perceptible off-flavour was found in kiwifruit-treated sausages, but at the same time they were rated as slightly bitter.

On the other side, the results of our study also showed that dry sausages tenderized with a pineapple juice, especially those after four weeks of drying, despite the high tenderness and juiciness, had the worst general attractiveness which was connected with a slimy texture (Figure 3). The ginger, and kiwifruit juice-treated samples received better scores for overall acceptability compared to others sausages. According to the study of Ionescu et al. (2008) bromelain showed hydrolytic activity on the connective tissue, leading to the better tenderization of the tough meat but sometimes lead to over- tenderization and to a product with a pasty texture (Miller et al., 1995). These results were confirmed by Żochowska-Kujawska et al. (2012), who have found that even very hard wild boar muscles such as biceps femoris and semimembranosus soaked in marinades made from fresh pineapple juice were characterized by the worst sensory properties as a consequence the deepest changes in structure elements. Whereas improvement in flavor, juiciness, tenderness and overall acceptability scores with ginger extract treatment in our experiment is consistent with some earlier reports (Mendiratta et al., 2000; Syed Ziauddin et al., 1995).

Also Lewis and Luh (1988) compared the effect of actinidin on the tenderization of bovine semitendinosus muscles found that this enzyme had a milder protyolytic activity compared with other tested and did not produce off flavors or odors in the meat or excessive surface tenderization.

Conclusions

The results obtained in this experiment indicate the tenderizing effect of pineapple, kiwifruit and ginger, regardless of drying time, even if these enzymes were added directly to the meat during the dry sausages production. In general, there was a significant increase in proteolysis, and a reduction in hardness, chewiness and improvement in sensory quality in all enzyme-treated samples compared to controls. Our results showed more pronounced effect of pineapple and kiwifruit juices on protein proteolysis and thus texture changes compared with other exogenous proteolytic plant enzymes.

In turn, sensory analysis showed that samples treated with ginger and kiwifruit were rated superior and most preferred by the panelists, which can be attributed to the desirable ginger flavor. Pineapple and mango-treated samples scored almost equally, but the first one probably by deepest changes in structure elements resulted in a product with the mashy texture. It follows that, kiwifruit and ginger could be effectively utilized at household or industrial level for tenderization of tough meat such as venison in dry traditional fermented sausages production.

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Full Length Research Paper

Extracellular β-D-fructofuranosidase from *Aspergillus parasiticus*: Optimization of the production under submerged fermentation and biochemical characterization

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The β-D-fructofuranosidases are enzymes with biotechnological potential that can be used in different industrial sectors as food and beverage. In this context, microorganisms are important producers of these biomolecules, especially filamentous fungi. The production of extracellular β-D-fructofuranosidase from *Aspergillus parasiticus* using sugarcane bagasse as a carbon source under submerged fermentation was optimized by factorial design and high levels of enzyme were obtained in 24 h-old cultures at 30°C using 1.5% sugarcane bagasse under agitation. The extracellular β-D-fructofuranosidase was purified 119-fold using diethyl aminoethyl (DEAE)-cellulose and Sephacryl S-200 chromatographic columns with recovery of 16%. The native molecular mass was estimated as 136 kDa with two subunits of 63 kDa determined by 7% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and 64% carbohydrate content. The purified enzyme showed optimal temperature of activity from 38-56°C and optimum pH from 4.5 to 6.2 determined by experimental design (CCRD), with half-life of 25 min at 50°C. It was stable from pH 5.0-10.0. The extracellular enzyme activity was stimulated by Ba^{2+} and Mg^{2+}, and it was not affected by urea, silver and ethylenediaminetetraacetic acid (EDTA). The $K_{0.5}$ and V_{max} values were 10 mM and 1565 U/mg of protein, and 19 mM and 1965 U/mg of protein for sucrose and raffinose, respectively.

Key words: Invertase, fructofuranosidase, *Aspergillus parasiticus*, sugarcane bagasse, factorial design.

INTRODUCTION

Microorganisms are recognized as sources of different molecules with biotechnological potential. Among these microorganisms, the filamentous fungi deserve attention. They are able to degrade many organic and inorganic substrates by action of secreted enzymes. According to this, in the last years, the interest in the use of agro-industrial residues as substrates in bioprocess has been increased as, for example, for the production of molecules with aggregate value using different fermentative processes. As carbon source, agro-industrial residues

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Abbreviations: FOS, Fructooligosaccharides; **PB,** Plackett and Burman; **CCRD,** central composite rotatable design; **DEAE,** diethyl aminoethyl; **DNS,** 3',5'-dinitrosalisilic acid; **BSA,** bouvine serum albumin; **SDS-PAGE,** sodium dodecyl sulfate poly-acrylamide gel electrophoresis; **EDTA,** ethylenediaminetetraacetic acid.

have been used for enzyme production as, for example, β-D-frutofuranosidases (EC 3.2.1.26) also known as invertase (Pandey et al., 2000).

These enzymes can be obtained from different animals and plant tissues as well as from microorganisms as bacteria (Awad et al., 2013), yeast (Kumar and Kesavapillai, 2012; Sainz-Polo et al., 2013) and filamentous fungi as *Aspergillus niger* (Taskin at al., 2013), *Rhizopus delemar* (Orikasa and Oda, 2013) and *Aspergillus niveus* (Guimarães et al., 2009). The β-D-fructofuranosidases catalyze the hydrolysis of the sucrose molecule to produce an equimolar mixture of D-glucose and D-fructose, known as invert sugar, which has important properties that are interesting for food and beverage (Alberto et al., 2004). In addition, some microbial β-D-fructofuranosidases are able to perform transfructosilation reaction to produce fructooligosaccharides (FOS). FOS have no considerable caloric value and can be used by diabetic people because they are not metabolized by the human organism. These saccharides also collaborate to reduce the cholesterol and triglycerides levels, and they are beneficial for intestine microorganism (Mussato and Mancilha, 2007).

Production of FOS by filamentous fungi has been mentioned as, for example, by *Aspergillus phoenicis* (Aziani et al., 2012) and *Penicillium expansum* (Prata et al., 2010). Taking into account the importance of the β-fructofuranosidases and the filamentous fungi as source of enzymes for biotechnological application, the search for new fungal strains that are able to produce enzymes with attractive properties is interesting, especially if the biodiversity is con-sidered. The knowledge on fungal diversity and biotechno-logical potential is reduced and deserves attention. Thus, this manuscript describes the optimization, using factorial design approach, of the production process of an extracellular β-D-fructofuranosidase by the filamentous fungus *Aspergillus parasiticus* under submerged fermenta-tion using agro-industrial residues, as well as some enzyme properties of the purified enzyme.

MATERIALS AND METHODS

Microorganism and culture conditions

The filamentous fungus *A. parasiticus* was isolated from Brazilian soil, identified by the Laboratory of Microbiology from Universidade Federal de Pernambuco using morphological analysis and maintained on PDA (Potato Dextrose Agar) slants, at 4°C, in the culture collection from the Laboratory of Microbiology from Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo.

The submerged fermentation cultures were obtained by adding 1 mL of aqueous spore suspension (10^5 spores/mL) in 25 mL of Khanna medium (Khanna et al., 1995) in 125 mL Erlenmeyer flasks containing sugarcane bagasse as carbon source, pH 6.0 previously autoclaved at 120°C, 1.5 atmosphere for 30 min. The cultures were maintained at 30°C, under orbital agitation (100 rpm) for different periods as determined for each experiment.

Optimization of the culture condition for extracellular β-D-fructofuranosidase

The influence of different independent variables (Table 1) on the production of the extracellular β-D-fructofuranosidase by *A. parasiticus* was analyzed using the Plackett and Burman (PB) factorial design considering the high (+1) and low (-1) levels for each variable. The matrix was composed by 15 assays (12 factorial assays and 3 central point assays). The independent variables selected using the PB design (temperature and period of cultivation, and proportion of carbon source) were used to perform a central composite rotatable design (CCRD). The matrix was composed by 17 assays (8 factorial assays, six axial assays and three central point assays) (Table 3). For both design analysis, the results were submitted to variance analysis (ANOVA) with *p* value fixed at 0.2 and 0.05 for PB and CCRD, respectively. The analysis and the response surface were obtained using the software Statistica 8.0 (StatSoft).

Obtainment of enzyme extract

The cultures were filtered using filter paper Whatmann no 1 with a vacuum pump. The filtrate without cells was named as extracellular crude extract and it was dialyzed overnight against distilled water at 4°C and used for enzymatic assay and purification procedure.

Determination of the β-fructofuranosidase activity and protein quantification

The β-D-fructofuranosidase activity was determined using sucrose (1%, m/v) as substrate in 100 mM of different buffers (sodium acetate pH 4.0-5.5; MES pH 5.5-7.0; Tris-HCl pH 7.0-9.0; and McIlvaine pH 4.0-7.0) and different temperatures (40-60°C). The reducing sugar was quantified using 3',5'-dinitrosalisilic acid (DNS) (Miller et al., 1959). The absorbance was determined at 540 nm. One unit of enzymatic activity was determined as the amount of enzyme necessary to produce 1 μmol of reducing sugar per minute under the assay condition. The protein was quantified according to Lowry et al. (1951), using bouvine serum albumin (BSA) as standard. The absorbance was determined at 660 nm, and the results expressed as mg of protein per mL of sample.

Purification

The extracellular crude extract was loaded in diethyl aminoethyl (DEAE)-cellulose (1.2 x 11.0 cm) chromatographic column previously equilibrated with 10 mM Tris-HCl buffer, pH 7.0. The 2 mL fractions containing β-D-fructofuranosidase were eluted using a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 1.54 mL min^{-1}. These fractions were pooled, dialyzed against distilled water overnight at 4°C, lyophilized, ressuspended in 2 mL of 20 mM Tris-HCl, pH 7.0 plus 100 mM NaCl and loaded in Sephacryl S-200 (1.0 x 80 cm) chromatographic column previously equilibrated with the same buffer. Fractions of 1.0 mL were collected at the flow rate of 0.37 mL min^{-1} and those with activity were pooled, dialyzed against distilled water overnight at 4°C and used for biochemical characterization.

Molecular mass and carbohydrate content determination

The native molecular mass of the extracellular β-D-fructofuranosidase from *A. parasiticus* was determined by gel filtration in Sephacryl S-200 chromatographic column using the same conditions described above. We used β-amylase (200 kDa), alcohol

Table 1. PB matrix with real values and encoded values using eight independent variables and the responses (enzyme activity).

Trial	Independent variable								Response	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	U/mL	U/mg of protein
1	7 (+)	30 (-)	150 (+)	1 (-)	0 (-)	0 (-)	1 (+)	96 (+)	13.1	47.5
2	7 (+)	40 (+)	80 (-)	3 (+)	0 (-)	0 (-)	0.001 (-)	96 (+)	7.6	22.2
3	5 (-)	40 (+)	150 (+)	1 (-)	1.5 (+)	0 (-)	0.001 (-)	48 (-)	2.1	7.5
4	7 (+)	30 (-)	150 (+)	3 (+)	0 (-)	1.5 (+)	0.001 (-)	48 (-)	4.7	2.9
5	7 (+)	40 (+)	80 (-)	3 (+)	1.5 (+)	0 (-)	1 (+)	48 (-)	8.7	19.6
6	7 (+)	40 (+)	150 (+)	1 (-)	1.5 (+)	1.5 (+)	0.001 (-)	96 (+)	6.2	3.7
7	5 (-)	40 (+)	150 (+)	3 (+)	0 (-)	1.5 (+)	1 (+)	48 (-)	10.6	6.8
8	5 (-)	30 (-)	150 (+)	3 (+)	1.5 (+)	0 (-)	1 (+)	96 (+)	16.3	25.1
9	5 (-)	30 (-)	80 (-)	3 (+)	1.5 (+)	1.5 (+)	0.001 (-)	96 (+)	6.6	5.1
10	7 (+)	30 (-)	80 (-)	1 (-)	1.5 (+)	1.5 (+)	1 (+)	48 (-)	13.5	10.2
11	5 (-)	40 (+)	80 (-)	1 (-)	0 (-)	1.5 (+)	1 (+)	96 (+)	9.3	5.6
12	5 (-)	30 (-)	80 (-)	1 (-)	0 (-)	0 (-)	0.001 (-)	48 (-)	8.8	29.6
13	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	18.7	32.1
14	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	18.9	27.1
15	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	20.2	29.4

X_1, pH; X_2, temperature (°C); X_3, rpm; X_4, sugar cane bagasse (%, m/v); X_5, KH_2PO_4 (%, m/v); X_6, peptone (%, m/v); X_7, spores (10^7); X_8, time of cultivation (h).

dehydrogenase (150 kDa), BSA (66 kDa), egg albumin (43 kDa) and carbonic anhydrase (29 kDa) as molecular markers. The molecular mass was also determined under denaturing condition (7% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Macroglobulin (169 kDa), β-galactosidase (112 kDa), lactoferrin (92 kDa), piruvate kinase (67 kDa), fumarase (60 kDa), lactic dehydrogenase (36 kDa) and triose phosphate isomerase (31 kDa) were used as mass molecular markers. The carbohydrate content was determined according to Dubois et al. (1956) using mannose as standard.

Determination of optimum of temperature and pH of activity and stability

The best conditions of temperature and pH of activity for extracellular β-D-fructofuranosidase from *A. parasiticus* were determined using an experimental design 2^2, where temperature and pH were considered as independent variables. We performed 11 assays (four factorial assays, four axial assays and three at the central point assays). The analysis of variance was carried with *p* value fixed at 0.05 using the software Statistica 8 (StatSoft).

The thermal stability was determined by the incubation of enzyme in aqueous solution at different temperatures (50, 60 and 70°C) and periods (0 - 60 min). The stability to pH was determined using different buffers (100 Mm) (KCl-HCl - pH 2.0; citrate - pH 3.0 and 4.0; sodium acetate - pH 5.0; phosphate - pH 6.0 and 7.0; Tris-HCl - pH 8.0 and 9.0; glicine-NaOH - pH 10.0) and periods (0, 30 and 60 min). Samples were taken off at a pre-determined time, kept in ice bath and after assayed for enzymatic activity as described previously using 200 µL of purified enzymatic sample (10 µg/mL).

Influence of different compounds and salts on enzyme activity

The influence of different compounds (β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) e urea) and salts ($AgNO_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$, KCl, $MgCl_2$, $MgSO_4$, NaCl, NaF NH_4Cl, $ZnCl_2$ e $ZnNO_3$) on the extracellular β-D-fructofuranosidase activity was investigated by adding of 1 mM of each in the enzymatic assay and the activity was determined as described previously using 200 µL of purified enzymatic sample (10 µg/mL).

Determination of kinetic parameters

The kinetic parameters V_{max}, $K_{0.5}$ and $V_{max}/K_{0.5}$ were determined using both sucrose and raffinose (0.25 - 100 mM) as substrates in 100 mM sodium acetate buffer pH 5.5, at 50°C. A sigmoid plot was obtained using the software Origin 8 (Origin Lab).

RESULTS AND DISCUSSION

Optimization of culture conditions for β-D-fructofuranosidase production

The enzyme production by microorganisms is directly influenced by the culture conditions, for example, temperature and period of cultivation, pH of the culture medium and carbon source, among others. The statistic methodology, as experimental design, is a powerful tool to optimize the culture conditions for enzymatic production by filamentous fungi. The PB matrix obtained for optimization of the extracellular β-D-fructofuranosidase production by *A. parasiticus* is presented in Table 1. Considering the enzyme production, the best condition was obtained at the assay 15, with 20.2 U mL^{-1}. According to the statistical analyses with *p* value fixed at 0.05, the influences of independent variables temperature (X_2), proportion of KH_2PO_4 (X_5) and peptone (X_6) and number of spores used (X_7), were significant ($p < 0.05$)

Table 2. Effects analyses for the β-fructofuranosidase activity (U/mg of protein).

