

A hand is holding a glass flask containing a red liquid. A green tube is inserted into the flask, extending from the top to the liquid level. The background is a light purple gradient.

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Implications for conservation**

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Full Length Research Paper

Genetic diversity of endangered populations of *Butia capitata*: Implications for conservation

Hélida Mara Magalhães^{1*}, Lucas Rezende Pinheiro², Flávia Aparecida Silveira¹, Mariney de Menezes¹, João Bosco dos Santos², Luciane Vilela Resende² and Moacir Pasqual²

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The flora and fauna of the Cerrado biome in central Brazil both show great diversity and high levels of endemism. *Butia capitata* is a palm native to this biome that has significant economic, social, and environmental value. We sought to identify and quantify the genetic diversity of four fragmented populations of *B. capitata* growing in northern Minas Gerais State, Brazil, as well as one located at the Institute of Agricultural Sciences (ICA) at UFMG, assessing 93 genotypes using 11 ISSR markers. The relationships among populations were evaluated by constructing dendrograms, principal coordinate analysis, genetic distances, as well as Bayesian inference, including and excluding the ICA population. High genetic diversity was found in the populations studied, with most of that diversity occurring within populations. Bayesian inference regrouped the original populations into four populations, redistributing the ICA individuals to the Abóboras and Cristália populations. The analysis that excluded the ICA population arranged the original populations into two groups - with the Abóboras and Cristália populations together in the same group. The ICA population was found to be a repository for future reintroductions into the Abóboras and Cristália regions, as they genetically resemble those populations. It should be noted that other management measures outlined in this study should be adopted before these palm populations enter critical decline phases, such as: stimulus to plants seedlings derived from seeds originated in each area (principally the Abóboras site); quantification of the genetic diversity of neighboring populations to the Mirabela site for future reintroductions as this population showed low intrapopulation diversity.

Key words: Arecaceae, inter simple sequence repeats (ISSR) molecular markers, Bayesian analysis, management measures.

INTRODUCTION

The Cerrado (Brazilian savannas) is the second largest biome in Brazil, but extensive areas of this natural vegetation

have been removed for grain production and pasture formation (Klink and Machado, 2005). The Ministry of the

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Environment (Ministério do Meio Ambiente, 2014) has estimated that almost 8,000 km² of this biome were converted between 2008 and 2009 and many species have become endangered due to habitat loss and the fragmentation of natural populations (Silva and Bates, 2002; Klink and Machado, 2005; Carvalho et al., 2009). *Butia capitata* Mart. Becc., is a palm tree native to the Cerrado biome that is considered as an endangered species (Lopes et al., 2011). Its fruits are widely consumed either fresh or processed to produce pulp, juices, jams and popsicles (Faria et al., 2008), constituting one of the main sources of income for small farmers and harvesters that supply local markets and schools. *B. capitata* also has ornamental value and is used in gardening and landscape projects. This species is usually found bordering rivers in northern Minas Gerais State, contributing to the formation of riparian forests and providing food for local wildlife (Mercadante-Simões et al., 2006). This species experiences intensive extractive exploitation of its fruits as there are no commercial orchards (Magalhães et al., 2012). As demand is always greater than natural supplies, collectors will harvest virtually everything that is produced. The fruits of *B. capitata* show great heterogeneity, coming in many different shapes, epicarp colors, scents, and flavors (Moura et al., 2008; Faria et al., 2008), indicating a high genetic diversity that is now threatened by over-exploitation. While the genetic diversity within populations of *B. capitata* is probably high, it is now threatened by habitat loss and fragmentation. Fragmentation is known to be a significant threat to the maintenance of biodiversity in palms (Bouzat, 2010; Shapcott et al., 2012; Avalos et al., 2013; Federman et al., 2013) as it reduces population sizes and increases the spatial distances between them (Young et al., 1996) – with serious implications for genetic drift, inbreeding, and gene flow (Biebach and Keller, 2010; Silva et al., 2011). The loss of heterozygosity can reduce the viability of the remaining population, and have long-term effects on the ability of a species to respond to environmental changes (Young et al., 1996; Bouzat, 2010). Intensive extraction of fruits/seeds will compromise the natural regeneration of a species (Byg and Balslev, 2001), and deplete its genetic reserves (Homma, 2012).

Genetic data can contribute to the quantification of genetic diversity and aid the management of conservation and recovery programs of endangered palm populations (Shapcott et al., 2009; Nazareno et al., 2013). Molecular information can complement ecological information (Nazareno et al., 2013) and morphological studies, increase the efficiency of collection processes and genetic enrichment, and aid in species classifications (Gaiero et al., 2011). There have been no studies yet for the genetic diversity of *B. capitata* populations growing in northern Minas Gerais State, and it is hoped that this study will be a useful starting point for the pro-active conservation and management programs of *B. capitata* palm trees (and other species) in the Cerrado biome.

One way to assess this genetic diversity is to examine (Inter Simple Sequence Repeats (ISSR) markers – which do not require prior knowledge of genome sequences (Kumar et al., 2009) and is relatively simple, quick, and inexpensive technique, with high reproducibility (Karim et al., 2010). ISSR include many polymorphic loci and generate a great number of informative bands per reaction that can be used to differentiate between even closely related individuals (Gaiero et al., 2011). These markers have been successfully employed in examining the genetic diversity and taxonomy of *Phoenix dactylifera* L. (Hamza et al.; 2012; Srivashtav et al.; 2013) and of species of *Butia* (Rossato et al. 2007 and Gaiero et al.; 2011).

Ecological restoration is a relatively young science, however, numerous criteria must be verified (Hufford and Mazer, 2003) regarding the genetic composition of plants before the use in restoration projects (Jones, 2003; Hufford and Mazer, 2003; McKay et al., 2005) and researchers have noted that molecular markers may provide guidelines for this process (McKay et al., 2005). That is why we also examined the genetic diversity of individuals of *B. capitata* growing on the campus of the Institute of Agricultural Sciences (ICA), UFMG, to evaluate potential as a genetic repository for the re-introduction of individuals into threatened populations of this palm. We therefore sought to evaluate the levels of genetic diversity in four fragmented natural populations of *B. capitata* in northern Minas Gerais State, Brazil, as well as specimens growing on the campus of the Institute of Agricultural Sciences (ICA) UFMG, using ISSR markers.

MATERIALS AND METHODS

Characterization of populations

The *B. capitata* populations studied here were located in the central region of northern Minas Gerais State, Brazil. Ninety-three individuals were sampled from four wild populations growing in four municipalities (Cristália, Bonito de Minas, Mirabela, and Montes Claros) as well as from a nine-year-old orchard at the Institute of Agricultural Sciences (ICA) of the Federal University of Minas Gerais State (which were grown from seeds derived from the Abóboras population) (Figure 1).

The Abóboras population

This population consists of 60 plants economically exploited. The landscape is dominated by a mosaic of small savannah fragments surrounded by croplands (sugar cane and pineapples) and pastures. The plants there were nearly all adults that were more than 27 years old. No juvenile plants were encountered (Figure 2A and 2B).

The Bonito de Minas population

The Bonito de Minas population is composed of approximately 100 plants (generally 25-year-old adults in their reproductive phase),