Variable	Effect	Standard error	t(21)	*p*-value
Media	18.7	1.5	12.8	0 [A]
X_1	3.7	3.3	1.1	0.272
X_2	-11	3.3	-3.4	0.003 [A]
X_3	0.2	3.3	0	0.964
X_4	-5.2	3.3	-1.6	0.127 [B]
X_5	-7.5	3.3	-2,3	0.032 [A]
X_6	-19.7	3.3	-6	0.000 [A]
X_7	7.2	3.3	2.2	0.039 [A]
X_8	4.1	3.3	1.3	0.221

A, Significant parameters ($p < 0.05$); B, significant parameters ($p < 0.15$).

Table 3. Matrix and responses for 2^3 experimental design with real and encoded values.

Trial	Independent variable			Response	
	X_2	X_4	X_8	U/mL	U/mg of protein
1	27 (-)	0.9 (-)	43 (-)	5.7	35.2
2	27 (-)	0.9 (-)	101 (+)	6.9	48.0
3	27 (-)	2.1 (+)	43 (-)	7.5	35.8
4	27 (-)	2.1 (+)	101 (+)	9.0	32.0
5	33 (+)	0.9 (-)	43 (-)	6.1	42.9
6	33 (+)	0.9 (-)	101 (+)	6.8	39.5
7	33 (+)	2.1 (+)	43 (-)	6.9	26.2
8	33 (+)	2.1 (+)	101 (+)	9.0	26.1
9	25 (-1.68)	1.5 (0)	72 (0)	7.7	30.3
10	35 (+1.68)	1.5 (0)	72 (0)	6.7	25.9
11	30 (0)	0.5(-1.68)	72 (0)	5.2	43.2
12	30 (0)	2.5 (+1.68)	72 (0)	8.7	31.3
13	30 (0)	1.5 (0)	24 (-1.68)	7.1	27.9
14	30 (0)	1.5 (0)	120 (+1.68)	9.6	30.5
15	30 (0)	1.5 (0)	72 (0)	7.7	29.9
16	30 (0)	1.5 (0)	72 (0)	7.7	30.6
17	30 (0)	1.5 (0)	72 (0)	7.7	31.4

X_2, Temperature (°C); X_4, sugar cane bagasse (% m/v); X_8 - time of cultivation (h).

with R^2 of 0.75 (Table 2). Only the variable X_7 showed a positive effect, suggesting that an increase in the number of spores would be favorable to the enzyme production. The variables X_2, X_5 and X_6 showed negative effect. Then, the variables X_2 (temperature) and X_4 (sugar cane bagasse) were selected for CCRD despite the effect of the latter to be considered not significant. In addition, the variable X_8 (period of cultivation) was also included because the production of metabolites by microorganisms is influenced by this aspect, what has been demonstrated by a lot of works. For CCRD the variables X_5 (KH_2PO_4), X_6 (peptone) and X_7 (number of spores) were not considered.

The matrix obtained for CCRD is presented in Table 2. Taking into account the R^2 value of 0.92, the influences of all variables were significant. The variable sugarcane bagasse (X_4) was significant in both levels, linear and quadratic while the temperature (X_2) was only in the quadratic level and the period of cultivation (X_8) was only in linear level (Table 4). The main interactions as well as the second-order interactions observed for experimental design are very important for the understanding of the behavior of the process (especially, in this case, the fermentative process).

Considering the variance analysis, the *F*-test showed that the model is predictive and the calculated *F* value (34.7) was 10-fold higher than the tabled *F* value (3.26). According to that, a global model of second order for β-D-fructofuranosidase production was established as function of these three variables (Equation 1). The reduced model

Table 4. Determination of coefficients of regression for the β-fructofuranosidase production.

Variable	Coefficient	Standard error	t(7)	*p*-value
Media	7.7	0.18		
X_2 (L)	-0.15	0.09	-1.8	0.1122 [B]
X_2 (Q)	-0.2	0.09	-2.4	0.0482 [A]
X_4 (L)	0.95	0.09	11	0 [A]
X_4 (Q)	-0.3	0.09	-3.4	0.0111 [A]
X_8 (L)	0.7	0.09	8.1	0.0001 [A]
X_8 (Q)	0.2	0.09	1.9	0.0974 [B]
$X_2.X_4$	-0.1	0.11	-1	0.3364
$X_2.X_8$	0.05	0.11	0.2	0.8214
$X_4.X_8$	0.2	0.11	1.8	0.1149 [B]

A, Significant parameters ($p < 0.05$); B, significant parameters ($p < 0.15$).

Table 5. Purification of the extracellular β-fructofuranosidase from *A. parasiticus*.

Step	Volume (mL)	Activity (Total U)	Protein (Total mg)	U/mg of protein	Yield (%)	Purification factor (fold)
Crude extract	350	1521	123	12.4	100	1
DEAE-cellulose	43	828	13	63.7	54.4	5.1
Sephacryl S-200	16	237	0.16	1481.2	15.6	119.4

was obtained using only the significant level of each variable (equation 2).

$$U/mL = 7.7 - 0.15X_2 - 0.2X_2^2 + 0.95X_4 - 0.3X_4^2 + 0.7X_8 + 0.2X_8^2 - 0.1X_2X_4 + 0.05X_2X_8 + 0.2X_4X_8 \quad (1)$$

$$U/mL = 7.7 - 0.2\,X_2^2 + 0.95\,X_4 - 0.3\,X_4^2 + 0.7\,X_8 \quad (2)$$

Using the reduced model, the surface response was obtained (Figure 1). The high level of enzyme production (> 8 U mL^{-1}) was obtained when we used 1.5-2.5% (m/v) sugar cane bagasse and temperature from 27 to 30°C (Figure 1A). When the period of cultivation was considered, the best results were obtained between 101 and 120 h in the same temperature interval (Figure 1B). Considering the proportion of the sugar cane bagasse and the period of cultivation (Figure 1C), the best production was obtained in the same intervals cited above. According to these observations, the reduced model obtained was validated using the encoded levels 0 (30°C) for temperature, 0 (1.5 m/v) for sugar cane bagasse and -1.68 (24 h) for period of cultivation.

Under these conditions, we obtained 6.92 U mL^{-1} while the calculated value (using the equation) was 7.08 U mL^{-1}. Then, the temperature of 30°C, proportion of sugar cane at 1.5% (m/v) and period of cultivation of 24 h were selected for enzyme production by *A. parasiticus* aiming at the puri-fication and characterization. Many studies determined the best culture conditions for β-D-fructofuranosidase production using one-factor analysis as presented by Alegre et al. (2009), which observed a temperature of 40°C and period of 72 h for cultivation of *Aspergillus caespitosus*. The same was observed for enzyme pro-duction by *A. niveus* and *A. phoenicis* (Rustiguel et al., 2010; Guimarães et al., 2009). The use of experimental design allows one to observe the interaction of factors, what does not occur when one factor analysis is used.

The use of agro industrial residues as sugar cane bagasse to produce biomolecules is interesting and has attracted the attention from different sectors. Many authors have mentioned the use of soybean meal, wheat bran and sugar cane bagasse as carbon sources for β-D-fructofuranosidase production by filamentous fungi (Guimarães et al., 2009; Alegre et al., 2009; Giraldo et al., 2011).

Purification

The extracellular crude extract obtained under the optimized condition was submitted to two chromategraphic steps. The β-fructofuranosidase was purified 119-fold with 16% recovery (Table 5). According to this procedure, it is possible to obtain around 10 mg/L of β-fructofuranosidase. The native molecular mass estimated by Sepha-cryl S-200 was 136 kDa, while a molecular mass of 63 kDa was obtained under denaturating condition

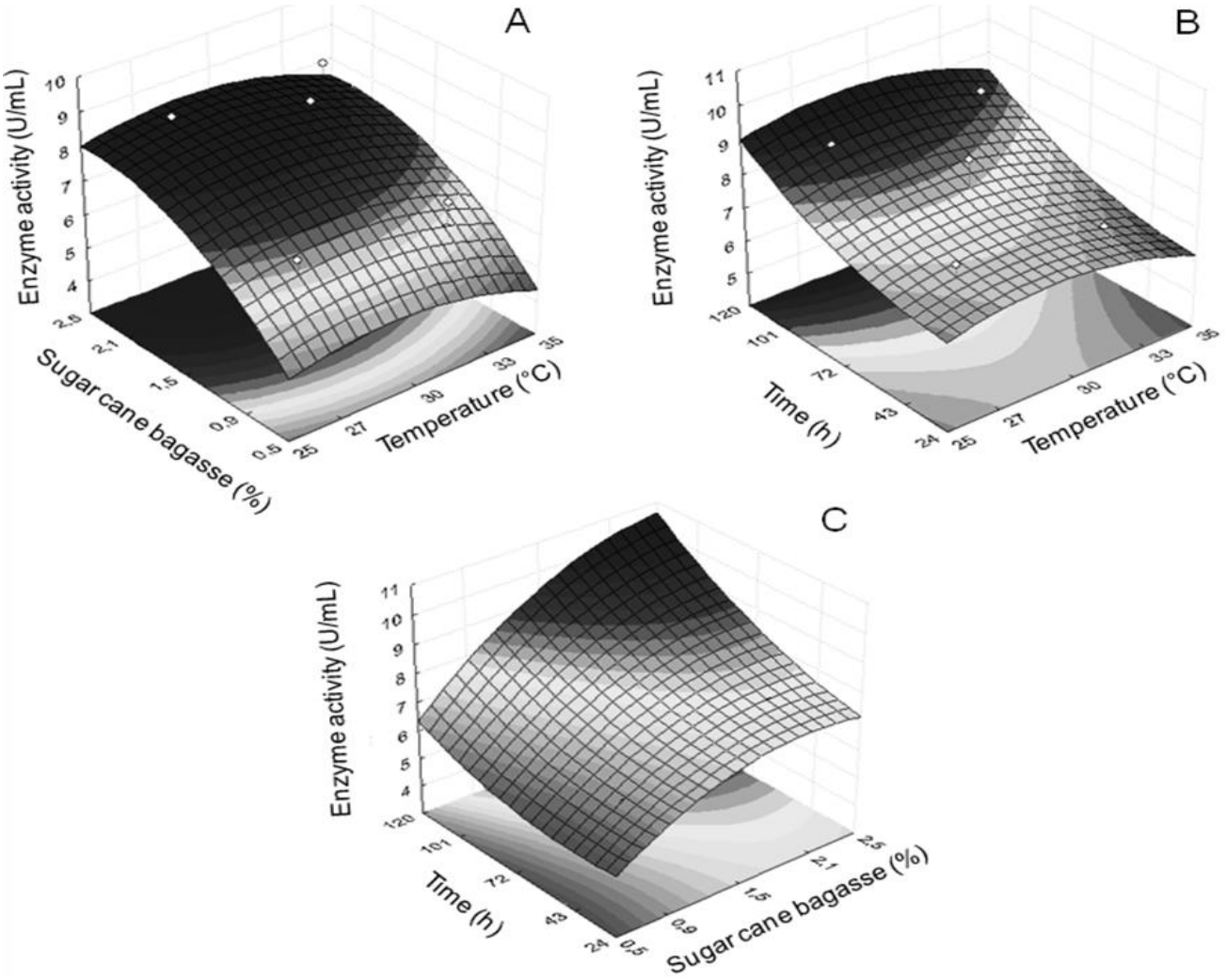

Figure 1. Surface responses for the extracellular β-fructofuranosidase production by *A. parasiticus* as function of temperature and proportion of sugar cane bagasse (A), temperature and time of cultivation (B), and proportion of sugar cane bagasse and time of cultivation (C).

(SDS-PAGE) (Figure 2), indicating a homodimeric structure with 64% carbohydrate content. The β-fructofuranosidases from *Aspergillus ochraceus* and *A. phoenicis* were also characterized as homodimers (Rustiguel et al., 2010; Guimarães et al., 2007). The carbohydrate content was higher than that observed for the enzymes produced by other filamentous fungi as, for example, *A. niger* (17%) (Nguyen et al., 2005) and *A. ochraceus* TS (30%) (Gosh et al., 2001), among others.

Optimization of the temperature and pH of reaction and stability

The influence of independent variable temperature (X_1) and pH (X_2) on the extracellular β-fructofuranosidase activity was analyzed using experimental design (Table 6). The highest levels of enzymatic activity were obtained when the reaction was conducted at 45°C and pH 5.0, which were defined as central point levels. The influence of both variables was significant in the linear and quadratic levels with *p* value fixed at 0.05 and with R^2 value of 0.96 (Table 7). According to the F-test, the calculated *F* value (135.7) was 52-fold higher than the tabled *F* value (2.57), allowing the obtainment of the second-order model, that was significant and predictive for *A. parasiticus* β-fructofuranosidase activity (equation 3).

$$\text{Relative activity (\%)} = 99.3 + 4.2X_1 - 21.9X_1^2 + 13.1X_2 - 37X_2^2 + 21.6X_1X_2 \quad (3)$$

The analysis of the response surface plot shows that the highest enzyme activity can be obtained between 38 and

Table 6. CCRD matrix using real and encoded values for relative β-fructofuranosidase activity.

Trial	X_1 (°C)	X_2 (pH)	Relative activity (%)
1	34 (-)	3.6 (-)	46.9
2	34 (-)	6.4 (+)	33.8
3	56 (+)	3.6 (-)	11.3
4	56 (+)	6.4 (+)	90.5
5	30 (-1.41)	5 (0)	22.2
6	60 (+1.41)	5 (0)	54.4
7	45 (0)	3 (-1.41)	5.6
8	45 (0)	7 (+1.41)	35.1
9	45 (0)	5 (0)	99
10	45 (0)	5 (0)	99.5
11	45 (0)	5 (0)	100

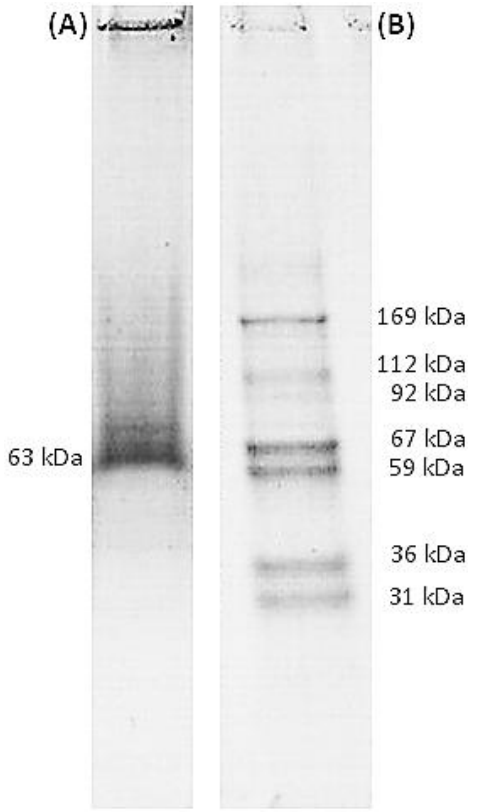

Figure 2. 7% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane A, β-fructofuranosidase; Lane B, molecular mass markers.

56°C, and pH from 4.5 to 6.2 (Figure 3). Generally, the influence of temperature and pH on enzyme activity has been determined using the one-factor approach, but the experimental design is a powerful approach that also allows the analysis of interaction of these both variables. The values of temperature obtained were similar if compared to the enzymes from *A. caespitosus* (Alegre et al., 2009) and *A. niger* PSSF21 (Reddy et al., 2010), but higher than that observed for the enzymes produced by *Fusarium solani* (Bhatti et al., 2006) and *A. niger* IMI303386 (Nguyen et al., 2005). In addition, the pH values were similar to the most fungal β-fructofuranosidases as, for example, from *Termitomyces clypeatus* (Chowdhury et al., 2009).

The extracellular β-fructofuranosidase produced by *A. parasiticus* had a half-life (t_{50}) of 25 min at 50°C, but when the temperatures of 60 and 70°C are considered, the t_{50} was reduced to 6 min (Figure 4). On the other hand, the enzyme was fully stable at temperatures below 50°C. This aspect is very interesting because the hydrolytic process can be conducted in mild conditions allowing the energy economy. Additionally, the enzyme was stable to wide pH range (5.0 to 10.0) as also observed for enzymes from *A. niger* IMI303386 (Nguyen et al., 2005) and *A. niger* AS0023 (L'Hocine et al., 2000). Modifications in the pH values of the enzyme environment can promote modifications in the lateral chains of the amino acids interfering with the protein conformation and consequently with the catalytic activity. Thus, an enzyme that is more resistant to pH variation is a good option for different biotechnological application.

Influence of different compounds on enzyme activity

The extracellular β-fructofuranosidase activity from *A. parasiticus* was increased in the presence of Ba^{2+} (+29%) and Mg^{2+} (+22%) (Data not shown). On the other hand, when $CuSO_4$ and $CoCl_2$ were used, the enzyme activity was reduced around 77 and 40%, respectively. Interesting, the enzyme activity was preserved in the presence of β-mercaptoethanol that are able to act on disulfide bonds, EDTA that is chelant of divalent cations, urea that is responsible to denature proteins and $AgNO_3$ that is able to precipitate proteins. Increase in the enzyme activity by addition of Ba^{2+} and Mg^{2+} was also observed for *Penicillium variotti* (Giraldo et al., 2011) and *A. niveus*

Table 7. Determination of the coefficients of regression for relative β-fructofuranosidase activity.

Variable	Coefficient	Standard error	t (27)	*p*-value
Media	99.3	2.42	41	0 [A]
X_1 (L)	4.2	1.48	3	0,008 [A]
X_1 (Q)	-21.9	1.76	-12	0 [A]
X_2 (L)	13.1	1.48	9	0 [A]
X_2 (Q)	-37	1.76	-21	0 [A]
$X_1.X_2$	21.7	2.09	10	0 [A]

A, Significant parameters ($p < 0.05$).

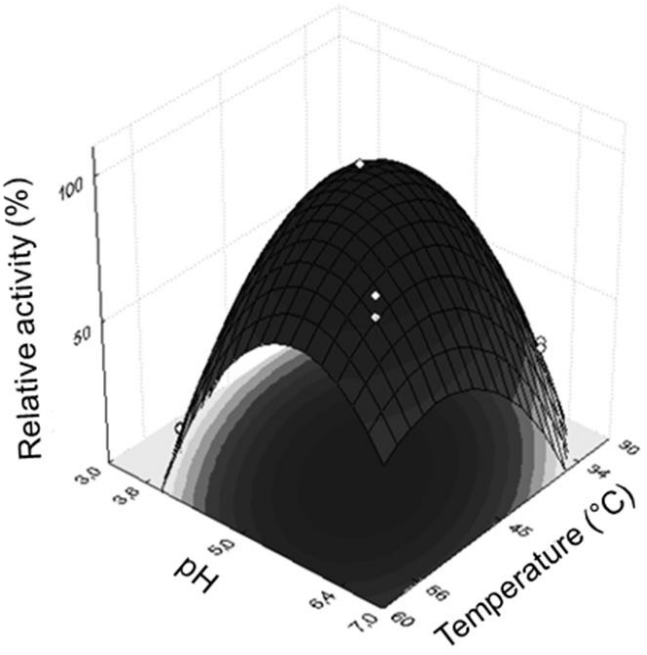

Figure 3. Response surface for the extracellular β-fructofuranosidase activity as function of temperature and pH.

enzymes (Guimarães et al., 2009). The enzyme produced by *A. phoenicis* had its activity increased by the addition of Ag^+, with suggestion of a new group of fructofuranosidases activated by silver (Rustiguel et al., 2010).

Determination of kinetic parameters

The extracellular β-fructofuranosidase from *A. parasiticus* was able to hydrolyze sucrose, inulin and raffinose, as well as the mixture of theses substrates (data not shown). The best activity was observed for the sucrose and raffinose mixture (1:1) (16.6 U/mL). The experimental values of hydrolysis obtained for the mixtures were approximately similar to that obtained for the hypothetical sum of the individual values observed for each substrate, indicating the possibility of the existence of different catalytic sites. Another aspect that should be highlighted is the hydrolysis on inulin. There is a wide discussion on the nature of the enzyme, that is, if β-D-fructofuranosidase or if inulinase. Many authors have considered the relation between the hydrolysis of sucrose on inulin (S/I value) to define the nature of the enzyme. The S/I value for *A. parasiticus* extracellular enzyme was 7.3, higher than that obtained for *A. phoenicis* enzyme (Rustiguel et al., 2010), indicating the enzyme as β-fructofuranosidase.

However, the S/I value cannot be considered as isolated for the distinction of the enzymes, but also kinetic and structural studies. The S/I value also depends on the inulin source used. Taking into account that the best hydrolytic activity was obtained on sucrose, the kinetic parameters using this substrate were determined. The purified extracellular enzyme showed $K_{0.5}$ of 10 mM, V_{max} of 1565 U/mg of protein and $V_{max}/K_{0.5}$ of 156.5 U/ mg of protein mM^{-1}. When used raffinose as substrate, the $K_{0.5}$ was 19 mM, with V_{max} of 1965 U/mg of protein and $V_{max}/K_{0.5}$ of 103.4 U/mg of protein mM^{-1}. For both substrates the coefficient of Hill (n) was higher than 1.0, indicating a positive cooperation.

Thus, the extracellular β-fructofuranosidase from *A. parasiticus* showed higher affinity by sucrose than by raffinose. The affinity by the former was higher than that observed for the enzymes produced by *A. ochraceus* (Guimarães et al., 2007), *A. phoenicis* (Rustiguel et al., 2010) and *A. pullulans* (Yoshikawa et al., 2006).

Conclusion

The filamentous fungi are important sources of bio-molecules with biotechnological potential as enzymes. The production of extracellular β-fructofuranosidase by *A. parasiticus* was optimized using an experimental design, as well as the temperature and pH of the activity. This is the first time that the experimental design was used to analyze the influence of temperature and pH on fungal β-fructofuranosidase activity.

The homodimeric glycoprotein showed attractive characteristics for application, as wide range of pH stability and mild conditions of temperature, minimizing the energetic expense.

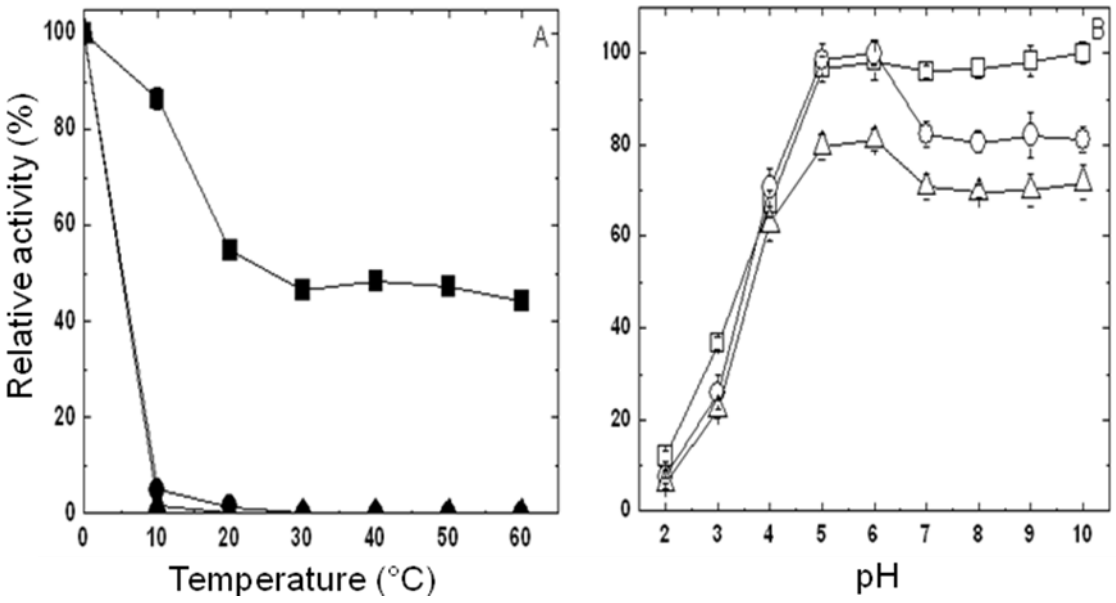

Figure 4. Thermal stability (A) at 50°C (■), 60°C (●) and 70°C (▲), and stability to pH (B) for 0 (□), 30 (○) e 60 (∆) minutes for extracellular β-fructofuranosidase activity.

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Full Length Research Paper

Thermal and pH stabilities of partially purified polyphenol oxidase extracted from *Solanum melongenas* and *Musa sapientum* fruits

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Enzyme activity depends largely on environmental conditions such as temperature and pH. The stability of polyphenol oxidase (PPO) extracted from *Solanum melongenas* and *Musa sapientum* fruits pre-incubated in varying thermal and pH conditions were carried out. Enzyme activity was measured by spectrophotometric methods. The reaction mixture contained 3.5 mL of 0.20 M phosphate buffer (pH = 6.8), 1.0 mL of 0.75 mM catechol, and 0.5 mL of enzyme solution. $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ gave different temperature and pH optima. The temperature-activity profile of $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ showed a strong positive correlation (r = 0.907363). At pH = 10.0, $PPO_{M.\ sapientum}$ activity represented 65.3% decay in enzyme activity, whereas $PPO_{S.\ melongenas}$ represented 79.3% decay in enzyme activity. $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ stability at pre-incubated temperatures of 20, 50 and 60°C and pH values of 3.5, 6.0 and 8.0 were measured. Residual activities of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ showed a strong positive correlations under the same experimental thermal conditions, with exception at 20°C (r = 0.693375). Specifically, pre-incubation of $PPO_{M.sapientum}$ for t = 90 min at 60°C caused 18.4% decay in relative activity of $PPO_{M.\ sapientum}$. At t = 90 min, pre-incubation of $PPO_{M.\ sapientum}$, at pH = 3.5 caused decay in activity within the range of 30.8-36.1%, whereas $PPO_{M.\ sapientum}$ pre-incubated in pH = 6.0 and pH = 8.0 gave decay in activity within the range of (1.5-9.8%) and (2.7-6.5%) respectively. $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ showed relatively higher stabilities as the incubation pH tended towards alkaline conditions, whereas the two experimental temperatures (20 and 60°C) promoted destabilization.

Key words: Polyphenol oxidase, temperature, pH, *Solanum melongenas* and *Musa sapientum.*

INTRODUCTION

Polyphenol oxidase (PPO) is a collection of ubiquitous plant enzymes [EC 1.10.3.2, catechol oxidase or diphenol oxygen oxidoreductase (Klabunde et al., 1998; Fawzy, 2005); EC. 1.14.18.1; monophenol oxidase, cresolase and tyrosinase (Mayer, 2006; Madani et al., 2011)] responsible for undesirable browning reactions of fruits and vegetables. However, studies have shown that many plant PPOs lack monophenol oxidase (cresolase) activity, restricting potential substrates of the enzymes to diphenolic compounds such as catechol, 3, 4-dihydroxyphenylalanine, and chlorogenic acid (Steffens et al., 1994; Escobar and Shilling, 2008). Enzymatic browning is associated with oxidation of phenolic compounds in the presence of molecular oxygen to corresponding quinone intermediates that polymerize to form melanin and off-colour pigments (da Silva and Koblitz, 2010). The kinetic properties of PPO extracted from various plant sources have been reported by several authors (Gowda and Paul, 2002; Chikezie, 2006; Gouzi et al., 2010).

PPO is a copper-metalloenzyme located in the chloroplast thylakoid membrane (Sommer et al., 1994) and can exist in an active or latent state (Mayer and Harel 1979). PPO enzymes extracted from various plant tissues exhibit different characteristics, and exit in multi-

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ple molecular forms (isoforms) (Marshall et al*.,* 2000; Altunkaya and Gokmen, 2011; Ünal et al., 2011). Isoenzymic forms of PPOs are identified according to their physical, chemical or enzymatic properties such as electrophoretic mobility, temperature and pH optima, substrate specificity and isoelectric index (pI) (Yoruk and Marshall, 2003).

Enzyme activity depends largely on environmental conditions such as temperature and pH. Thermal and pH stabilities of PPO, as being reported here, describes the capacity of pre-incubated enzymes to withstand thermal and pH induced unfolding at specified experimental temperature and pH conditions. Fruits of *Solanum melongenas* (eggplant) and *Musa sapientum* (Banana) are highly cherished and consumed in Nigeria and the world over. The economic benefits from the sales of these fruits are profound. However, spoilage of these fruits with financial losses is most often associated with the initiation of browning reactions associated with post-harvest activities.

Unavoidably, most of the fruits are bruised and injured during the course of transportation, storage and preservation. In an effort to control the browning process, the present study sought to establish the thermal and pH conditions that promote stability of the PPOs extracted from *S. melongenas* and *M. sapientum* fruits. Insights into the nature of environmental factors that promote stability of PPO could serve as point of reference for the conception of environmental conditions as instruments for control and mitigation of the browning process that has been implicated in fruit spoilage, deterioration and unacceptability.

MATERIALS AND METHODS

Collection and preparation of fruit samples

Fresh and disease free fruits of *S. melongenas* and *M. sapientum* were harvested from a private botanical garden in Umuoziri-Inyishi, Imo State, Nigeria between 17th -30th of July, 2012.The fruits were identified and authenticated by Dr. F. N. Mbagwu at the Herbarium in the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. The two fruits were washed under continuous current of distilled water for 5 min and air dried at room temperature. The stalk (*S. melongenas*) and rind (*M. sapientum*) were removed manually. The samples were stored at -4°C until used for analyses.

Extraction and purification of PPO

Extraction and partial purification of PPO was according to the methods of Madani et al*.* (2011) with minor modifications. Ten grams (10 g) of the sample was homogenized in external ice bath, using Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) homogenizer set in 80 mL of 0.1 M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid for 180 s at intervals of 60 s. The homogenate was quickly squeezed through two layers of clean cheesecloth into a beaker kept in ice. The crude extract was rinsed with 200 mL of acetone (-20°C) to eliminate phenolic compounds (Liu et al*.,* 2007; Ünal et al*.,* 2011). The sample was centrifuged at 32000 *g* for 20 min at 4°C. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% $(NH_4)_2SO_4$ saturation and precipitated proteins were separated by centrifugation at 32000 *g* for 30 min at 4°C. The precipitate was re-dissolved in 10 mL distilled water and ultra-filtered in a Millipore stirred cell with a 10-kDa membrane (Millipore 8050, Milan, Italy). The filtrate was dialyzed (cellulose membrane, Medicell Intl. Ltd., 6-27/32) at 4°C against distilled water for 24 h with 4 changes of the water during dialysis. The dialyzed sample constituted the partial purified PPO extract and was used as the enzyme source from the corresponding segments of the two fruits. Protein concentrations were determined by the methods of Bradford (1976) using bovine serum albumin as standard at λ_{max} = 595 nm. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 mL^{-1} min^{-1} under the condition of the assay (Oktay et al*.,* 1995).