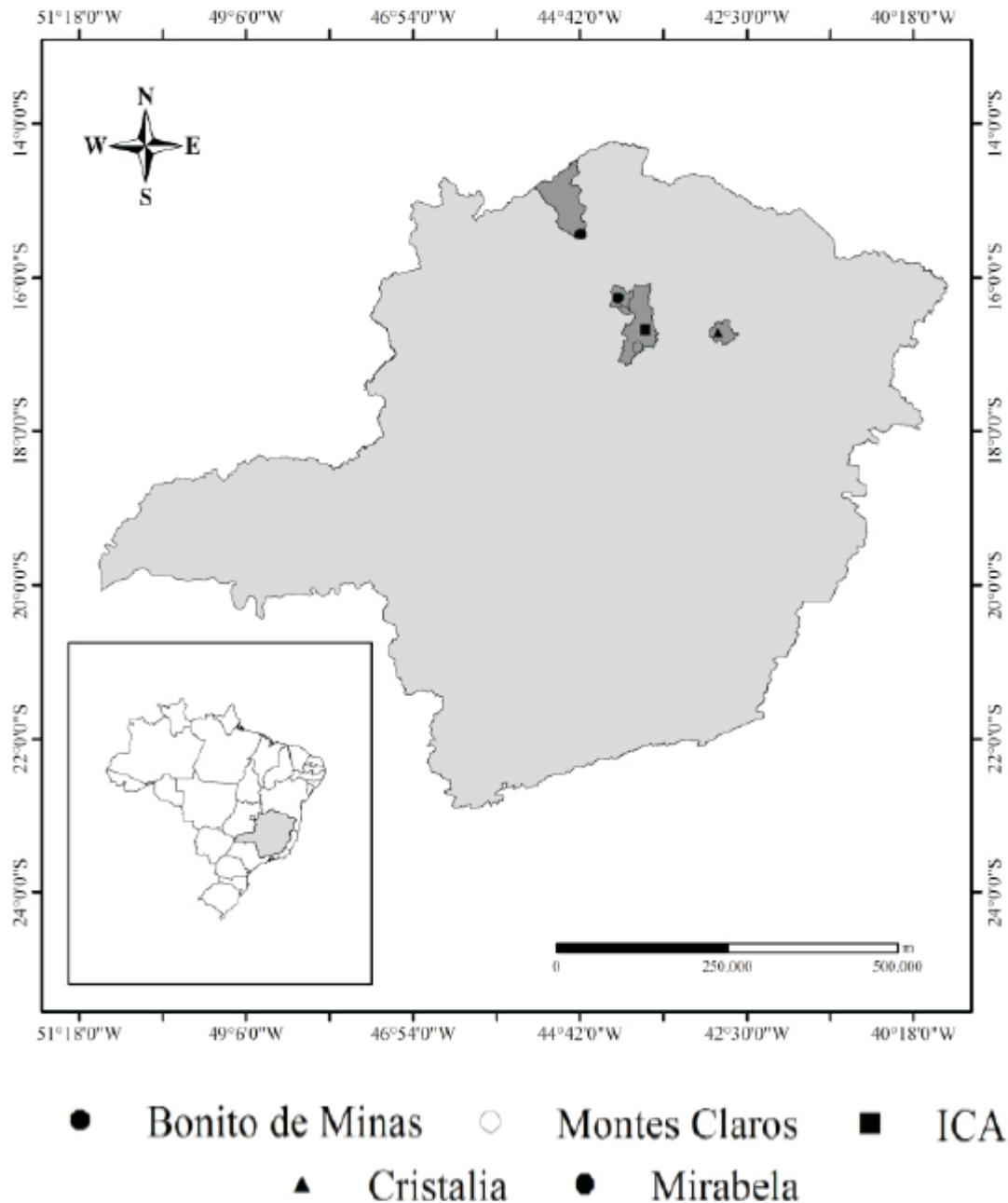


Figure 1. Geographical distributions of *Butia capitata* populations in the municipalities of Bonito de Minas (B), Mirabela (M), Abóboras (A), and Cristália (C), and at ICA (I) in northern Minas Gerais State, Brazil. The dark gray areas indicate the municipal boundaries.

although juveniles were observed in their initial seedling stages. The area is also used as pasture for cattle. The fruits of this population are not commercially harvested for human consumption but used to feed pigs, chickens and cattle (Figure 2D).

The Mirabela population

This population comprises approximately 150 adult plants of reproductive age; seedlings were also observed. The area is used for cattle grazing and the palm fruits are collected to be sold for

human consumption. The mean age of this population is approximately 20 years old (Figure 2E).

The Cristália population

This population is composed of approximately 80 plants in their reproductive phase (average of 20 years old). The area is used for grazing, and the palm fruits are harvested to feed pigs, chickens, and cattle (Figure 2F).

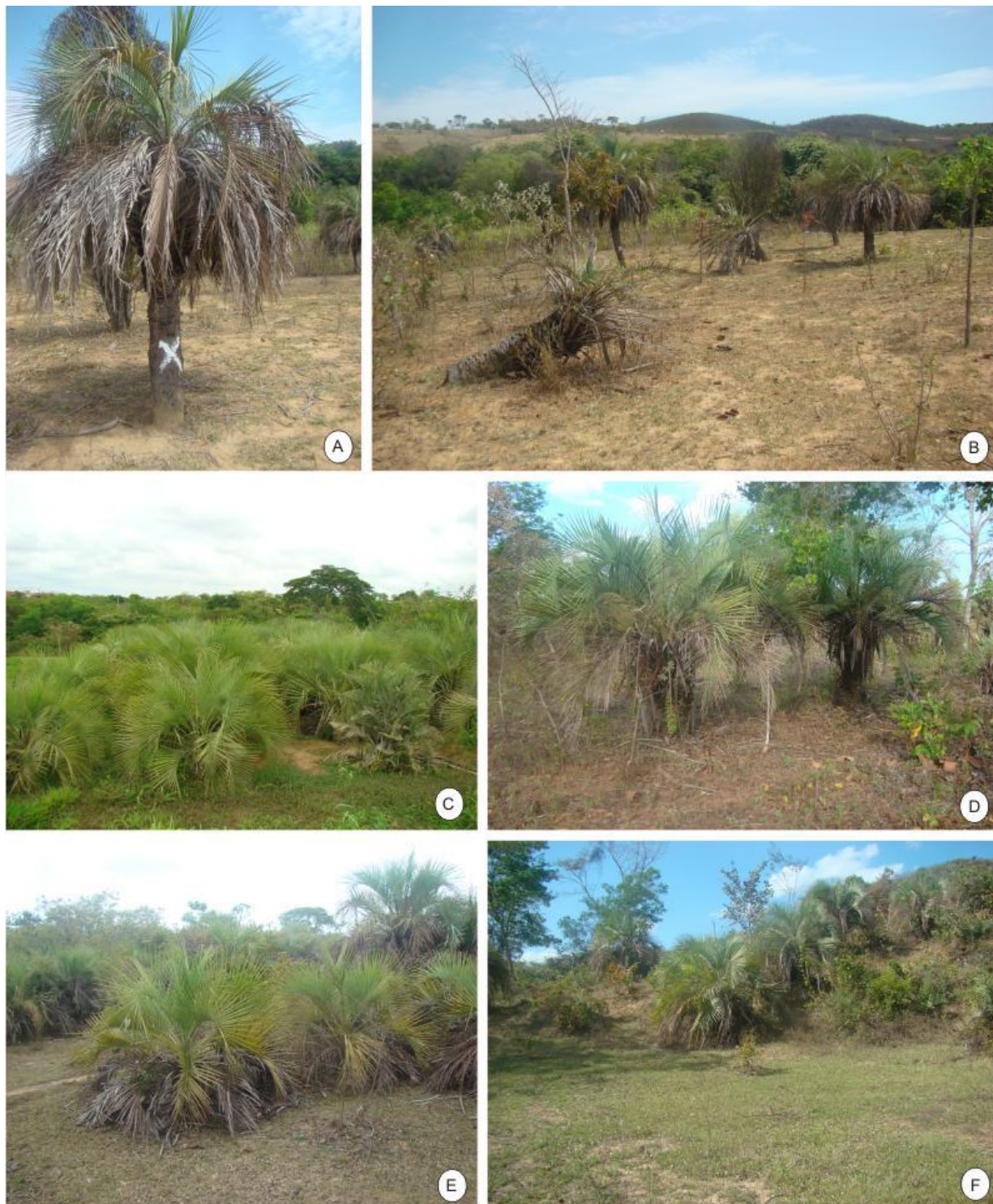


Figure 2. Populations of *Butia capitata* studied in the municipalities of Abóboras (A,B), Bonito de Minas (D), Mirabela (E), and Cristália (F), and ICA (C) in northern Minas Gerais State, Brazil.

The ICA population

The ICA orchard was planted in 2006 by researchers at the Institute of Agricultural Sciences, UFMG, in Montes Claros. The seeds used were collected at the Abóboras site, and 40 seedlings were planted

with 2.5 m x 3.0 m spacing. This population is now in its reproductive phase after approximately 8 years (Figure 2C). All plants sampled in these populations were geo-referenced using a GPS (Garmin III Plus). Their geographical coordinates and the numbers of plants sampled are shown in Table 1. The distances (in

Table 1. Geographical coordinates of the populations of *Butia capitata* and the numbers of plants sampled in five locations in northern Minas Gerais State, Brazil.

Population	Latitude	Longitude	Samples	Approximate population sizes
Bonito de Minas (B)	15.434.037	44.691.877	21	~ 100
Abóboras (A) - Montes Claros	16.915.687	43.937.155	17	~150
ICA (I) - Montes Claros	16.682.370	43.839.108	20	~80
Cristália (C)	16.723.888	42.879.871	21	40
Mirabela (M)	16.266.282	44.691.977	14	60

Table 2. Sequences of the ISSR primers used and the annealing temperatures of the PCR reactions.