Determination of PPO activity

PPO activity was measured immediately after the extract was partially purified. Enzyme assay was according to the methods of Qudsieh et al*.* (2002) with minor modifications (Chikezie, 2006). Enzyme activity was determined by measuring the increase in absorbance using a spectrophotometer (U-2000 Hitachi, Japan) at 24°C. The reaction mixture contained 3.5 mL of 0.20 M phosphate buffer (pH = 6.8), 1 mL of 0.75 mM catechol, and 0.5 mL of enzyme solution in a final volume of 5 mL. The mixture was quickly transferred into a cuvette and the change in absorbance was monitored at λ_{max} = 540 nm at a regular interval of 30 s. The rate of the reaction was calculated from the initial linear slope of activity curves.

Measurement of temperature and pH optima of PPO activity

Activity of PPO was measured in assay mixture containing 0.75 mM catechol under varying temperatures within the range of 20-70°C. The enzyme activity was measured using 0.20 M phosphate buffer under varying pH conditions within the range of 5-10.

Effect of temperature and pH on PPO activity and stability

Purified enzymes extracted from *S. melongenas* and *M. sapientum* fruits were pre-incubated in varying temperatures of 20, 50 and 60°C. At regular time intervals of 30, 60 and 90 min, aliquots of the enzyme solution was withdrawn and assayed for PPO activity. The residual PPO activity was measured according to the following experimental conditions ($PPO_{S.\ melongenas}$: $pH_{optimum}$≈ 7.0 at T °$C_{optimum}$≈ 30; $PPO_{M.\ sapientum}$: $pH_{optimum}$ ≈ 7.0 at T °$C_{optimum}$≈40), at the given time intervals. At the same time intervals, measurement of PPO activity pre-incubated in varying pH values of 3.5, 6.0 and 8.0 were carried out. The residual PPOactivity was measured according to the following experimental conditions ($PPO_{S.\ melongenas}$: $pH_{optimum}$≈ 7.0 at T °$C_{optimum}$≈ 30; $PPO_{M.\ sapientum}$: $pH_{optimum}$ ≈ 7.0 at T °$C_{optimum}$≈40). Residual PPO activity was determined in the form of percent residual PPO activity at the temperature and pH optima.

RESULTS

The fractionation steps and corresponding purification indices of the two PPO extracts is summarized in Table 1. At the end of the purification steps, specific enzyme activity of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ increased

Table 1. Properties of PPO extracted from *S. melongenas* and *M. sapientum* fruits at various purification steps.

Enzyme fraction	*S. melongenas*				*M. sapientum*			
	E_A (U)	T_P (mg)	Specific E_A (U/mg)	% Yield	E_A (U)	T_P (mg)	Specific E_A (U/mg)	% Yield
Crude homogenate	0.308	0.980	0.314	100	0.234	0.802	0.292	100
Centrifuged at 32000 *g*	0.215	0.072	2.99	69.8	0.167	0.082	2.04	71.4
80% $(NH_4)_2SO_4$	0.194	0.032	6.06	62.9	0.151	0.043	3.51	64.5
Ultra-filtration	0.162	0.022	7.36	52.6	0.133	0.029	4.59	56.8
Dialysis	0.154	0.019	8.11	49.9	0.130	0.017	7.65	55.6

E_A, Enzyme activity; T_P, total protein.

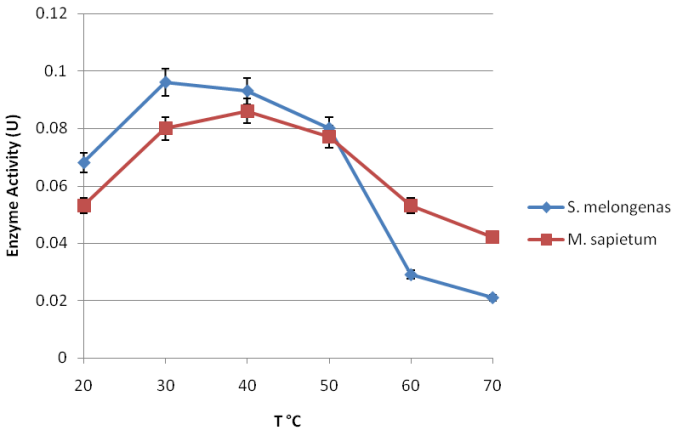

Figure 1. Temperature-activity profile of PPO activity extracted from *S. melongenas* and *M. sapientum* fruits.

within the range of 0.314 to 8.11 U/mg protein and 0.292 to 7.65 U/mg protein respectively. Final enzyme activity of $PPO_{S.melongenas}$ was 0.154 U, whereas $PPO_{M.\ sapientum}$ gave 0.130 U (Table 1).

Temperature-activity profile of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ is presented in Figure 1. The temperature-activity profile of the two PPOs showed a typical bell-shaped curve. The $PPO_{S.melongenas}$ T °$C_{optimum}$ ≈ 30; $PPO_{S.\ melongenas}$ activity = 0.096±0.02 U, whereas $PPO_{M.\ sapientum}$ T °$C_{optimum}$ ≈ 40; $PPO_{M.\ sapientum}$ activity = 0.086±0.02 U. The temperature-activity profile of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ showed a strong positive correlation (r = 0.907363). At experimental temperature of 70°C, $PPO_{S.melongenas}$ activity = 0.021±0.01 U; $PPO_{S.\ melongenas}$ = 0.042±0.03 U, which represented 78.1 and 51.1% decay in enzyme activity, respectively.

The pH-activity profile of the two PPOs showed a typical bell-shaped curve. The $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ gave $pH_{optimum}$ ≈ 7.0. However, pH-activity profile of $PPO_{S.\ melongenas}$ exhibited two peak values; pH ≈ 7.0 and pH ≈ 8.5. At experimental maximum pH = 10.0, $PPO_{M.\ sapientum}$ activity = 0.034 U, representing 65.3% decay in enzyme activity, whereas $PPO_{S.\ melongenas}$ = 0.025 U representing 79.3% decay in enzyme activity. Tables 2 and 3 show the residual activities of $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ incubated at varying temperature and pH. The residual activity of $PPO_{S.melongenas}$ ranged between 0.86±0.02 and 0.067±0.03 U; $PPO_{M.\ sapientum}$ was between 0.080±0.03 and 0.070±0.02 U. A cursory look at Table 2 shows that the decreasing levels of $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ depended on temperature and duration of incubation. Residual activities of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ showed a strong positive correlations under the same experimental temperature conditions, with exception at 20°C, which gave a weak positive correlation (r = 0.693375) (Table 4).

Similarly, $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ incubated at varying pH conditions exhibited decreasing residual levels of PPO activity with the progress of experimental time. Specifically, residual level of $PPO_{S.melongenas}$ activity under the three experimental pH was in the order pH = 6.0 > pH = 8.0 > pH = 3.5 within the duration of the experiment (30 <t < 90). Table 4 shows $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ activities displayed a strong positive correlations under the same pH conditions.

Pre-incubation of $PPO_{S.melongenas}$ at 50°C caused the lowest decay in relative activity compared to $PPO_{S.\ melongenas}$ pre-incubated at 20 and 60°C. $PPO_{S.melongenas}$ pre-incubated at 60°C exhibited lower decay in relative activity at incubation period t = 30 min and t = 90 min, compared to $PPO_{S.\ melongenas}$ pre-incubated at 20°C. Conversely, $PPO_{S.melongenas}$ pre-incubated at 60°C showed higher decay in activity compared to the enzyme pre-incubated at 20°C; at t = 60 min (Figure 3). Pre-incubation of $PPO_{M.sapientum}$ at the three experimental temperatures caused increasing decay in the relative activity of the enzyme with the progress of time, which was in the order 20 >60 >50°C (Figure 3). However, the increasing decays in activities of $PPO_{M.\ sapientum}$ pre-incubated at 20 and 60°C was not significantly different (p< 0.05). Specifically, pre-incubation of $PPO_{M.sapientum}$ for t = 90 min at 60°C caused 18.4% decay in relative activity of $PPO_{M.\ sapientum}$ (Figure 4). Pre-incubation of $PPO_{M.sapientum}$ in pH = 3.5 caused decay in relative enzyme activity between the range of 56.3-78.8% within the experimental time (30 <t < 90) min. Decay in relative activity was significantly different among the $PPO_{M.\ sapientum}$ incubated at the three experimental pH conditions,

Table 2. Residual activity of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ incubated in varying temperature.

Time (min)	Enzyme activity V_o(U)					
	S. melongenas			*M. sapientum*		
	30	60	90	30	60	90
20°C	0.079±0.03	0.076±0.02	0.067±0.03	0.077±0.02	0.072±0.03	0.072±0.03
50°C	0.086±0.02	0.083±0.01	0.082±0.02	0.080±0.03	0.079±0.02	0.076±0.02
60°C	0.082±0.01	0.075±0.02	0.073±0.02	0.076±0.03	0.073±0.02	0.070±0.02

Values are means of three determinations ± S.D.

Table 3. Residual activity of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ incubated in varying pH.

Time (min)	Enzyme activity V_o(U)					
	S. melongenas			*M. sapientum*		
	30	60	90	30	60	90
pH = 3.5	0.067±0.03	0.052±0.03	0.033±0.03	0.068±0.03	0.065±0.03	0.063±0.03
pH = 6.0	0.143±0.02	0.140±0.02	0.138±0.02	0.097±0.02	0.092±0.02	0.088±0.02
pH = 8.0	0.127±0.01	0.124±0.01	0.122±0.01	0.095±0.01	0.094±0.01	0.092±0.01

Values are means of three determinations ± S.D.

Table 4. Correlation coefficient between residual activities of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ incubated in varying temperature and pH.

Correlation coefficient (*r*)					
Temperature (°C)			pH		
20	50	60	3.5	6.0	8.0
0.693375	0.846154	0.952217	0.983342	0.984018	0.953821

which was in the order pH = 3.5 > pH = 8.0 > pH = 6.0 (Figure 5).

The decay in relative activity of $PPO_{M.sapientum}$ pre-incubated in pH = 6.0 and pH = 8.0 were not profound compared to pH = 3.5 pre-incubation. At the end of experimental *t* = 90 min, pre-incubation of $PPO_{M.\ sapientum}$, in pH = 3.5 caused decay in relative activity within the range of 30.8-36.1%, whereas $PPO_{M.\ sapientum}$ pre-incubated in pH = 6.0 and pH = 8.0 gave moderate decays in relative activities, which was within the range of 1.5-9.8% and 2.7-6.5% respectively (Figure 6).

DISCUSSION

From the present study, PPO extracted from the two fruits showed different pH and temperature optima ($PPO_{S.\ melongenas}$: $pH_{optimum}$≈ 7.0 at T $°C_{optimum}$≈ 30; $PPO_{M.\ sapientum}$: $pH_{optimum}$ ≈ 7.0 at T $°C_{optimum}$≈40) (Figures 1 and 2). Worthwhile to note, $PPO_{M.sapientum}$ T$°C_{optimum}$ ≈ 40, was same as PPO extracted from lily *Carica papaya* and *Cucurbita pepo* (Ying and Zhang, 2008). Other reports by several authors (Liu et al*.,* 2007; Bello et al*.,* 2011; Yemenicioglu et al*.,* 1999; Mizobutsi et al*.,* 2010; Mahmood et al*.,* 2009; Gaoa et al*.,* 2011) gave diverse temperature and pH optima of PPOs extracted from various plant tissues. Specifically, Zheng et al*.* (2012) using 10 mM catechol as substrate reported that *Vitis vinifera Thompson Seedless* PPO activity $pH_{optimum}$≈ 6.0 and $temperature_{optimum}$≈ 25. Nakamura et al. (1983) stated that T$°C_{optimum}$ and $pH_{optimum}$ of PPO extracted from Koshu *V. vinifera* were approximately 25 and 6.0°C, respectively. Alyward and Haisman (1969) and Sellés-Marchart et al. (2006) reported that differences in optimum pH for PPO activity depended on plant sources, extraction methods, and purities of enzyme, buffers, and substrates. However, most plants show maximum PPO activity near neutral pH values (Jime´nez-Atie´nzar et al*.,* 2004; Dogan and Dogan, 2004). These previous reports are consistent with the present report ($PPO_{S.\ melongenas}$: $pH_{optimum}$≈ 7.0; $PPO_{M.\ sapientum}$: $pH_{optimum}$ ≈ 7.0) (Figure 2). Remarkably, pH-activity profile of $PPO_{S.melongenas}$ exhibited two peak values; pH ≈ 7.0 and pH ≈ 8.5, which was an indication of the presence of isoenzyme based on similar reports by Bello et al*.* (2011). Using catechol as substrate, Bello and Sule (2012), reported variable T $°C_{optimum}$ of PPO extracted from wide varieties of tropical fruits and vegetables. Accordingly, *S. aethiopicum*: T $°C_{optimum}$≈ 50°C; *C. papaya*: T $°C_{optimum}$≈ 40°C; *C. pepo* T $°C_{optimum}$ ≈ 50°C; *Psidium guajava*: T $°C_{optimum}$ ≈ 30°C;

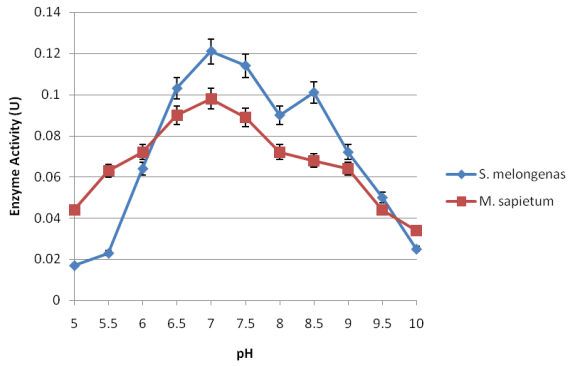

Figure 2. pH profile of PPO activity extracted from *S. melongenas* and *M. sapientum*fruits.

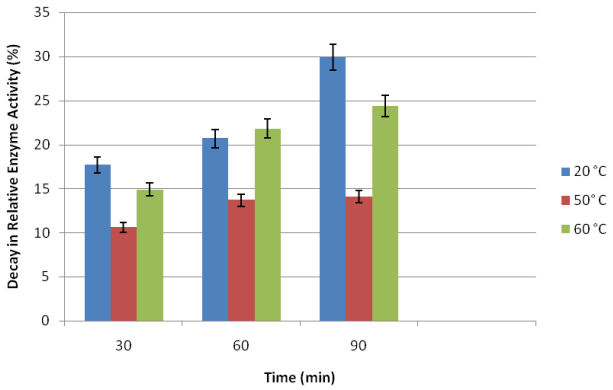

Figure 3. Percentage decay in enzyme activity of PPO extracted from *S. melongenas* incubated at varying temperature.

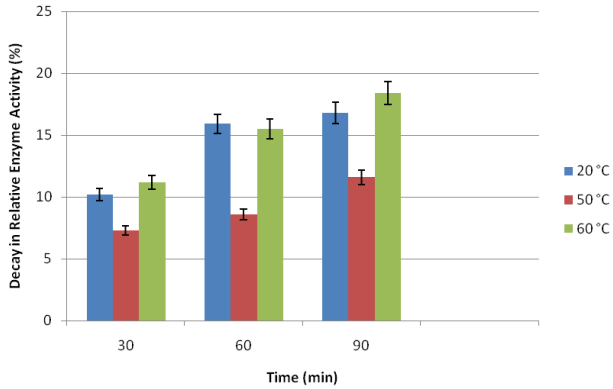

Figure 4. Percentage decay in enzyme activity of PPO extracted from *M. sapientum* incubated at varying temperature.

Irvingia gabonnensis: T °$C_{optimum}$≈ 50°C. It is worthwhile to note that PPO T °$C_{optimum}$ is dependent on substrate type (Mahmood et al., 2009). Notably, Ziyan and Pekyrdimic (2004) had earlier reported the effect of seven different substrates on the T °$C_{optimum}$ of *Pyrus communis* PPO.

Pre-incubation of $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ under the two experimental conditions of temperature and pH gave diverse activity, which was a reflection of divergent level of PPO stability. The time dependent decay in PPO activity of two fruit extracts (Figures 3, 4 5 and 6) showed also the divergent capacities of the enzymes to withstand destabilizing effects of unfavourable temperature and pH conditions. This finding was an obvious indication that the three dimensional structure and functionality of enzymes are inextricably connected with pH and temperature conditions (Rodwell and Kennelly, 2003). In a similar study, Lacki and Duvnjak (1999) reported that changes in pH level from 5.0 to 3.2 caused loss of PPO stability of white-rot fungus *Trametes versicolor*, which was comparable to that observed when the pre-incubation temperature was increased from 50 to 70°C. In another study, Yemenicioğlu and Cemeroğlu (2003), showed the effect of ripening on thermal stability of *P. armeniaca* PPO and posited that thermal stability of PPO depended on the cultivar and stage of ripening and the presence of isoenzyme as reported by Yemenicioglu et al. (1999).

The study by Mahmood et al. (2009) showed that PPO from different plant sources exhibited different thermal stabilities. The present study shows that decreasing levels of $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapientum}$ activity depended on temperature and duration of incubation (Table 2), which was a reflection of level of thermal stability of the two PPO extracts (Mizobutsi et al., 2010; Bello and Sule, 2012; Zheng et al., 2012). The decay in $PPO_{S.melongenas}$ and $PPO_{M.\ sapientum}$ activity was more profound at 20 and 60°C (Figures 3 and 4).

The present finding is in similarity with the reports of Marcos et al. (2008) in which they noted that PPOs from melon varieties (*Amarillo* and *Charentias*) were nearly com-pletely inactivated after 30 min of incubation at 60°C (94% loss of enzyme activity). Mizobutsi et al. (2010) reported that *Litchi chinensis* pericarp incubated at temperature of 60°C for 10 min reduced the enzyme activity to scarcely detectable level.

Furthermore, studies by Zheng et al. (2012) reported that Thompson seedless grape PPO exhibited thermal stability between 10 and 25°C, but showed significant activity loss at temperatures higher than 40°C and was completely inactivated at 70°C for 10 min. They further stated that thermal inactivation of PPO showed a first-order kinetic with an activation energy (E_a) of 146.1±10.8 kJ/mol at pH = 6.0. Therefore, it is worthwhile to note that PPOs from different plant sources exhibited divergent thermal stabilities (Bello and Sule, 2012).

Similarly, the relationship between stability of PPO and pH showed a time depended decay in enzyme activity (Figures 5 and 6). Nakamura et al. (1983) had earlier noted that PPO extracted from Koshu *V. vinifera* was

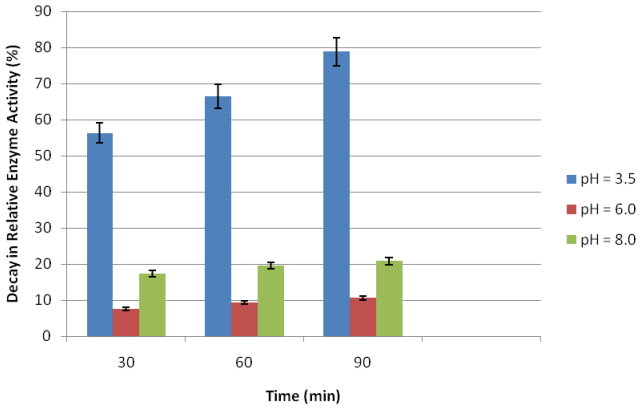

Figure 5. Percentage decay in enzyme activity of PPO extracted from *S. melongenas* incubated at varying pH.

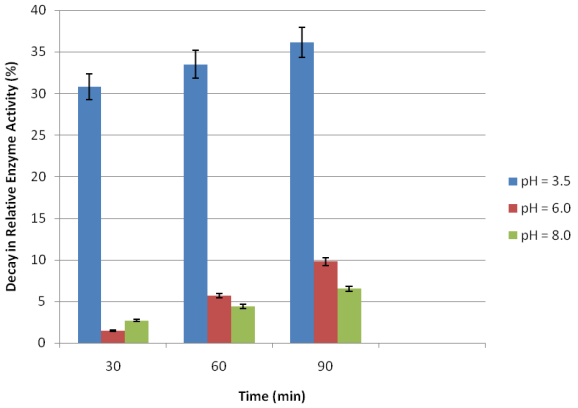

Figure 6. Percentage decay in enzyme activity of PPO extracted from *M. sapientum* incubated at varying pH.

stable in the alkaline pH range (between pH = 7.0 and pH = 11.0). Again, Mizobutsi et al. (2010) reported that *L. chinensis* pericarp pre-incubation up to 35 min, at pH 2.5 or 9.5 caused complete inactivation of the enzyme. They further stated that the acid pH was more an effective destabilization agent. Likewise, reports of Gaoa et al. (2011) showed that PPO of leaf extract of *Cleome gynandra L* exhibited optimal activity at pH = 8.0, and further noted a progressive PPO stability from pH 3.0 to 9.0. In similar characteristics, $PPO_{S.melongenas}$ and $PPO_{M. sapientum}$ showed relatively lower decay in activity when pre-incubation pH tended towards alkaline conditions, whereas decay in activity was profound at acidic pH conditions. The relatively high decay of $PPO_{S.melongenas}$ and $PPO_{M. sapientum}$ activities pre-incubated at pH = 3.5 (Figures 5 and 6) was an indication that acidic pH promoted enzyme destabilization, which provided strong evidence that denaturalization pH of the PPOs was near pH ≈ 3.5. However, the propensity of acidic pH to cause destabilization of the enzyme extracts was in the order $PPO_{S. melongenas} > PPO_{M. sapientum}$. Comparable reports on characterization of PPO from *L. chinensis* pericarp according to Liu et al. (2007) showed that incubation of the enzyme at pH = 3.1 for 1 day caused 49.50% loss in PPO activity, and only 2.43% of the activity remained after 12 days of incubation, indicating that *L. chinensis* pericarp PPO was very unstable at pH = 3.1. They further posited that the PPO activity decayed more moderately when incubated at pH = 4.5 than when incubated at pH = 3.1. Furthermore, Bello et al. (2011) reported that crude PPO extracted from *S. aethiopicum, C. papaya* and *C. pepo* showed instability in acidic pH but was more stable near neutral pH, whichis in agreement with the findings of Kavraya and Aydemir (2001) in which *Mentha piperita* PPO was found to be stable between pH 6.0 and 7.0.

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Full Length Research Paper

Induction of thermotolerance through heat acclimation in lablab bean (*Dolichos lablab*)

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The acclimation of plants to moderately high temperature plays an important role in inducing plant tolerance to subsequent lethal temperatures. This study was performed to investigate the effects of heat acclimation and sudden heat stress on the antioxidant and metabolic profile of lablab bean (*Dolichos lablab*). Following separate pretreatments with heat acclimation (35°C) and NaCl (100 mM), seedlings of lablab bean were exposed to heat stress at 45°C for 5 h and then recovered at 25°C for five days. Pre-treated seedlings performed better under heat stress than the control and it could be associated with the observed increased levels of sugars, proline, glutathione and ascorbate; and increased activities of Peroxidase (POX), glutathione reductase (GR) and ascorbate peroxidase (APX) than just heat shocked seedlings. Seedling growth was dramatically reduced under heat stress but heat acclimation and NaCl pre-treatment were effective in imparting thermoprotection against the lethal heat shock.

Key words: Acclimation, antioxidants, catalase, *Dolichos lablab*, glutathione reductase, heat stress, peroxidase, proline, sugar.

INTRODUCTION

Dolichos lablab, a member of *Fabaceae*, is an ancient crop and has been documented by archaeo-botanical finds in India prior to 1500 BC at Hallur, India's earliest Iron Age site in Karnataka (Fuller, 2003). Despite its label as 'underutilized', substantial cultivation of Lablab bean is seen in certain tropical regions, either as a sole crop or in mixed production systems. Remarkable morphological variations have also been reported throughout India (Sankaran et al., 2007). It also has considerable physiological diversity; a range of adaptation to acidity, low soil phosphorous and drought has been reported for the plant (Mugwira and Haque, 1993; Karachi, 1997). Transitory or constant high temperatures cause an array of morphoanatomical, physiological, and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. Plants have evolved various mechanisms for thriving under higher prevailing temperatures. These include short term avoidance/acclimation mechanism or long term evolutionary adaptations. In case of sudden heat stress, short term response that is, leaf orientation, transpirational cooling and changes in membrane lipid composition are more important for survival (Wahid et al., 2007). Different tissues in plants show variations in responses in terms of developmental complexity, exposure towards the prevailing or applied stress types (Queitsch et al., 2000).

The stress responsive mechanisms established by an initial stress signal are in the form of ionic and osmotic effects or changes in the membrane fluidity. This helps

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Abbreviations: AMY, Amylase; **ASC,** ascorbate; **APX,** ascorbate peroxidase; **CAT,** catalase; **GR,** glutathione reductase; **GSH,** reduced glutathione; **INV,** invertase; **POX,** peroxidase; **ROS,** reactive oxygen species; **RWC,** relative water content; **TBARS,** thiobarbituric acid reactive species; **TSS,** total soluble sugars.

to reestablish homeostasis and to protect and repair damaged proteins and membranes (Vinocur and Altman, 2005). However, irreversible changes in cellular homeostasis may occur due to inadequate response during signaling and gene activation processes that result in the destruction of structural and functional proteins and membranes, ultimately leading to cell death (Vinocur and Altman, 2005; Bohnert et al., 2006). Plants lacking the ability to display rapid heat acclimation responses may be more prone to thermo-damage. Here, acquired thermotolerance may have significant role. Since plants have to face temperature fluctuations during day/night cycle, the acquisition of thermotolerance reflects a more general mechanism that contributes to homeostasis of metabolism on a daily basis (Hong et al., 2003). Some major mechanisms, which make plants thermotolerant include ion transporters, free radical scavengers, late embryogenesis abundant (LEA) proteins, osmoprotec-tants and factors involved in signaling cascades and transpirational control (Wang and Luthe, 2003). Heat stress effects are of greater concern at various levels including plasmalemma, biochemical pathways operative in the cytosol or organelles (Sung et al., 2003).

Studies have revealed that the first target of heat stress is the plasmalemma that shows increased fluidity (Wahid et al., 2007). This leads to the induction of Ca^{2+} influx and reorganization of cytoskeleton and eventually the upregulation of calcium dependent protein kinase (CDPK) and mitogen activated protein kinase (MAPK). Nuclear signaling of such cascades shows the synthesis of cytosolutes and antioxidants. The cytosolutes help to maintain cellular water balance; while the antioxidants scavenge reactive oxygen species (ROS) and are correlated with acquisition of thermotolerance (Maestri et al., 2002). The accumulation of ROS can cause peroxidation of membrane lipids, denaturation of proteins and damage of nucleic acids, ultimately upsetting homeostasis (Mittler, 2002). It is known that plants resist stress-induced production of ROS by increasing the activity of their ROS induced scavenging system (Ali et al., 2008; Goyal and Asthir, 2010). The major ROS-scavenging mechanisms include enzymatic system, which consists of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic system, which consists of ascorbic acid (ASC) and glutathione (GSH). Previous studies have indicated that the changes in antioxidant enzymes and antioxidants contribute to the plants resistance to high temperature (Almeselmani et al., 2006).

Heat acclimation, during which the plants develop heat tolerance, is a genetically controlled process that is triggered by exposing plants to mild or sublethal temperatures or by the application of compounds or biomolecules to the growth medium (Charng et al., 2006). The processes involved in temperature acclimation are initiated by the perception of temperature signals and transduction of these signals into biochemical processes that finally lead to the development of heat tolerance (Xu et al., 2006).

The proteins thus expressed facilitate growth and survival of plants not only at transient temperatures, but also under conditions of severe heat stress, whereby lethal temperature can be tolerated for short periods. The present work was initiated to study the effect of high temperature stress on antioxidants and antioxidant enzymes, as well as other parameters, and the role played by these factors in protecting the plant cell from damage occurring due to high temperature stress.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of *D. lablab* (cv. HA-4) were purchased from National Seed Project, University of Agricultural Science, GKVK, Bangalore, India. Seeds were surface sterilized with 0.1% (w/v) mercuric chloride for 30 s, rinsed immediately with large volume of distilled water and imbibed overnight in distilled water. The overnight-soaked seeds were sown in plastic trays (3 seeds per pot) containing vermiculite and acid-washed sand (1:1 w/w) and irrigated daily with distilled water. The germination was carried out under natural greenhouse conditions; day/night temperature and relative humidity were 25/20°C and ~70%, respectively. The average photoperiod was 12 h light/12 h dark.

Heat acclimation and heat stress treatments

Five day old seedlings were subjected to heat treatments in 1X Hoagland medium (Allen, 1968). For heat acclimation (HA), plants were maintained at 35°C for 2 h and then exposed to heat stress (HS) at 45°C for 5 h. For sudden heat shock (HS), plants were exposed only to 45°C for 5 h. A combination of salt stress and heat stress (SS + HS) was carried out by subjecting salt-stressed plants (100 mM NaCl at 25°C for 24 h) to the heat shock treatment at 45°C for 5 h. All plants, that is, HS, HA + HS, HS + SS were subjected to a recovery period at ~25°C for 3 days in 1X Hoagland media and then sampled. Seedlings kept entirely at 25°C without subjection to any treatment were taken as control (C). Ten seedlings were used in each experiment and each experiment was done in triplicate.

Relative water content (RWC)

The relative water content was estimated according to the method of Turner and Kramer (1980) using the equation:

$$RWC = (FW-DW) \times 100 / (TW-DW)$$

Leaf discs of 10 mm diameter were weighed to determine the fresh weight (FW), soaked in distilled water at 25°C for 4 h to determine the turgid weight (TW), then oven dried at 80°C for 24 h to determine the dry weight (DW). Similarly, entire shoot and root was taken for analysis and RWC was computed as aforementioned.

Assay of metabolite and antioxidant enzymes

The frozen samples were homogenized with pre-chilled 50 Mm

sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM EDTA using pestle and mortar. L-ascorbate was raised to a final concentration of 2 mM for extraction of APX. The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was used as a source of enzymes. Soluble protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

β-Amylase (AMY, E.C. 3.2.1.1)

Activity of β-amylase was measured using the DNS method (Bernfield, 1955). The reaction mixture consisted 0.5 ml of 2% starch solution in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract.

Invertase (INV, E.C. 3.2.1.26)

Invertase activity was determined by the method of Sridhar and Ou (1972). 4.0 ml reaction mixture containing 0.025 M sodium acetate buffer (pH 5.0), 0.625% sucrose and appropriate volume of enzyme extract was incubated at 37°C for 24 h. The reaction was arrested by adding equal volume of DNS reagent. The reducing sugars present were estimated using the method of Miller (1959).

Catalase (CAT, E.C. 1.11.1.6)

Catalase activity was assayed by following the decline in absorbance of H_2O_2 at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) according to the method of Aebi (1984). The reaction mixture consisted of 50 µl of enzyme extract in 50 mM sodium phosphate buffer (pH 7.0). The reaction was started by addition of H_2O_2 to a final concentration of 10 mM, and its consumption was measured for 2 min. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of H_2O_2 per min under the assay conditions.