Marker code	ISSR Sequence (5' → 3')	Annealing temperature (°C)	Nab	Npb
1	(AC) ₈ T	50	10	9
1a	(GACA ₃)RG	48	14	12
7	GAG(CAA ₄)	55	15	14
817	(CA ₈)A	55	14	11
849	(GT ₈)YA	55	12	10
851	(GT ₈)YG	55	15	15
853	(TC ₈)RT	48	23	20
855	(AC ₈)YT	50	14	10
857	(AC ₈)YG	50	13	10
864	(ATG) ₆	48	14	10
876	(GATA ₂)(GACA ₂)	48	22	20

Nab = Number of amplified bands; Npb = number of polymorphic bands.

Table 3. Estimates of genetic (below) and geographic distances (km) (above) of different populations of *Butia capitata* from the municipalities of Bonito de Minas, Mirabela, Abóboras, ICA, and Cristália in northern Minas Gerais State, Brazil.

Population	Bonito de Minas (B)	Abóboras (A)	ICA (I)	Cristália (C)	Mirabela (M)
Bonito de Minas (B)	0.000	263.0	218.0	377.0	149.0
Abóboras (A)	0.078	0.000	45.0	210.0	114.0
ICA (I)	0.082	0.060	0.000	165.0	69.0
Cristália (C)	0.121	0.094	0.065	0.000	277.0
Mirabela(M)	0.162	0.177	0.137	0.109	0.000

km) between the populations are listed in Table 3.

DNA extraction and amplification using ISSR

DNA was extracted from the youngest leaves (still contained within the leaf sheaths and characterized by their yellow color). Leaf samples were ground in a porcelain mortar in the presence of liquid nitrogen (N₂) and 0.1 g polyvinylpyrrolidone (PVP) to prevent oxidation, and then stored at -80°C in an Ultrafreezer in 1.5 ml tubes. DNA extraction was performed according to the methodology described by Ferreira and Grattapaglia (1995). A NanoDrop ND-1000 spectrophotometer was used to quantify the DNA samples, and they were subsequently diluted in TE (TRIS - 10 mM HCl; 1 mM EDTA; pH 8.0) to a final concentration of 10 ng/μL. After standardization of the DNA concentrations for each PCR reaction, 10 ng of DNA, 100 μM of each dNTP, 1U of Taq DNA polymerase buffer composed of 50 mM TRIS, pH 8.3, 20 mM KCl, 2 mM MgCl₂,

10 mg BSA, 0.25% Ficoll 400, 10 mM tartrazine and pure water was added to a final reaction volume of 12.01 μL. The amplification reactions were performed in a Master Cycler Gradient thermocycler model 5331. Sixty-one primers (© Life Technologies) were tested to examine polymorphism, and 11 were selected based on producing high numbers of fragments and good quality bands. Their respective base sequences and quantities of bases are shown in Table 2. The amplification programme consisted of an initial denaturation of DNA at 95°C for 5 min followed by 40 cycles of 20 s at 94°C (denaturation), 20 s annealing at varying temperatures (depending on the primer – Table 2), and 20 s at 72°C (polymerization), followed by one cycle of 4 min at 72°C, with final stabilization at 10°C. The amplified fragments were separated on 1.5% agarose by gel electrophoresis in 1X TBE buffer with 10 μL of GelRed™ (Uniscience®) fluorescent dye, at 90 V from 2.5 h to 4 h (depending on the primer used). Comparisons of fragment sizes were based on a standard 100 bp DNA ladder. The fragments were visualized under ultraviolet light (Fotodyne) and the images were

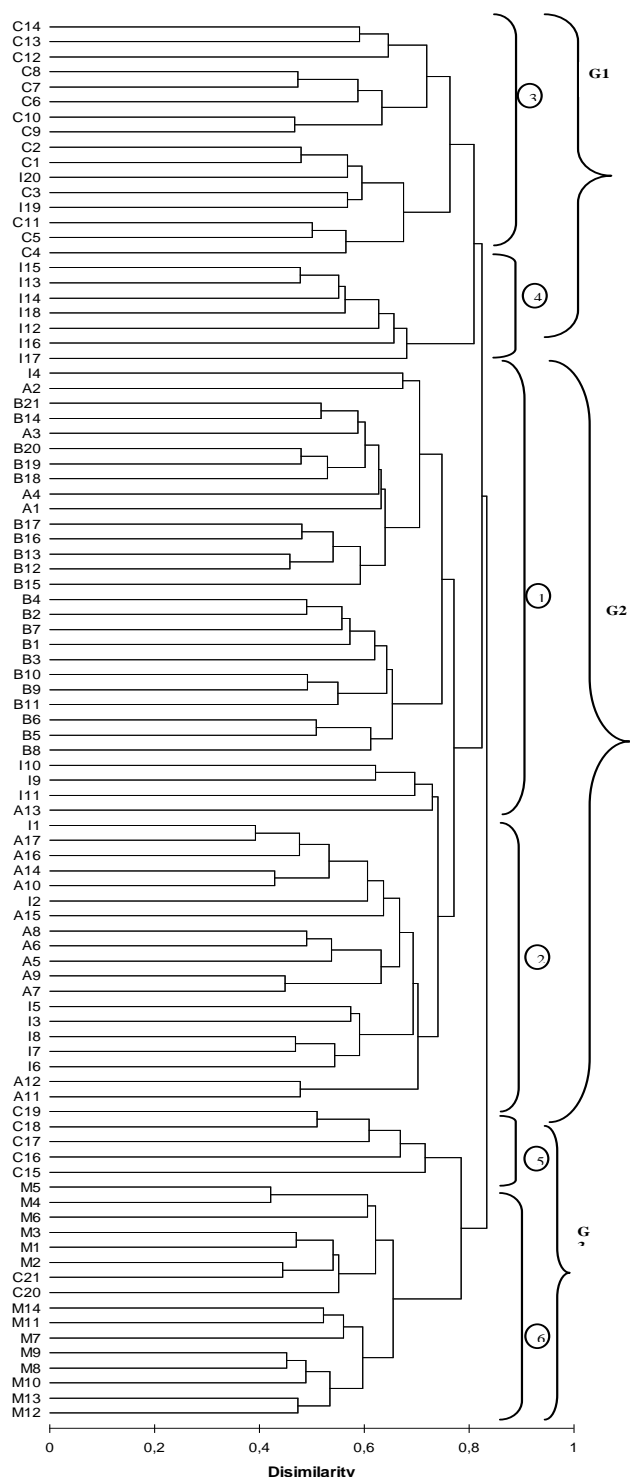


Figure 3. UPGMA grouping and the Jaccard (1908) genetic similarity coefficient of different genotypes of *Butia capitata* in the municipalities of Bonito de Minas (B), Mirabela (M), Abóboras (A), Cristália (C), and at the ICA experimental orchard (I) in northern Minas Gerais State, Brazil. G1= group 1; G2 = Group 2; G3 = Group 3.

Statistical analyses of the data

The electrophoretic profiles of each gel were transformed into a binary matrix, with the presence of a fragment being represented by (1) and its absence by (0). Binary data was used to evaluate all subsequent analyzes. The genetic dissimilarity between each pair of individuals was determined using the Jaccard coefficient (Jaccard, 1908). The Unweighted Pair Group Method Arithmetic Average (UPGMA) was used to group the genotypes according to their genetic similarity, using XLSTAT software (Addinsoft © version 2011.2.04, 2009). The genetic similarities between populations were calculated by the Nei index using Genalex v.6.3 software (Peakall and Smouse, 2006). Population groupings using principal coordinates analysis (PCoA), with the help of XLSTAT software (Addinsoft, 2009) was also performed.

The Shannon index (I) (Brown and Weir, 1983) and expected heterozygosity (H_e) were calculated as described by Lynch and Milligan (1994) and Maguire et al. (2002), using Genalex v.6.3 software (<http://www.anu.edu.au/BoZo/GenAlEx/>). The genetic variation within and among populations was estimated by AMOVA using the GenAlEx program, version 6.43 (Peakall and Smouse, 2006) like the Mantel test was performed based on the genetic and geographic distances of individuals. The significance of variance components and of the Φ statistics and the Mantel test was estimated using permutation procedures (5000 permutations). Inferences about genetic structures were performed using Structure version 2.2 software (Pritchard et al., 2000; Falush et al., 2007). We estimated the most likely cluster (K) number, and the number of reconstructed panmictic populations (RPP) using values ranging from 1 to 10 and assuming that the sampled genotypes were of unknown origin. The burn-in was 20,000, and a Markov chain Monte Carlo (MCMC) clustering was performed with 20,000 iterations, with five replicates. This program estimates the most likely number of clusters (K) by calculating the log likelihood of the data for each value of (K). We evaluated the best (K) value using the method proposed by Evanno et al. (2005).