Guaiacol peroxidase (POX, E.C. 1.11.1.7)

Guaiacol peroxidase activity was measured in a reaction mixture of 3.0 ml consisting of 50 mM phosphate buffer (pH 7.0) containing 20 mM guaiacol, 10 mM H_2O_2 and 100 µl enzyme extract (Chance et al., 1955). The formation of tetraguaiacol was followed by an increase in A_{470} nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). One unit of peroxidase is defined as the amount of enzyme needed to convert 1 µmol of H_2O_2 min^{-1} at 25°C.

Glutathione reducatase (GR, E.C. 1.6.4.2)

GR activity was determined by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) according to the method of Carlberg and Mannervik (1985). The reaction mixture contained 50 mM tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH and 250 µl of enzyme extract in a total volume of 1.5 ml. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH per min under the assay conditions.

Ascorbate peroxidase (APX, E.C. 1.11.1.11)

The activity of APX was determined spectrophotometrically as described by Allen (1968). The assay mixture contained 50 mM HEPES buffer (pH 7.0), 1 mM EDTA, 1 mM H_2O_2, 0.5 mM sodium ascorbate and 50 µl of enzyme extract in a total volume of 2.0 ml. The reaction was initiated by addition of H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290} ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of ascorbate peroxidase is defined as the amount of enzyme necessary to oxidize 1 µmol of ascorbate per min at 25°C.

Determination of H_2O_2 and antioxidants

Hydrogen peroxide content in control and stressed seedlings were determined according to the study of Velikova et al. (2000). Ascorbic acid estimation was carried out according to the procedure of Sadasivam and Manickam (1997). Glutathione (GSH) was estimated according to the study of Beutler (1963). Total phenols were estimated by the method of Slinkard and Singleton (1977) using catechol as an authentic standard.

Determination of stress response factors

Proline content was estimated using ninhydrin reagent according to the study of Bates et al. (1973). The amount of total soluble sugars was estimated colorimetrically at 540 nm using anthrone reagent, according to Roe (1955). The extent of lipid peroxidation was determined according to Heath and Packer (1968). The TBARS content was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

The experiment was performed using a randomized design. All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using PrismGraph version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by least significant difference (LSD) test. Comparisons with $P < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Effect of stress treatments on growth and RWC

The efficacy of various pre-treatments like heat acclimation and use of salt was studied by inducing thermotolerance in Lablab bean. Growth is an irreversible increase in volume and structural biomass involving cell division, cell enlargement, maturation and specialization to form tissues and organs. A quantitative understanding of the plant growth dependence on temperature is essential for the selection of cultivars to optimize growth in different climates, to understand the physiological responses to climate change and to identify and quantify thermotolerant species. Direct analysis of plant growth rates involves the measurement of seedling length, fresh/ dry weights and RWC. Exposure to heat shock (HS) alone caused inhibition of shoot growth in terms of seedling length, fresh/ dry weights and RWC (Figure 1). HS severely limits water uptake causing a reduction in growth. Both pre-treatments that is, HA + HS and SS + HS helped seedlings to recover from heat stress wherein the best heat tolerance based on morphological analysis was conferred by the former. The fresh/dry weights of pre-treated seedlings increased when compared to control

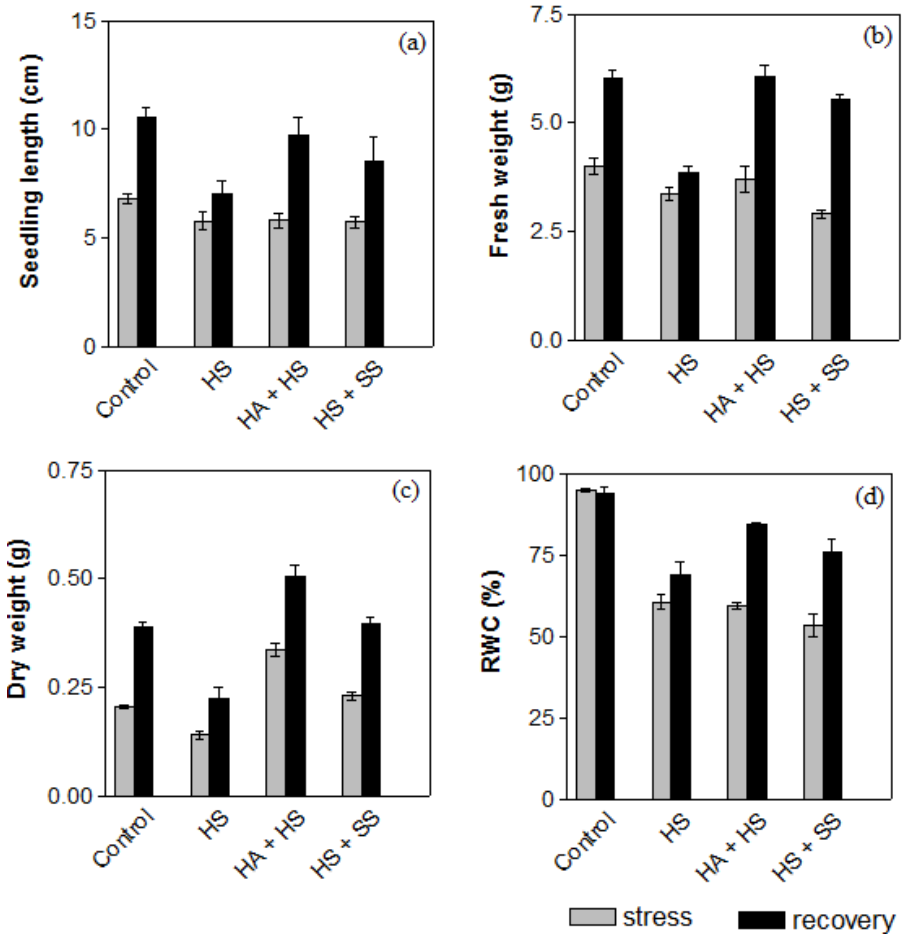

Figure 1. Seedling length (a), fresh weight (b), dry weight (c) and RWC (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

(Figure 1b and c). The increase in dry weight in these seedlings may have been due to accumulation of osmolytes, TSS and proline. Accumulation of osmolytes either active or passive is an important adaptation mechanism for stressed plants to protect cellular components form the injury caused by dehydration (Wahid and Close, 2007; Ashraf and Foolad, 2007). Several studies have reported biomass accumulation in heat acclimated *Brassica* (Kaur et al., 2009) and wheat (Asthir and Deep, 2011) under heat stress conditions.

Plant RWC status is the most important variable under changing ambient temperatures (Mazorra et al., 2002). In general, plants tend to maintain stable RWC regardless of temperature when moisture is abundant; however, high temperatures results in limited availability of water (Simoes-Araujo et al., 2003). In Lablab bean, elevated temperatures caused reduction in RWC in all treated samples when compared to control, the decrease being greater in HS treated seedlings (Figure 1d). A decrease in RWC in relation to raised temperature was also reported in *Lotus creticus* (Anon et al., 2004) and tomato (Morales et al., 2003). Reduction in tissue water causes a decrease in water potential thereby leading to perturbation of many physiological processes (Tsukaguchi et al., 2003) such as reduction in rate of transpiration, protein synthesis, enzymes and ion uptake and transport (Khalil et al., 2009). This explains the growth inhibition observed in HS treated seedlings even after the removal of the stressing conditions.

Response of hydrolytic enzymes and soluble sugars

Metabolites have a number of functions in addition to those of intermediary metabolism. They act as signaling/regulatory agents, compatible solutes, antioxidants or defense molecules against pathogens. The results obtained with Lablab bean provide an insight into the roles of two known signaling molecules and protectants, namely total soluble sugars (TSS) and proline.

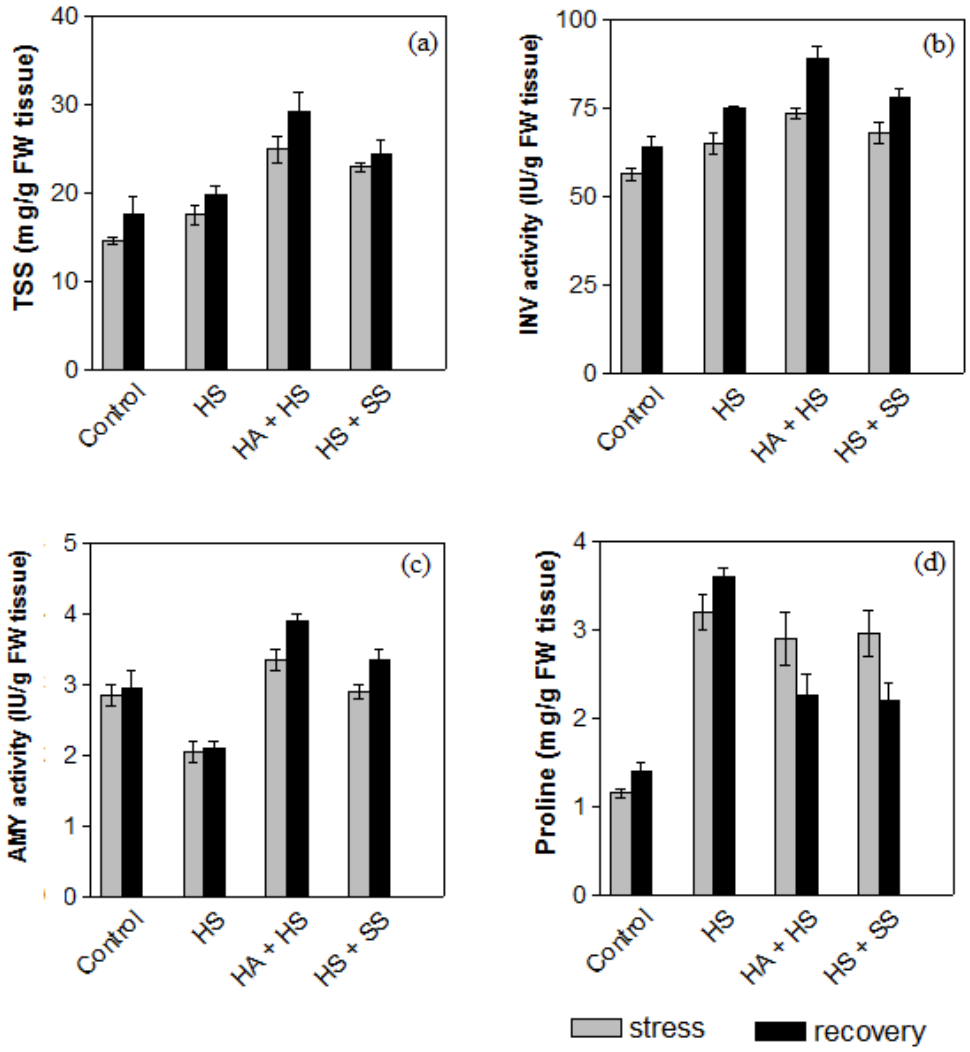

Figure 2. Total soluble sugars (a), invertase activity (b), amylase activity (c) and proline (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

Accumulation of total soluble sugars (TSS) under heat stress has been implicated in the establishment and maintenance of thermotolerance (Wahid and Close, 2007; Rizhsky et al., 2004). Sugars serve as signalling molecules during abiotic stress in stress-tolerant phenotypes (Rosa et al., 2009). Sugar signaling pathways interact with stress pathways in a complex network to modulate the metabolic responses of plants (Gill et al., 2003; Tran et al., 2007). The effect of HS on carbohydrate metabolism in Lablab bean showed a small increase in the TSS while HA and SS + HS pre-treatments resulted in a significant increase (Figure 2a) implying better signalling in place in pre-treated seedlings. Accumulation of TSS has also been reported in heat acclimated grape (Greer and Weston, 2010) and sugarcane (Wahid and Close, 2007) as a means of establishing thermotolerance. Invertase (INV) plays an important function in cell elongation and plant growth through carbon allocation (Gibeaut et al., 1990). Secondly, it also helps in sucrose metabolism, which in turn, has a crucial role in germination, seedling growth and in increasing the osmotic potential of the stressed cell (Ruan et al., 2010).

Increased INV in HS and pre-treated seedlings (Figure 2b) corroborates the higher level of TSS in these seedlings. An increase in INV was also reported in Brassica (Kaur et al., 2009) and potatoes (Lorenzen et al., 2002) where it was shown to contribute to theromtolerance of these plants. β-Amylase (β-AMY) is important in the transitory starch breakdown (Scheidig et al., 2002) needed to combat heat stress (Mansoor and Naqvi, 2012). The activity of β-AMY was reduced under HS;

however, HA treatment prior to HS (HA + HS) induced AMY (Figure 2c). Lethal temperatures retard seedling growth as well as β -AMY in winter wheat (Sultana et al., 2000). Kaplan and Guy (2004) demonstrated the appearance of maltose after β-AMY induction and also highlighted the contribution of stress-induced maltose accumulation towards the protection of the photosynthetic electron transport chain, proteins and membranes inside the chloroplast during acute temperature shock.

Response of the osmolyte, proline

Proline accumulation is a widespread phenomenon in higher plants in response to various environmental stresses and is demonstrated to be protective for plants under adverse conditions. Proline so accumulated is proposed to act as a compatible osmolyte, free radical scavenger, cell redox balancer, potential inhibitor of programmed cell death (PCD), cytosolic pH buffer and stabilizer for subcellular structures during various stresses (Kavi Kishore et al., 2005; Trovato et al., 2008; Gill and Tuteja, 2010). Under supra optimal temperature, free proline is known to accumulate in different crops (Rasheed et al., 2011). It is therefore, considered to be a useful component for evaluating the degree of heat stress (Kou et al., 1986). Proline content in leaves was significantly higher in HS treated Lablab bean while its levels declined considerably in HA + HS (Figure 2d) after recovery. This suggests that the HS seedlings were still unable to recuperate from stress even after 3 days of recovery.

The exact physiological function of proline is still controversial, and several researchers have attributed its beneficial function to the process of proline metabolism rather than to the proline molecule itself. The inter conversion of pro and pyrroline-5-carboxylate (P5C) in different cellular compartments might be involved in metabolic signaling, regulation of intracellular redox potential in higher plants and generation of ATP required for recovery from stress (Miller et al., 2009). Compared with other stresses; however, only a few reports demonstrated proline accumulation during heat stresses (Chakraborty and Tongden, 2005; Rasheed et al., 2011).

Response of antioxidants and antioxidant enzymes

In plants, ROS has been demonstrated to cause oxidative damage leading to cellular injury during various environmental stresses, including extreme temperature (Larkindale and Knight, 2002; Apel and Hirt, 2004). Even very short heat stress is able to bring about the increase in ROS, among which hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are believed to be the most important components (Apel and Hirt, 2004). The scavenging of O_2^- by superoxide dismutase (SOD) results in the production of H_2O_2, which is then removed by POX or CAT. H_2O_2 is primarily associated with the stress-induced stomatal closure that, in turn, causes a decrease in the CO_2/O_2 ratio in the chloroplasts (Cavalcanti et al., 2004). It has been demonstrated that this CO_2/O_2 ratio reduction in leaves inhibits CO_2 fixation, increasing the rate of ROS formation by enhancing electron leakage to oxygen molecules, and also, therefore, increasing the photorespiration process (Foyer and Noctor, 2003). To explore whether increased heat tolerance in HA seedlings is related to ROS generation during the acclimation phase, seedlings were analyzed for H_2O_2 levels upon recovery from heat stress. In Lablab bean, even after recovery, H_2O_2 levels were highest in HS treated seedlings than others (Figure 3a). Pre-treatment of seedling that is, HA + HS and SS + HS resulted in lower H_2O_2 content indicating that pre-treatments induced antioxidative components more efficiently than direct HS.