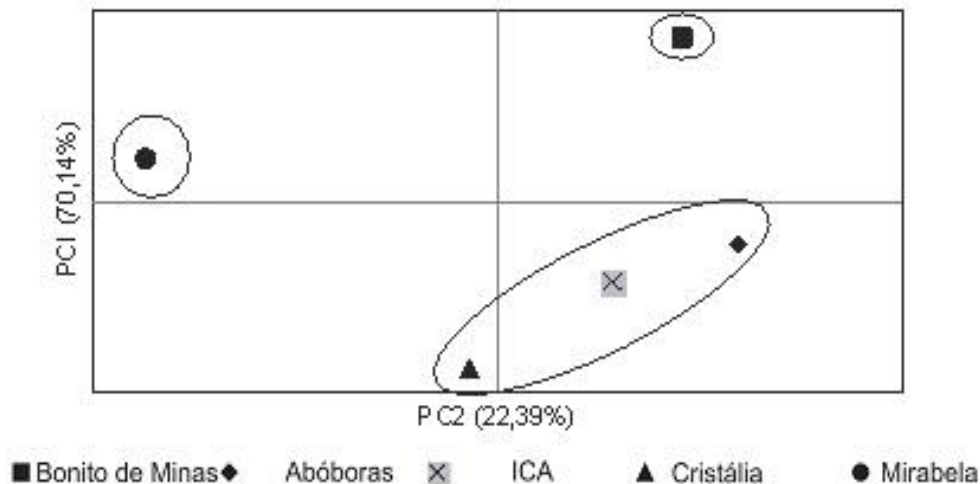
Genetic distance analysis of molecular variance (AMOVA) and Bayesian inference analyzes were performed in two ways: excluding and including the ICA samples. The same programs described above were used in both analyses.

RESULTS

Eleven primers were selected for this study that generated 141 polymorphic bands. The numbers of polymorphic bands ranged from 9 to 20 per primer, with a mean of 12.81. The ISSR 876 and 853 primers produced most fragments (20 each) (Table 2). The UPGMA analyses revealed high genetic diversity in the *B. capitata* populations, distributing them into three groups. The largest group comprised 49 genotypes, with 100% of the individuals sampled in Bonito de Minas, 100% of the Abóboras individuals, and 55% of the ICA specimens (Figure 3). The second largest group consisted of 23 plants from the populations at Cristália (71.42%) and from ICA (45%). Group 3 was the smallest group, with 21 individuals, comprising 33% of the population of genotypes sampled at Cristália and 100% of those from Mirabela (Figure 3). The Mantel test revealed a significant positive correlation between genetic and geographic distances among populations of *B. capitata* ($r = 0.472$; $P < 0.005$). The genetic distances varied when the five populations were analyzed together and, in this

Table 4. Analysis of molecular variance (AMOVA) of populations of *Butia capitata* in the municipalities of Bonito de Minas, Mirabela, Abóboras, ICA, and Cristália in northern Minas Gerais State, Brazil.

Origin	Degrees of freedom	Estimate of variation	Percentage of variation
between populations	4	5.561	19
within populations	88	23.415	81
Total	92	28.975	100

**Figure 4.** Principal Coordinates Analysis (PCoA) of different populations of *Butia capitata* in the municipalities of Bonito de Minas, Mirabela, Abóboras, and Cristália, and at ICA (I) in northern Minas Gerais State, Brazil.

case, the population of Mirabela was the farthest from the others (Table 3). The largest genetic distance was found between the Mirabela and Abóboras populations (0.177), followed by Bonito de Minas, ICA, and Cristália. The smallest distances were observed between the ICA and Abóboras population (0.060) and between ICA and Cristália (0.065) (Table 3). When the ICA population was excluded, the results were similar to the first analysis. The Mirabela population showed the greatest genetic distance in relation to others. The largest genetic distance was found between the Mirabela (M) and Abóboras (B) populations (0.084), and the smallest distance was observed between the Abóboras and Cristália populations (0.041). Diversity between populations was lower than within populations. This result was confirmed by AMOVA, which showed variations of 19% between populations and 81% within populations (Table 4) when excluding the ICA individuals, the diversity variations were 22% between populations and 72% within populations.

The graph generated by principal coordinate analysis (PCA) explained 92.53% of the observed diversity (Figure 4). Populations of *B. capitata* from different municipalities formed three distinct groups. The first was composed only of the Mirabela population, the second of the Bonito

de Minas population, and the third comprised the Abóboras, Cristália, and ICA populations together (Figure 4). The Shannon index (I) identifies genetic diversity, and can range between 0 and 1. The closer its value is to 1, the greater diversity among the genotypes (Perry and McIntosh, 1991). The Shannon index (I) values of the *B. capitata* populations ranged from 0.374 to 0.432, with a mean of 0.41. The lowest value was seen with the Mirabela population (0.374), which also showed a lower percentage of polymorphic loci (67.38%) (Table 5). The PCA and UPGMA analyses indicated that this population was the most divergent from the others, although it had a lower internal diversity. The highest Shannon index (I), genetic diversity (He), and percentage of polymorphic loci values were observed with the Cristália and ICA populations (Table 5).

Bayesian inference was performed to evaluate the genetic structure of the genotypes of *B. capitata*. The best (K) to represent the number of reconstructed panmictic populations including the ICA individuals was K = 4 (RPP1 to RPP4), and K = 2 (RPP1 to RPP2) when excluding the ICA population. The results were mostly in agreement with the UPGMA method described above, although they provide some interesting insights into these populations. Twenty-seven individuals were grouped in

Table 5. Shannon index (I), genetic diversity (He), and percentage of polymorphic bands (P%) of different populations of *Butia capitata* in the municipalities of Bonito de Minas, Mirabela, Abóboras, ICA, and Cristália in northern Minas Gerais State, Brazil.

Population	I	Ĥe	P%
Bonito de Minas	0.409	0.275	76.60
Abóboras	0.414	0.277	78.01
ICA	0.432	0.287	83.69
Cristália	0.420	0.276	85.82
Mirabela	0.374	0.254	67.38
Mean	0.410	0.274	78.30
Standard deviation	0.010	0.007	3.22

RPP1 (15 from Cristália and 12 from ICA). Of the genotypes found in the Cristália population, 14 had adherence probabilities of over 90% for RPP1. Of the 11 genotypes identified in the ICA population, seven had at probabilities of over 90% for RPP1 (Figure 5a). These results were generally consistent with the UPGMA analysis (Figure 3), which grouped the Cristália genotypes and part of the ICA genotypes together (1) (Figure 3). RPP2 was reconstructed with 21 individuals – 12 from the Abóboras population and 9 from ICA. There was more than 95% adherence of Abóboras genotypes to RPP2 and more than 80% adherence of ICA genotypes (Figure 5a). The results of this analysis show that these two populations are connected only through the ICA population (Figure 5a). RPP3 was composed of all of the genotypes from Bonito de Minas and four from Abóboras, totaling 24 individuals; more than 85% of the Abóboras individuals showed adherence to this reconstructed population (Figure 5a). Finally, RPP4 comprises all 14 individuals from Mirabela and 6 from Cristália (total of 20 genotypes), this being the smallest reconstructed population compared with the others.

The best (K) number of panmictic populations (excluding the ICA specimens) was $K = 2$ (RPP1 and RPP2). RPP1 included all individuals of the Abóboras population, Bonito de Minas, and the vast majority of Cristália specimens with more than 90% adherence, as RPP2 included all of the Mirabela individuals and four from Cristália (C18, C19, C20, and C21) (Figure 5b).

DISCUSSION

In general, the number of polymorphisms found in *B. capitata* was comparable to other studies of diversity in the family Arecaceae, and in some cases superior. A number of populations of the genus *Butia* were characterized in terms of their diversity in the study undertaken by Nunes et al. (2008), with 77 polymorphic bands being observed in *Butia odorata* using 21 RAPD primers. Rossato et al. (2007) and Gaiero et al. (2011)

obtained 150 and 74 polymorphic loci, respectively using ISSR primers in various species of *Butia*. In both studies the primer sequence (AC)₈T generated the highest number of polymorphic fragments (26 and 17, respectively); this primer was also efficient in generating polymorphic fragments in the present study. The mantel test indicated positive correlations between geographical and genetic distances, with spatial patterns influencing genetic variability between populations, so that closer populations tend to be more genetically similar - with genetic differences tending to increase with geographic distance. Similar results were reported by Gaiero et al. (2011) for species of *Butia* ($r = 0.652$) in Uruguay. Shapcott et al. (2012) and Rossetto et al. (2004) did not, however, find significant correlations using the mantel test with the palm species *Lemurophoenix halleuxii* and *Elaeocarpus grandis* in fragmented environments. Estimates of genetic distance in the analyses (including or excluding the ICA population) showed that the Mirabela population was the most divergent, although the genetic distance values decreased when the ICA population was excluded. Among all of the populations sampled, Mirabela was the only one that showed fruits with different colors (ranging from purple through different shades of red to yellow), reinforcing the expectation that this population was actually more divergent than the others, although only minor intrapopulational variations were, in fact, encountered. The results of genetic distance analyses were similar to those of other species of the genus *Butia* (Gaiero et al., 2011) and *Euterpe edulis* (Cardoso et al., 2000). Principal coordinates analysis (Figure 4) reinforced the positioning of both nearby populations as well as the more distant populations identified by UPGMA (Figure 3). The group formed by the Abóboras, ICA, and Cristália populations in the dendrogram showed that the ICA individuals were partly grouped with the Cristália population while a larger number and grouped with the Abóboras population, a fact that is probably related to the source of this plant population, since the ICA orchard was planted with seeds collected in Abóboras. This can be seen in Figure 5a, as the Abóboras and Cristália populations are linked

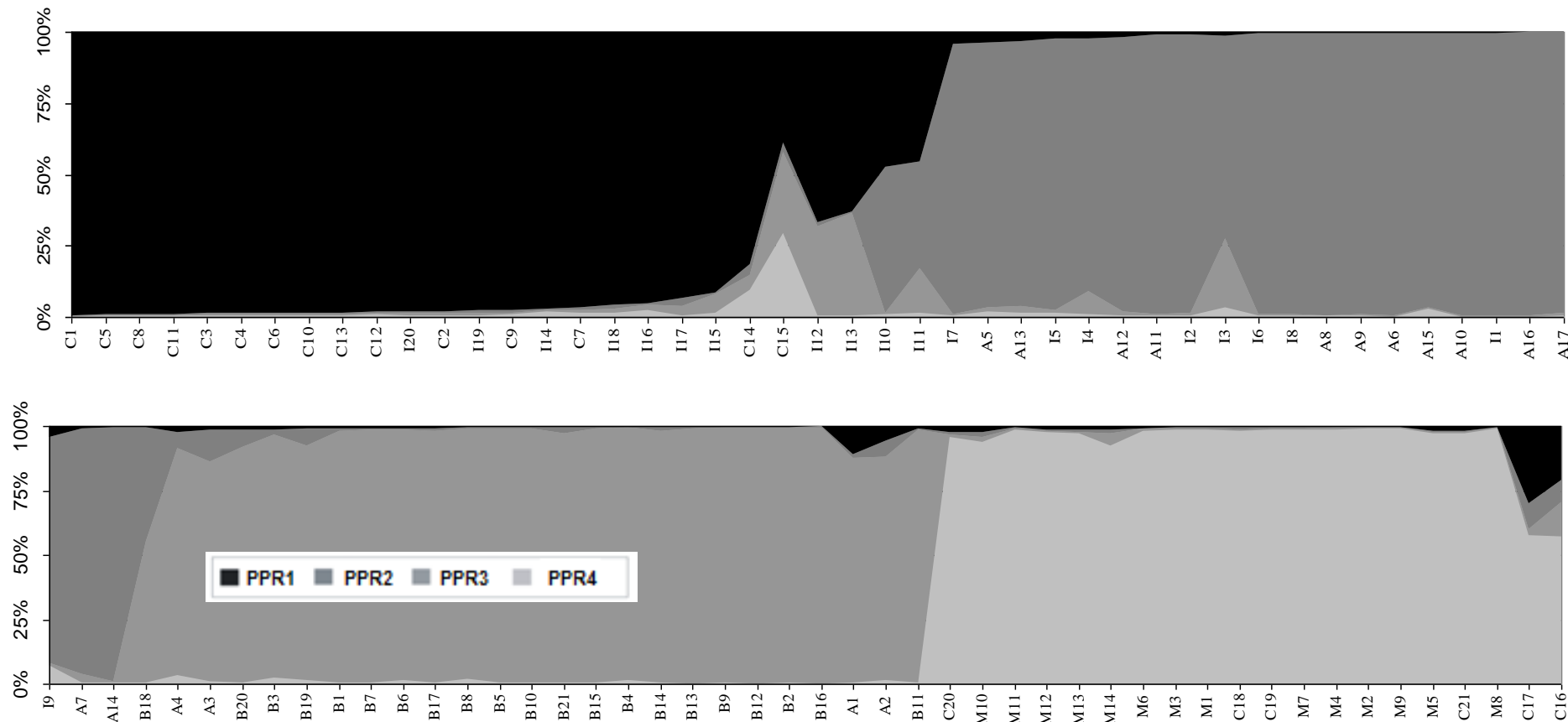


Figure 5a. Bayesian Inference for populations of *Butia capitata* from the municipalities of Bonito de Minas (B), Mirabela (M), Abóboras (A), and Cristália (C), and at ICA (I) in northern Minas Gerais State, Brazil (using K = 4).

by the ICA population and, when it is removed, appear grouped into a single population (Figure 5b).

Genetic diversity in a population is determined by several well-known factors, including gene flow (Lenormand, 2002), genetic drift and habitat fragmentation (Lienert 2004), reproductive isolation (Charlesworth and Charlesworth, 2000), biotic and abiotic factors, topographic relief

(Escudero et al., 2003), human interference, mutation (Lande, 1995) and reproductive biology. Most of the genetic diversity of *B. capitata* was found to be distributed within populations (Table 4), in agreement with the work of Gaiero et al. (2011) who reported that 94% of the diversity within the genus *Butia* was at the population level, with only 6% between populations. Similar results were

reported by Nunes et al. (2008) for *B. odorata*, and with palm trees of other species, such as *Calamus thwaitesii* Becc. and *Acrocomia aculeata* (with 79.79 and 82.8% intra-population genetic diversity, respectively) (Sreekumar and Renuka, 2006; Oliveira et al., 2012.). These results can be explained by hypothesizing that populations of *B. capitata* had a common origin, but geographical

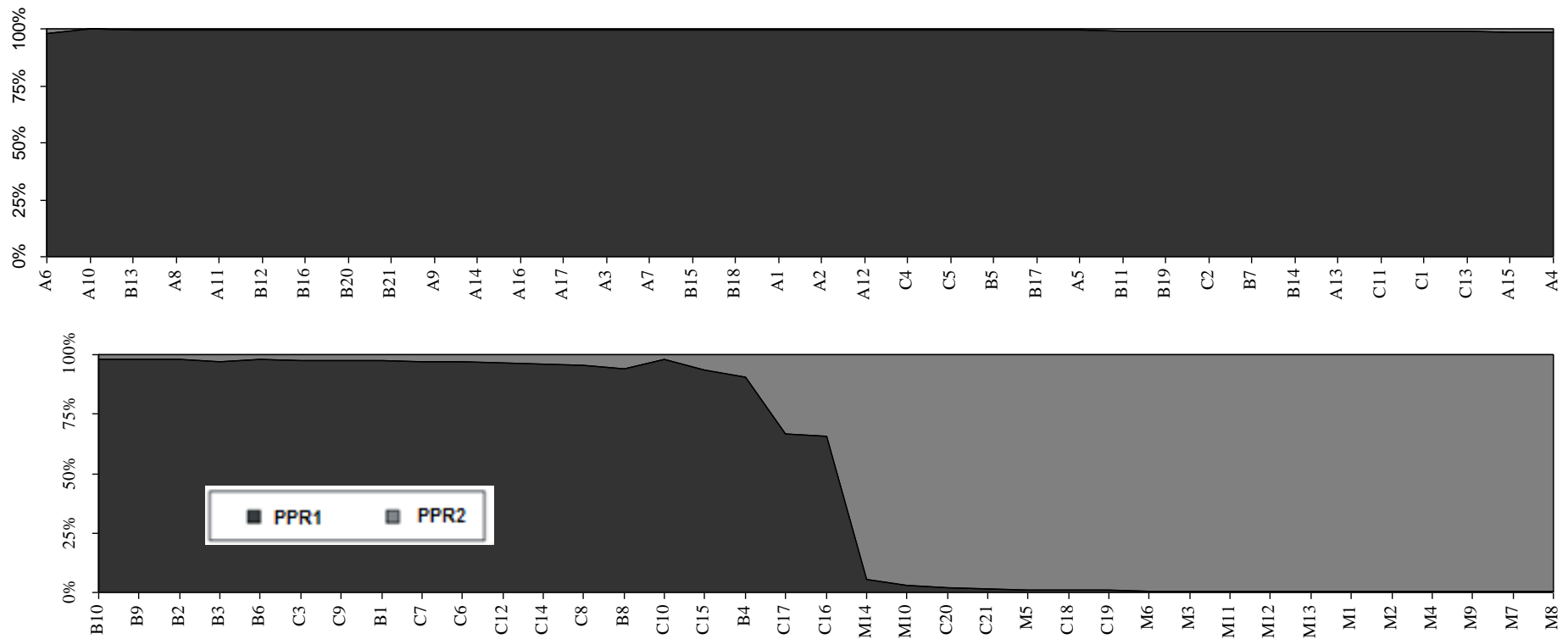


Figure 5b. Bayesian Inference for populations of *Butia capitata* in the municipalities of Bonito de Minas (B), Mirabela (M), Abóboras (A), and Cris tália (C), in northern Minas Gerais State, Brazil (using $K = 2$).

isolation processes, including selection, drift, and historical process of landscape and human activities across the savanna region caused the observed differences between them, structuring them into subpopulations (Buttow et al., 2010). Studies have indicated that the vegetation of this biome originally consisted of a mosaic of large areas of different vegetation types (from rocky, open fields to dense forests) (Klink and Machado, 2005). In the 60s and 70s stimulus for the production of grain and pasture in the central areas of the country caused large areas of natural vegetation to be removed or modified (Klink and Machado,

2005; Sano and Brito, 2010).