The accumulation of free radicals in stressed plants cause oxidation of polyunsaturated fatty acids in the plasma membrane resulting in the formation of thiobarbituric reactive species (TBARS) (Garg and Manchanda, 2009). TBARS level is used as an index of lipid peroxidation of cell membranes (Gechev et al., 2002). Temperature regulates membrane fluidity based on its composition and the degree of unsaturation (Los and Murata, 2004). Saturation of membrane lipids as a means of acclimation to high temperature is known to enhance thermal stability of PSII in thylakoid membranes (Sato et al., 1996). During the recovery stage after heat treatment, TBARS levels mimicked those of H_2O_2 (Figure 3b). These results demonstrate that oxidative stress is an important component of heat stress injury in Lablab bean and that HS induced more severe oxidative damage than pre-treated seedlings which were better equipped to scavenge ROS upon removal of heat stress. The increase in the content of lipid peroxides commonly associated with high temperature stress could serve as an activation signal for the expression of heat-shock genes which code for proteins and enzymes needed for the cell to tolerate high temperature (Vigh et al., 1998). Plants have multiple strategies to prevent oxidative damage to cells, employing enzymatic and nonenzymatic antioxidants. Superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) are among the enzymatic antioxidants. It is a well known fact that dismutation of O_2^- catalyzed by SOD produces H_2O_2 and O_2 (Asada and Takahashi, 1987). CAT exterminates H_2O_2 and is thought to be one of the most important antioxidant enzymes.

GR and APX act in the stress-regulated glutathione-ascorbate cycle. The activities of these enzymes have been proved to be inducible by the rise in intracellular ROS levels (Apel and Hirt, 2004). In Lablab bean, POX was found to be higher in HA + HS followed by SS + HS (Figure 3d). The maintenance of higher POX activity may provide further oxidative protection by detoxifying H_2O_2.

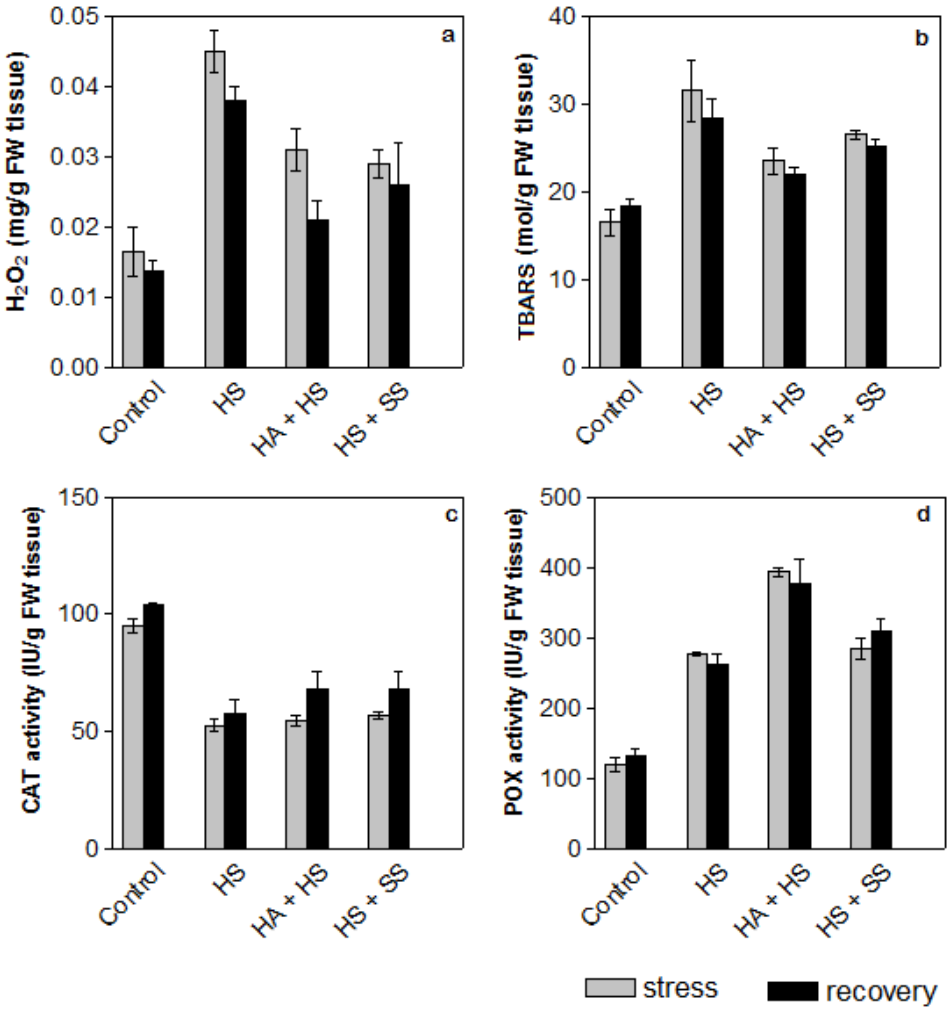

Figure 3. H_2O_2 (a), TBARS (b), CAT activity (c) and POX (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

These results are in consonance with POX activity reported in heat acclimated wheat (Asthir and Deep, 2011) and turfgrass seedlings (Du and Wang, 2009).

The reduction in CAT activity indicated that CAT may not be involved in antioxidant defence against heat stress in the Lablab bean (Figure 3c). A decrease in CAT activity was also reported in turfgrass (Du and Wang, 2009). The protective action of CAT is limited because it has relatively poor affinity for its substrates and is sensitive to light-induced inactivation compared with other antioxidant enzymes (Engel et al., 2006). Peroxisomal CAT is known to be sensitive to high temperature stress (Foyer and Noctor, 2000) probably because of an imbalance that occurs between its synthesis and degradation.

Also, as CAT has a rapid turnover, conditions inhibiting its synthesis will lower the steady-state level of this enzyme (Scandalios et al., 1997). Thus, heat shock and oxidative stress will enhance inactivation of CAT by preventing synthesis of new enzyme (Feierabend and Dehne, 1996), resulting in a decline in CAT activity. On the other hand, the absolute absence of recovery in leaf CAT activity in the recovered plants, even after 3 days, suggests that the enzyme suffered irreversible damage to its structure and/or that very low rates of *de novo* synthesis occurred. Available data suggests that signaling molecules like H_2O_2 may cause an increase in the antioxidant capacity of cells (Gong et al., 2001) by raising levels of GSH and ASC (Xu et al., 2006). GSH plays an important role in physiological functions such as redox regulation, conjugation of metabolites, detoxification of xenobiotics, homeostasis and cellular signaling that trigger adaptive responses (Foyer and Noctor, 2005; Rouhier et al., 2008). Nieto-Sotelo and Ho (1986) were the first to show that elevated synthesis of GSH occurs during temperature stress in plant cells.

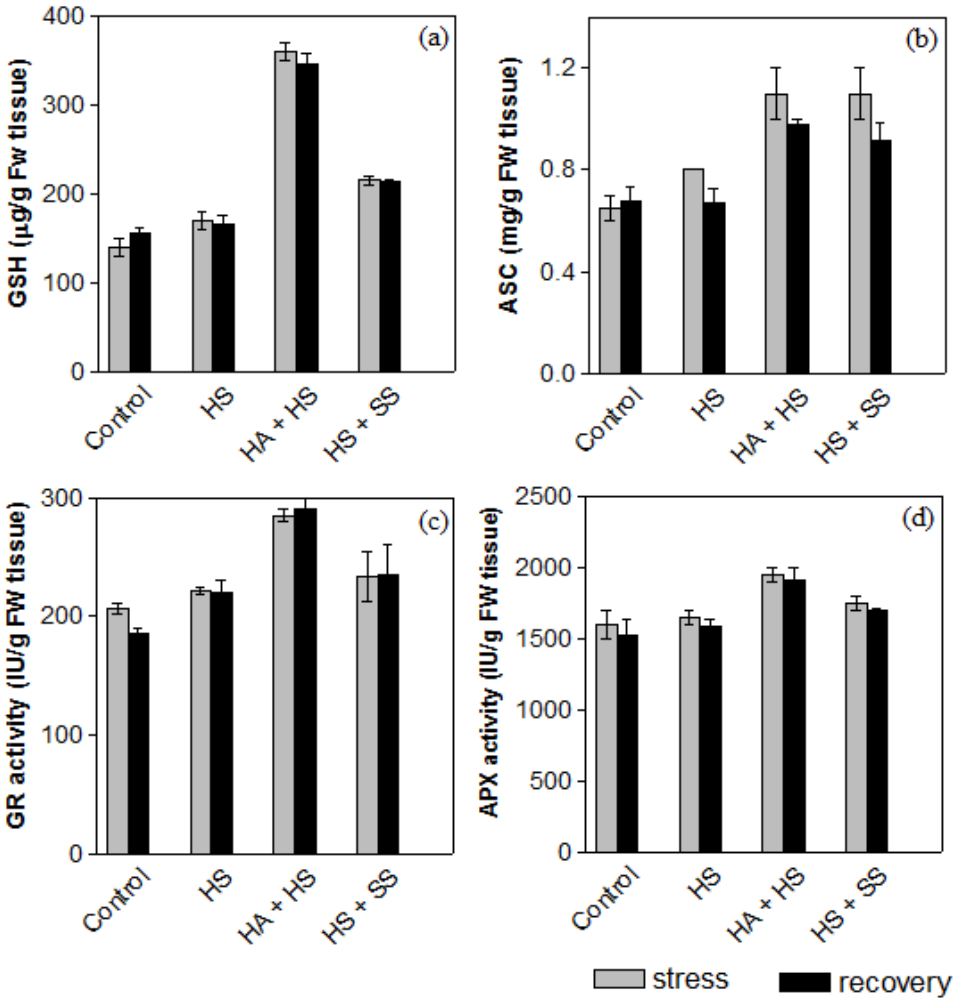

Figure 4. GSH (a), ASC (b), GR activity (c) and APX activity (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

Studies with heat-stressed Lablab bean suggested that seedlings that were pre-treated with heat stress had lower H_2O_2 levels as a result of enhanced synthesis of GSH (Figure 4a) and ASC (Figure 4b). Several authors have shown that elevated GSH content is correlated with the ability of plants to recover from heat stress when acclimated using sub-lethal temperatures (Nieto-Sotelo and Ho, 1986; Chao et al., 2009). Chao et al. (2009) have also demonstrated that HS signals lead to an early accumulation of H_2O_2 which in turn prevented rice seedlings from oxidative damage by Cd. The ASC pool in the chloroplast is regulated by the ascorbate-glutathione cycle involving four enzymes; APX, GR, DHAR and MDHAR (Noctor and Foyer, 1998). Our results show that GR and APX activities were significantly elevated in HA + HS (Figure 4c and d) resulting in a higher ASC content in these seedlings. It has been reported that the over-expression of GR leads to an increase in the ASC pool (Foyer et al., 1995) while overexpression of thylakoid membrane-bound APX functions to maintain the ASC content and the reduced status of ASC under stress conditions (Yabuta et al., 2002). In addition, enhanced choroplastic GR activity in transgenic plants have shown increased protection against oxidative stress (Melchiorre et al., 2009).

The increase in activity of POX, GR and APX in the recovery period of Lablab bean was in keeping with the work of Almeselamni et al. (2006) who have proposed that this type of response is characteristic of heat tolerant wheat genotypes upon recovery from high temperatures. The enhanced activities of GR and APX, concomitant with the enhanced content of ASC and GSH observed in this study could help to quench ROS and prevent cellular damage. According to the results obtained, it can be opined that Lablab bean plants may develop tolerance against superoptimal temperature stress caused at 45°C,

a temperature well above the optimal growth temperature of ~30°C through exposure to sub-lethal temperature of 35°C for 2 h. Thermotolerance acquired by plants through autonomous synthesis of pertinent compounds or induced through gradual exposure to sub-lethal temperatures (HA + HS), though cost intensive, is an important and potentially vital strategy. This phenomenon is principally related to display of heat shock response by antioxidants, antioxidant enzymes and compatible solutes; and accomplished by reprogramming of gene expression, allowing plants to cope with the heat stress.

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Full Length Research Paper

Determination of ulcer protecting effect of ethanol extract of *Gongronema latifolium* in rats

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Ethanol extract of dietary vegetable, *Gongronema latifolium*, was evaluated for anti-ulcer activity. The extract was obtained from air-dried, pulverized leaves of the plant following its maceration in ethanol, filteration with Whatman No. 1 filter paper and drying at 110°C. Fractionation of the dry crude ethanol extract was stepwisely carried out in with n-hexane, chloroform and ethylacetate, respectively, and their residual ethanol extract washed several times in ethanol. The four fractions were dried at low temperature and stored for use. The anti-ulcer activity of the crude extract was tested on indomethacin-induced and acid/ethanol-induced models of ulcer induction in rats. The activities of the fraction of the crude ethanol extract with respect, to reduction of ulcer index was evaluated only on indomethacin-induced ulcer model. The crude ethanol extract significantly ($p<0.05$) inhibited ulceration dose - dependently in the two ulcer models. This inhibition was higher in acid/ethanol model than in the indomethacin-induced ulcer model. The sub-fractions from the crude extract also inhibited ulcer with the chloroform fraction exhibiting the highest ulcer protection.

Key words: *Gongronema latifolium*, ulcer, protection, indomethacin, acid/ethanol.

INTRODUCTION

The epithelial lining of the gastric and intestinal mucosa is continually exposed to varied changes in chemical substances arising from intake of foods, drugs and drinks (Banks et al., 1976). As a result, tremendous disturbances that may culminate in pathological conditions such as ulcers, cancers could arise (Rang et al., 1995; Piper and Stiel, 1986). Ulcers are open sores or wounds appearing on the skin or mucus membrane caused by destruction of surface tissue (BMA, 2002; Owu et al., 2012). Peptic ulcer is one of the most rampart gastrointestinal (GIT) diseases creating a lot of pain and discomfort (Singh et al., 2008; Owu et al., 2012). Pathogenesis of ulcer has been attributed to effect of acid/ethanol (Goulart et al., 2005), non-steroidal anti-inflammatory drugs (NSAID), such as aspirin and indomethacin, used to inhibit pains, arthritis and inflammation (Vane, 1971) which can be complicated by *Helicobacter pylori* infection (Calam and Baron, 2001). Oxidative disturbances in the digestive system have also been implicated in ulcers especially, that of the activities of reactive oxygen species (ROS) (Repetto and Llesuy, 2002). Mucosal protection has been attributed to endogenous prostaglandin synthesis that stimulates the secretion of mucosa and bicarbonate layer along the GIT (Lanza, 1998).

Pharmacological intervention utilizing histamine H_2 blockers, antacids and anticholinergics have not succeeded to confer immunity from recurrence of disease or total restoration due to a number of limitation (Akhtar et al., 1992; Calam and Baron, 2001; Singh et al., 2008). Many indigenous plants used as food and spices are associated with bioactivities against ulcer and protection

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Abbreviations: NSAID, Non-steroidal anti-inflammatory drugs; **ROS,** reactive oxygen species; **GIT,** gastrointestinal.

of gastric mucosa (Ubaka et al*.,* 2009; Ukwe et al., 2010). Plants such as *Rodina rhomifilia* containing c-glycosyl flavones (Montanha et al., 2009 and Akudor et al*.*, 2012) or *Piper nigrum* with antioxidant property (Singh et al., 2008) have been shown to exhibit ulcer protecting as well as ulcer healing properties.