Another hypothesis is that the reproductive biology of *B. capitata* may have contributed to the high diversity seen within populations (Zehdi et al., 2004), as female flowers are found only on the basal portion of the rachillae while male flowers are formed only on the distal portion. Dichogamy of the protandry type was also observed, with anthesis of male flowers occurring before maturation of the female flowers. The low occurrence of synchrony between the male and female phenol-phases on the same plant thus favors xenogamic pollination in this species (Mercadante-Simões

et al., 2006).

Aguilar et al. (2008) and Shao et al. (2009) suggest that high genetic diversity within populations may also reflect the effects of time, in the sense that more recent habitat fragmentation may not yet be reflected in the current genetic diversity of long-lived species. Thus, habitat fragmentation may be a relatively recent event compared to the lifespans of these species, perhaps involving less than a few generations (Collevatti et al., 2010). Cluster analysis, ordination, and other indexes revealed a large genetic diversity in *B. capitata*, although we initially thought that its diversity might already have

been compromised due to anthropogenic impacts on the cerrado biome. The vast majority of fragmentation studies have reported negative effects on vegetation (Lowe et al., 2005; Aguilar et al., 2006), including decreasing areas of occupation and increasing isolation – leading to genetic drift and increased inbreeding (Young and Brown, 1996) and impairment of seed dispersal (as dispersers encounter difficulties in gaining access to other areas) (Ghazoul, 2005). Fragmentation was found to be detrimental to *Dipteryx alata* in the cerrado, as it showed very low levels of diversity and high levels of inbreeding (Collevatti et al., 2010; Collevatti et al., 2013). *Mauritia flexuosa* (Federman et al., 2012), *Acrocomia aculeata*, and other native Cerrado palm tree populations growing in Mirabela showed lower heterozygosity and lower percentages of polymorphic loci (Oliveira et al., 2012). Other studies, however, have shown that fragmentation may promote more gene flow between populations (Fore et al., 1992; Born et al., 2008) than seen when they are growing fairly close to each other. The effects of fragmentation may not be detectable or not initially compromise the genetic diversity in some species of palms, as noted by Nazareno et al. (2013) in *Butia eriospatha* and Shapcott et al. (2012) in *Voanioala gerardii*. These species showed increased coefficients of inbreeding, however, and reduced numbers of rare alleles (Nazareno et al., 2013; Shapcott et al., 2012). Generally, adults individuals are used to access genetic diversity, while the anthropogenic disturbances and fragmentation were recent events in evolutionary time, so this does not, however, rule out the possibility that these populations in fact have been damaged by fragmentation even though it is not yet detectable by the analyses used (Collevatti et al., 2010; Collevatti et al., 2014). It was not always possible to evaluate inbreeding or the presence of rare alleles in our studies with *B. capitata*, so that future studies should consider these analyzes due to the danger of these populations becoming extinct (Collevatti et al., 2010). While the results of the present study are encouraging in terms of the high genetic diversity observed in their populations, conservation programs of species conservation that must be initiated, as deforestation in the Cerrado biome (MMA 2014) and the extraction of *B. capitata* continues, and this species may soon cease to exist outside of protected reserves (Homma, 2012). Therefore, one should not wait for further population declines before taking action. Conservation and management measures that can be adopted for the preservation and maintenance of genetic diversity of *B. capitata* are discussed here based on the information generated in this study.

The Abóboras population exhibited high heterozygosity and Shannon diversity compared to the other areas, although it is one of the populations that most suffers from harvesting, fragmentation, and farmland conversion; steps should be taken to encourage awareness among local communities that some palm seeds should be planted (Gaiero et al., 2011; Nazareno et al., 2013),

especially in forest areas near crop fields. Cattle also feed on the inflorescences of this palm, and it is suggested that these areas be fenced off, at least until fruit set. The formation of small palm orchards and the restoration of native forests by planting seeds or seedlings originating from local palm populations will help reduce extraction pressures on native populations (Lopes et al., 2011; Gaiero et al., 2011). Overcoming dormancy and promoting seedling formation, however, is currently not a serious obstacle (Lopes et al., 2011; Magalhães et al., 2012; Oliveira et al., 2013; Dias et al., 2013). The ICA Orchard proved to be a viable repository for future conservation activities in the Abóboras and Cristália sites as our analyses showed high similarities between those three populations. Furthermore, our results showed that the ICA individuals demonstrated high heterozygosity and Shannon diversity. The advantage of this collection is that it is irrigated and produces fruits throughout the year (which does not occur with natural populations). However, the use of this material for restoring other populations (principally Mirabela) should be undertaken with great caution – as Hufford and Mazer (2003) and McKay et al. (2005) warn of the dangers of introducing external genes that can affect plants specifically adapted to local conditions.

The Mirabela population had the lowest Shannon index, heterozygosity, and numbers of polymorphic loci, and was the most divergent from the others, even though it showed genotypes with varying colors of fruits (from purple through red in different shades and, most commonly, yellow). In the future, new populations of *B. capitata* in the region Mirabela should be evaluated for their genetic diversity, and if they were found to be similar, genetic material could be exchanged between these populations – as has been suggested for the ICA, Abóboras, and Cristália populations – thus avoiding the introduction of extraneous genes that could jeopardize their populations Hufford and Mazer (2003) and McKay et al. (2005). These steps were suggested for *D. alata* by Diniz-Filho et al. (2012) for *Annona crassiflora* (Collevatti et al., 2013), and for different species of the genus *Butia* in Uruguay (Gaiero et al., 2011). The formation of genebanks by institutions may also be assisted by this study, as it may serve as a guide for the collection of different genotypes and protecting the greatest possible diversity and representivity of native populations. In the future, new populations should be visited to collect unique genotypes from each region to be used to strengthen their local populations (Collevatti et al., 2010), similar to how the ICA collection can be used. These measures have already been adopted for other species of the Cerrado Biome, such as *D. alata*, *Calophyllum brasiliense* and *Tibouchina papyrus* (Collevatti et al., 2010; Collevatti et al., 2013).

Our results indicate the urgent need for action and long-term studies to preserve the remaining genetic diversity of *B. capitata*. There are still several issues that

raise concerns about the future of this species and about collecting information about other populations across the range of threatened taxa. Our results showed that populations of *B. capitata* show high genetic diversity in spite of the high degree of fragmentation and disturbance of the Cerrado biome in northern Minas Gerais State. Intensive deforestation and extraction should serve as a warning of population declines – and other factors should be investigated, including complementary studies of demography, the genetic diversity of young individuals, gene flow, and inbreeding. The ICA collection has excellent potential for restoring populations at Cristália and Abóboras as they show high genetic diversity similarities; however, their use for restoring other populations should be avoided, and additional populations investigated with a view to designing more effective restoration strategies.

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Full Length Research Paper

Modulation of the transcriptional activity of the AP2/ERF family (DREB genes) in orange (*Citrus sinensis*) leaves subjected to drought stress

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Ethylene response factor (ERF) play important role in the development and expression of genes that regulate plant response to biotic and abiotic stresses. In this study, six *Citrus sinensis* genes belonging to AP2/ERF family, dehydration-responsive element-binding protein (DREB) genes, (*CitsERF01* to *CitsERF06*) were identified and five of them had their expression pattern evaluated in leaves of 18 month-seedling orange (*C. sinensis*) subjected to increasing intervals of drought stress. Two gene transcription patterns were identified. In the first pattern, transcription of *CitsERF01* and *CitsERF03* genes was delayed for many days and did not occur until the 12th day. In the second pattern, transcription of *CitsERF04*, *CitsERF05* and *CitsERF06* was immediate, and their relative value increased steadily, also reaching its peak at the 12th day. However, in both cases, transcription of *CitsERF* genes was down regulated with water recovery. These patterns suggest that *CitsERFs* genes may be involved in regulation mechanisms of drought response of *C. sinensis* and are controlled by factors acting in cascade. The data from this study will help understand the genetic mechanisms of drought tolerance, which could contribute to breeding programs of orange.

Key words: Gene expression, transcript factor; dehydration-responsive element-binding protein genes, semi-quantitative analysis RT-PCR.

INTRODUCTION

During their life cycle, plants are exposed to different adverse environmental events that affect their development and growth (Lee et al., 1999). To survive, the plants respond and adapt to these conditions with an array of biochemical and physiological changes. Many adaptation processes

are regulated by stress responsive gene expression. Transcription factors play central roles in gene expression by regulating the expression of downstream genes, and transcription factors have important functions in the transcriptional regulation of a variety of biological

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Abbreviations: ERF, Ethylene response factor; DREB, dehydration-responsive element-binding protein; ORF, open reading frame.

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processes, including various responses to the environment (Riechmann, 2000; Nakano et al., 2006; Chen et al., 2012). Among the different transcript factors, the ones from the AP2/ERF family play important role in the transcription regulation for increasing tolerance to drought, salinity, low and high temperatures, and plant diseases (Dubouzet et al., 2003; Zhuang et al., 2011). The ethylene response factor (ERF) family belongs to the AP2/ERF superfamily which is defined by the presence of preserved AP2/ERF domain consisting of a sequence of 60 to 70 amino acids involved in DNA binding (Jofuku et al., 1994; Riechmann et al., 2000; Hu and Liu, 2011). Proteins of ERF family have only one AP2 domain, and are classified into two subfamilies, ERF and DREB (dehydration-responsive element-binding protein) (Jofuku et al., 1994; Sakuma et al., 2002). The members of ERF family bind to the cis-regulatory element called GCC-box, in the gene-promoting regions related to pathogenesis and regulate the expression in response to dehydration and low temperature (Sharma et al., 2010). The superexpression of DREB genes in *Arabidopsis* activate the expression of many genes related to abiotic stress, improving tolerance to drought, salt and cold (Liu et al., 1998). Dobouzet et al. (2003) verified that the superexpression of *OsDREB1A* in transgenic *Arabidopsis* induced the expression of *DREB1A* genes, resulting in plants with greater tolerance to drought, salinity and frosting. Likewise, Bouaziz et al. (2012) revealed that the superexpression of *StDREB2* in transgenic potato resulted in greater tolerance to salt stress.

Orange fruit (*Citrus sinensis*) play important role in the economy of many countries around the world, but mainly in Brazil, USA and China (FAO, 2012). Adversely, the productivity and good quality of orange have been negatively affected by abiotic stresses like drought and salinity (Ben-Hayyim and Moore, 2007). Due to the importance of abiotic and biotic stress responses, AP2/ERF transcription factors represent interesting gene pools to be investigated for breeding and genetic engineering purposes (Xu et al., 2011). In this way, considering the importance of the subfamily DREB in citrus in relation to response and tolerance to abiotic stress, this study aimed to characterize the transcriptional modulation of five *CitsE*. The results of this study could be used to analyze the molecular mechanism underlying the stress tolerance of orange.

MATERIALS AND METHODS

Identification of AP2/ERF genes in *C. sinensis* genome

Protein sequences of DREB subfamilies belonging to group I, identified in *Arabidopsis thaliana* from TAIR database (<http://www.arabidopsis.org/>) were utilized for research in the Citrus Genome Database of *C. sinensis*, (<http://www.citrusgenomedb.org/species/sinensis>) using BlastP software (Altschul et al., 1997). The sequences were compared to other sequences stored in the GenBank database, utilizing BlastP

and BlastX (National Center for Biotechnology Information - NCBI - <http://www.ncbi.nlm.nih.gov>), to confirm their identity. The deduced sequences of amino acids were obtained through Open Reading Frame Finder (ORF Finder - NCBI - <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) software. The initially collected sequences that presented incomplete AP2 domain or whose ORFs were incorrect were excluded from the analysis. The protein sequences containing AP2 domain were aligned utilizing ClustalW algorithm, version 2.0 (Larkin et al., 2007) and redundant inputs were removed.

Characteristics of predicted AP2/ERF proteins in citrus

The basic physical and chemical characteristics of citrus AP2/ERF proteins were calculated using the online ProtParam tool (<http://web.expasy.org/protparam>), including the number of amino acids, molecular weight and theoretical isoelectric point (pI). Analysis of the 3-D structure was completed on the online server Phyre v.2 (Kelley and Sternberg, 2009).

Phylogenetic analysis

The phylogenetic analysis was done by aligning AP2 domains of *C. sinensis* using ClustalW algorithm, version 2.0 (Larkin et al., 2007). Searches for other similar proteins were done with BlastP software (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree was built by the neighbor-joining (NJ) method using the pair-wise deletion option with the help of MEGA software, version 5.10 (Tamura et al., 2011). To test the reliability of the analysis, 1,000 bootstrap replicas were utilized.

Plant material and stress treatment

In this study, we measured the transcription of five out of the six *CitsERFs* genes in leaves of orange (Pêra variety) subjected to increasing intervals of drought stress. In September 2012, 30 vigorous 18-month-old orange seedlings were brought from a nursery field to the greenhouse and received water at soil field capacity regularly for 15 days until the treatments started. The greenhouse was set up to maintain air temperatures of $25\pm 5^{\circ}\text{C}$, relative air humidity of $80\pm 10\%$ and the light: dark periods of 16: 08 h. After the period of acclimation, we set up the 'control' pool treatment by harvesting all young mature leaves from three seedlings. Following that, the drought treatments consisted of not watering the seedlings for three, six and twelve days, consecutively. At each interval, we similarly harvested all young mature leaves from another group of seedlings. After 12 days of drought, three seedlings received water as in the start of the experiment for another three days and were harvested as the 'recovery' treatment. Each biological replicate consisted of a pool of leaves from three different orange seedlings. Harvested material was immediately immersed in liquid N and stored in freezer at -80°C .

RNA extraction and cDNA synthesis

Total RNA extraction of *C. sinensis* leaves was done for the drought stress experiment. Total RNA was extracted by utilizing SV Total RNA Isolation System commercial kit (Promega, Madison, USA) following the manufacturer's instructions. RNA integrity was analyzed in electrophoresis in agarose gel 1.2%. The first cDNA tape was synthesized utilizing 4 μL of total purified RNA and GoScript™ Reverse Transcription System commercial kit (Promega, Madison, USA) and the oligo primers (dT)₁₅ following the

Table 1. Sequence of primers utilized for semi-quantitative analysis by RT-PCR of *C. sinensis* genes.

Genes	Forward primers 5'-3'	Reverse primers 5'-3'	Tm °C	Nº of cycles
<i>ACTB</i>	CCAATTCTCTCTTGAACCTGTCTT	GAAGACCGTCAAGAGTAGTCAGT	56	32
<i>CitERF01</i>	TGACAGCTCTAACAACTTTCTC	GGTTTCATAGGACAAGGAGG	56	30
<i>CitERF03</i>	GCCAAGATTCAAGCAATATGTC	CTCCACTTTGTCCTCTCAG	56	30
<i>CitERF04</i>	TACTCTCCAACCACTTCTTCAG	TGAGATGATTTAGCCCAAGAG	56	30
<i>CitERF05</i>	TTACCAGTCTTACCCACCTC	TCATCGACAACCCATTAGCA	56	30
<i>CitERF06</i>	GAAAGAGAAGCAAAGTAACTCC	CTGACCCATCACTCAAACCTG	56	30

manufacturer's instructions.

Semi-quantitative analysis by RT-PCR

The sequences of primers utilized for semi-quantitative analysis RT-PCR of *CitsERFs* genes and the *ACTB* gene (Yan et al., 2012) used as control, are given in Table 1. The reactions of RT-PCR considered a final volume of 15 µL, containing: 1.5 µL of 10x PCR buffer, 0.8 µL de MgCl₂ (50 mM), 0.5 µL of dNTP's (10 mM), 0.5 µL of each primer (10 mM), 0.4 µL of *Taq DN* thermocycler, model AG A *polymerase* (5U), 1 µL of diluted cDNA 1:10. The reactions occurred in a 22331 (Eppendorf, Hamburg, Germany), under the following conditions: 1 initial cycle at 94°C for 5 min and 30 to 32 cycles of 94°C for 30 s for denaturation, 56°C for 40 s for annealing, 72°C for 30 s for extension and 1 final cycle at 72°C for 10 mi. The amplified PCR product was submitted to electrophoresis in agarose gel 1.2% stained with ethidium bromide. The gel images were captured utilizing a photo documenting systems, L-PIX - Molecular Imaging (Loccus Biotecnologia, Cotia, Brazil) and analyzed densitometrically (Freschi et al., 2009). To quantify the band intensity, IMAGEJ 1.46 (<http://rsbweb.nih.gov/ij/download.html>) software was used.