Gongronema latifolium is an edible, perennial climbing vegetable located in many parts of Africa and Nigeria where it is called "Utazi" in Ibo and "Orokeke" in Yoruba. It contains bitter principle and has many folkloric attributes as a healing herb (Dalziel, 1937; Ayodele, 2008). Several studies have revealed that its leaves and stem possess anti-diabetic properties (Ugochukwu and Babbady, 2003; Ezekwe 2005) and intestinal muscle relaxant (Gamaniel and Akah, 1996; Edet et al., 2011) and anti-inflammatory properties (Morebisi et al. 2002; Etim et al*.,* 2008). It is with these characteristics in mind that this work was undertaken to ascertain the efficacy, if any, of the vegetable on drug-induced ulceration.

MATERIALS AND METHODS

Plant

Fresh leaf samples of *G. latifolium* were detached from the stem and dried under shade and pulverized.

Extraction

The pulverized leaves (1 kg) were macerated in 5 L of 96% ethanol for 48 h. The extract was filtered with Whatman No.1 filter paper and dried at 40°C.

Fractionation of crude ethanol extract

Serial fractionation of the crude ethanol extract (75 g) on silica gel using successive volumes of n-hexane (5 L), chloroform (4.6 L) and ethylacetate (2.3 L) was carried out and the residual fraction was washed in ethanol (3.7 l); each fraction was dried and samples stored for further analysis.

Animals

Albino mice (13) weighing between (17 - 30 g) were selected for the acute toxicity study. Wistar albino rats (60) weighing (150 - 250 g) were also used for the anti-ulcer activity. All were housed in metallic cages, fed standard diet and water *ad libitum* and acclimatized for seven days before the study.

Acute toxicity test

The evaluation of toxicity of the crude ethanol extract was determined on mice by the Lorke (1983) method. A two-phase assay involving initial, low dose (10, 100, 1000) mg/kg b.w of crude were administered to three groups of 3 mice each and observation made for 48 h. Then, a second phase using 1600, 2900 and 5000 mg/kg b.w was administered to three groups of one mice each and a fourth group (saline). The mice were also observed for signs of toxicity or fatality for 24 h.

Effect of crude ethanol extract on Indomethacin-induced ulcer

The method of Urishidani et al. (1979) was utilized. Gastric ulceration was induced in four groups of four rats each using oral administration of 20 mg/kg b.w. indomethacin, 30 min after each group had received oral administration of its respective extract, standard drug or saline. After 7 h, the animals were sacrificed in ether chamber and stomachs excised, dissected, washed and fixed in formal saline and mounted on slab. Ulcer craters or wounds were counted, rated from 1-3 and used to compute the ulcer scores. The ulcer indices of a group are summation of ulcer scores (number of ulcer spots × their rating and divided by the magnification). The percent ulcer inhibition was calculated.

The effect of acid/ethanol-induced ulcer (0.3 N HCl/60% ethanol)

The method of Goulart et al. (2005) was employed. An identical set of four groups of four rats each as above, that is, extract (100 /300) mg/kg, control (saline 3 ml) and ranitidine (100 mg/kg) were set up. Each group received its respective dose of extract and control orally 30 min before 25 ml/kg of the acid/ethanol solution was administered orally. After 1 h, the rats were sacrificed and their stomachs prepared using the procedure above. Ulcer indices were calculated.

Effect of crude ethanol extract and its fractions on indomethacin-induced ulcer

The effect of the crude ethanol fractions (400 mg/kg) and crude were tested at a higher level of induction using indomethacin (40 mg/kg). Seven groups of four rats each were administered their respective extracts and the same procedures as above were followed to determine ulcer indices.

Statistical analysis

The results obtained were expressed as mean± standard error of the mean (SEM) for the ulcer indices and also as percentage ulcer inhibition. Differences between means were considered significant at $p<0.05$ using students t-test.

RESULTS AND DISCUSSION

The result obtained from the acute toxicity study showed no fatality so then the crude ethanol extract of *G. latifolium* was safe for consumption up to 5,000 mg/kg b.w. Figure 1 shows that the standard reference drug significantly ($P < 0.05$) inhibited ulcer in rats induced with indomethacin. In the same manner, the crude extracts dose-dependently inhibited ulceration in rats. The highest inhibition of ulcer was 46.5% in the rats administered 300 mg/kg ethanol extract and this was better than the inhibition from ranitidine (42.0%). Figure 2 also shows that in the acid/ethanol-induced ulcer, the reference drug significantly ($P<0.05$) inhibited ulceration in rats. Similarly, the extracts dose- dependently and significantly ($p<0.05$) inhibited ulceration in rats. However, the reference drug had a higher ulcer inhibition (64.12%) than the highest extract dose of 300 mg/kg (62.46%). Table 1 shows the effect of the crude ethanol extract and its fractions had on

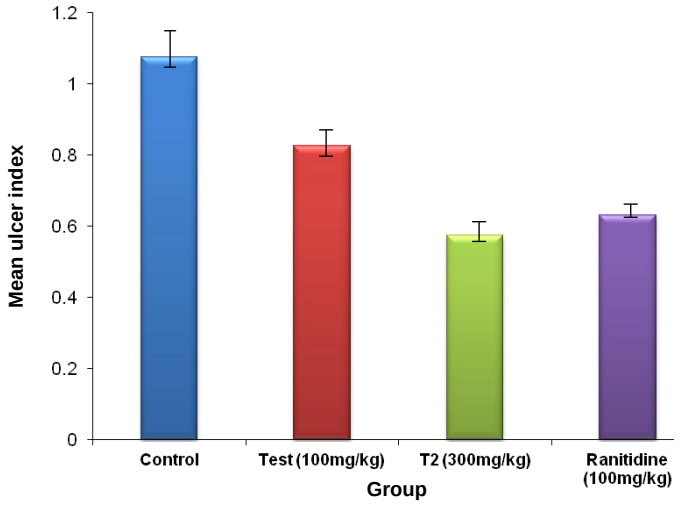

Figure 1. Effect of ethanol extract on *G. latifolium* (100, 300 mg/kg), control (3 ml/kg, 0.9%Nacl) and ranitidine (100 mg/kg) on indomethacin - induced gastric ulcer. ANOVA and students t-test were used to determine significant difference from control.

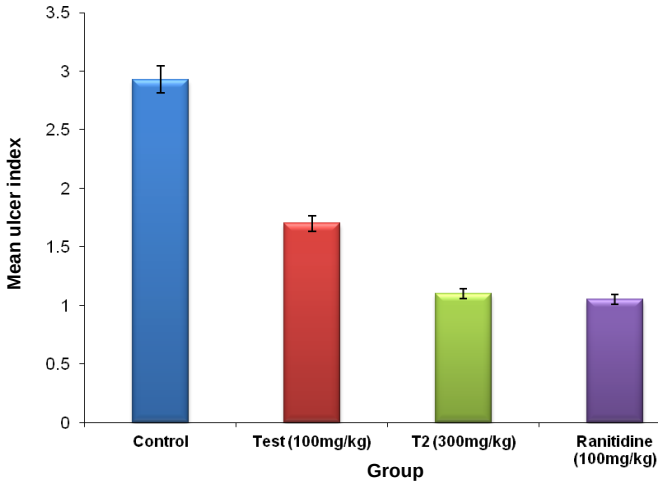

Figure 2. Effect of ethanol extract of *G. latifolium* on (100, 300 mg/kg), control (3 ml 0.9% Nacl), and ranitidine 100 mg/kg on acid/ethanol (25 ml) - induced gastric ulcer. ANOVA and Dunnets test were used to determine significant difference from the control.

severe ulceration induced by 40 mg/kg b.w indomethacin. The highest inhibition of ulcer was given by the chloroform fraction (78.2%) and the least by the residual ethanol fraction (29.02). In comparison, the reference had the highest ulcer inhibition (87.7%) while the crude exhibited only (62.3%).

The outcome of this study revealed that the crude ethanol extract and its fractions inhibited ulceration of the gastric mucosa. Oral administration of these extracts prior to the exposure of the gastric mucosa to necrotizing agents (indomethacin and acid/ethanol) resulted in significant protection from ulceration. The crude extract dose-dependently inhibited ulcer formation in indome0-thacin and for acid/ethanol models. The extract was not hindered by the mode of inducing the ulcer but compared favourably with the reference drug ranitidine for the two models, respectively. The crude extract also showed significant inhibition of ulcer in a more severe ulcer induction using 40 mg/kg indomethacin with ulcer inhibition of 62%. In effect, the ulcer inhibition increased from 23% (100 mg/kg), 46% (300 mg/kg) to 62.3% (400 mg/kg). This showed a case of consistent dose- dependent activity.

The performance of the fractions of the crude in ulcer inhibition at a severe ulcer induction of 40 mg/kg indomethacin was more impressive. The fraction with the highest ulcer protection was the chloroform extract followed by the n-hexane extract. The least was the residual ethanol extract. This trend seems to suggest that the components of the fractions must have a contributory effect on their ulcer inhibition and these are located in the non-polar phase. Phtochemical study by Ezekwe (2005) showed that chloroform fraction contained alkaloid, flavonoid in addition to steroids, terpenoid, fats and oils.

The mechanism of ulcer inhibition by these extracts is not very obvious. The maintenance of mucosal integrity is achieved by a number of mucosal protective devices among which are secretion and action of mucus and bicarbonate (Rang et al., 1995; Shlafer and Marieb, 1989). Prostaglandins stimulate secretion of mucus and bicarbonate (Rang et al., 1995) and especially prostaglandin E_2 and I_2 are implicated in maintaining gastric integrity (Hogan et al., 1994; Akudor et al., 2012) and also mucosal integrity and regeneration (Lanza, 1998). However NSAID, such as indomethacin are potent inhibitors of prostaglandin synthesis thereby promoting ulceration (Vane, 1971; Shlafer and Marieb, 1989). From this study, it can be seen that by inhibiting indomethacin induced ulceration of the mucosa, the extracts may be preventing the hindering generation of prostaglandin.

Another possible mechanism of ulcer protection is that observed in the inhibition of acid/ethanol induced injury to the mucosa. Ethanol challenge elicits production of oxygen free radicals, that is, reactive oxygen species, which generate lipid peroxidation that cause damage to cell and cell membrane (Cheeseman, 1993; Pihan, 1987; Owu et al., 2012). This result in lesions on the mucosal membrane (Singh et al., 2008) and the gastric mucosal ulceration causes severe damage to the system (Goulart et al., 2005) which can be alleviated by antioxidant especially from natura sources (Cetto and Llesuy, 2002). The crude extract exhibited as much inhibition of ulcer from acid/ethanol as the standard reference drug, ranitidine. This tends to suggest that the crude ethanol extract may be an inhibitor of reactive oxygen species generation and may possess strong antioxidant property. This needs to be investigated.

Table 1. Effect of sub-fractions and crude ethanol extract on indomethacin-induced ulcer.

Group	Dose (ml/kg)	Mean ulcer index	Ulcer inhibition (%)
Control	3	6.10±0.94	0.00
n-Hexane	400	1.83±0.47*	70.00
Chloroform	400	1.33±0.37*	78.20
Ethylacetate	400	3.13±0.54*	48.69
Residual ethanol extract	400	4.33±0.49*	29.02
Ranitidine	100	0.75±0.16	87.70
Crude ethanol extract	400	2.30±0.47*	62.30

Ulcer indices are expressed as mean + SEM. *Statistical significance against control at $P < 0.05$ with ANOVA test, followed by Dunnet posthoc analysis.

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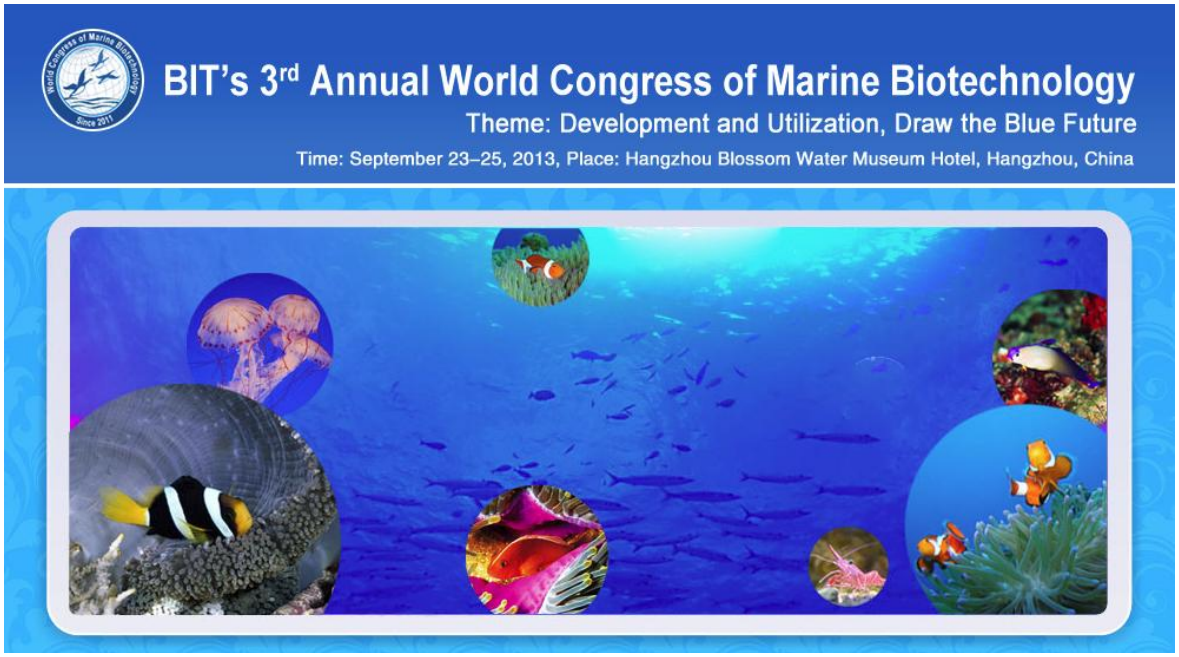

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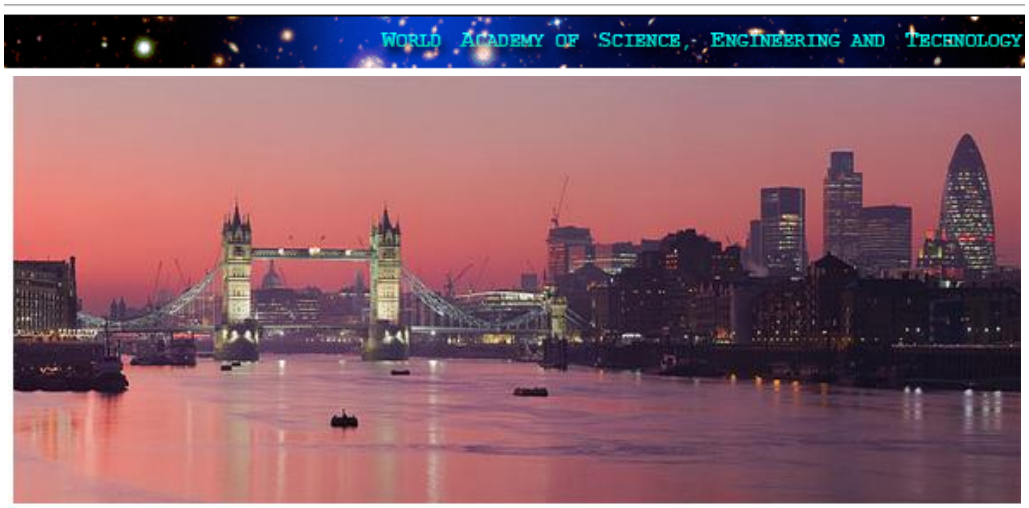

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