Statistical analysis

The results were evaluated and submitted to variance analysis and the averages were compared by Tukey's test at 5% probability, utilizing statistical software, SISVAR (Ferreira, 1999).

RESULTS

Molecular characteristics of ERFs of *C. sinensis*

From the *in silico* analysis of the Genome Database of *C. sinensis*, six ERFs belonging to DREB subfamily of group I were identified and named *C. sinensis ethylene-responsive element binding factor* (*CitsERF01-06*). The complete cDNA sequence of *CitsERF01* (orange1.1g039976m), *CitsERF02* (orange1.1g015775m), *CitsERF03* (orange1.1g045091m), *CitsERF04* (orange1.1g018103m), *CitsERF05* (orange1.1g017798m) and *CitsERF06* (orange1.1g021983m) is 1401, 1203, 939, 2071, 1985 and 1985 bp and codify proteins of 438, 400, 312, 360, 365 and 304 amino acid residues, respectively. The estimated molecular weight is 48.79,

44.73, 34.52, 39.97, 39.97 and 33.64 kDa, and the isoelectric point of 5.48, 8.27, 8.33, 6.32, 7.62 and 8.48 for *CitsERF01* to *06*, respectively. The 3-D structures of predicted from group I to AP2/ERF family proteins in citrus were built through the on online server Phyre 2. As shown in Figure 1, each 3-D structure of the citrus AP2/ERF family protein contains one α-helix and three β-sheets. The group I of AP2/ERF family generally has similar 3-D structures, with slight differences in the length and amino acid composition of units that make up the tertiary structures. This is due to the number of amino acids in each β-sheet of each 3-D structure was different, thus creating slight variations in the length of β-sheets of each tertiary structure. Research done in NCBI by BLAST indicated that the six ERF proteins have great similarity with the AP2/ERF transcription factors of other perennial species. *CitsERF01*, *CitsERF02* and *CitsERF04* share 62, 53 and 62% of identity with the sequence of AP2/ERF EOX99382.1, EOY05285.1 and EOX92151.1 transcription factors, respectively in *Theobroma cacao*. *CitsERF03* and *CitsERF05* share 70 and 60% of identity with XP_002304554.1 and AFY98895.1 sequences in *Populus trichocarpa* and *Jatropha curcas*, respectively. *CitsERF06* shares 63% of identity with DREB1p (ADX97444.1) identity in *Hevea brasiliensis*. The prediction of amino acid sequences of *C. sinensis* in relation to the complete sequence is indicated in percentage of identity and similarity (Table 2). The identity ranged from 22.76% (*CitsERF01* and *CitsERF03*) to 100% (*CitsERF05* and *CitsERF06*) and presented the average of 35%. For similarity, the variation was 17.25% (*CitsERF01* and *CistERF02*) and 100% (*CitsERF05* and *CitsERF06*) and the average of 33% in all ERFs of the studied group.

The homology research by Blastp showed that the alignment of ERFs sequences of *C. sinensis* has a conserved region of DNA binding, AP2/ERF domain with 58 amino acids, which is characteristic of the family of ERF genes in plants. Moreover, the ERF domain of citrus proteins showed high sequence homology with other characterized species (Figure 2). DREB gene subfamily includes two main amino acid residues in AP2 domain, valine (V) and glutamic acid (E) in positions 14 and 19, respectively (Sakuma et al, 2002). All genes presented

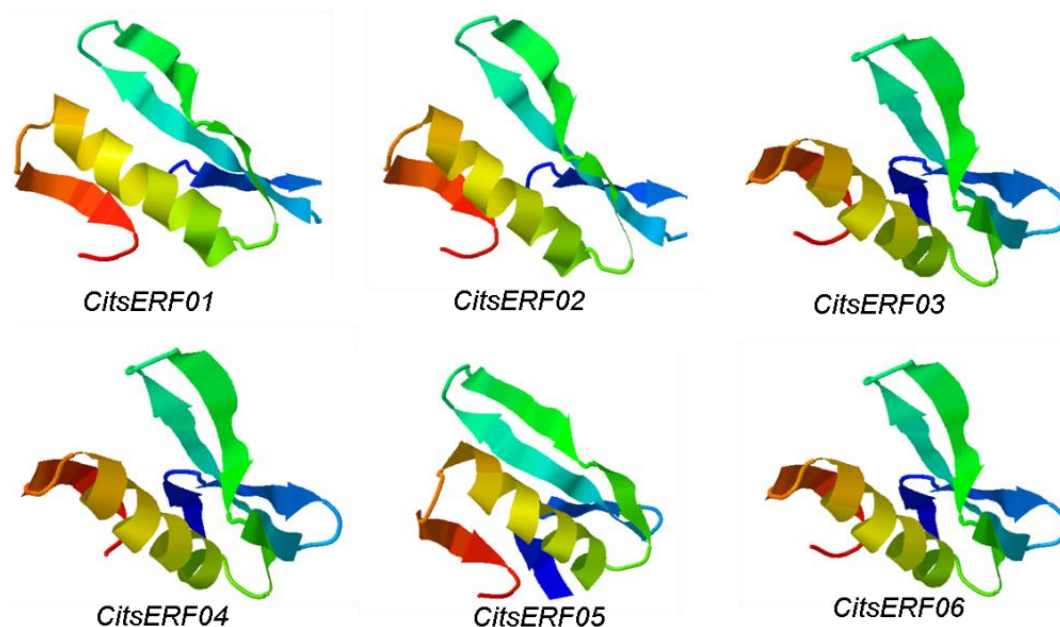


Figure 1. 3D structures of six AP2/ERF family proteins of group I in citrus. *CitsERF01* (orange1.1g039976m), *CitsERF02* (orange1.1g 015775m), *CitsERF03* (orange1.1g045091m), *CitsERF04* (orange1.1g018103m), *CitsERF05* (orange1.1g017798m) and *CitsERF06* (orange1.1g021983m); yellow-green = 1 alpha helix; green-blue = 3 beta sheets extended strand.

Table 2. Comparison among the predicted complete sequences of ERFs amino acids of *C. sinensis* (*CitsERFs*) cDNAs. The similarity values are over the diagonal whereas the identity values are under the diagonal.

	No. aa	<i>CitsERF01</i>	<i>CistERF02</i>	<i>CitsERF03</i>	<i>CitsERF04</i>	<i>CitsERF05</i>	<i>CitsERF06</i>
<i>CitsERF01</i>	438	-	17.25	22.76	21.11	24.66	26.64
<i>CistERF02</i>	400	22.82	-	24.04	23.89	25.21	25.66
<i>CitsERF03</i>	312	22.76	27.02	-	24.36	29.17	29.28
<i>CitsERF04</i>	360	24.29	29.94	29.53	-	48.33	51.97
<i>CitsERF05</i>	365	25.28	25.00	30.20	53.67	-	100.00
<i>CitsERF06</i>	304	27.30	28.57	30.30	55.89	100.00	-

No. aa: Number of amino acids

the preserved residue V14, and most genes presented leucine in position 19. In addition, alanine was found in position 37, and it was present in consensus in all protein sequences, including Arabidopsis, peach, grape, apple, rice and populus.

Phylogenetic analysis of *CitsERF* proteins

A phylogenetic analysis was done based on the sequence of ERF amino acids to determine the relation between citrus ERF and other characterized plant species (Figure 3). The analysis showed a distribution of protein sequences in four groups belonging to DREB subfamily and six *CitsERF* proteins were classified as members of group I; the other characterized proteins

were divided into groups I, II, III and IV according to the classification proposed by Nakano et al. (2006).

CitsERF expression in response to stress

To determine the response of *CitsERF* subjected to drought stress, the corresponding mRNAs levels were quantified. Out of six *CitsERFs* identified in group I, five had their transcriptional activity evaluated. When subjected to stress, *CitsERF01* and *CitsERF03* (Figure 4) presented a similar response pattern, where it was observed that the expression was constant between the control (day 0), three and six days after the beginning of the stress, and there was a significant increase in transcripts at 12 days of stress, reducing to basal levels

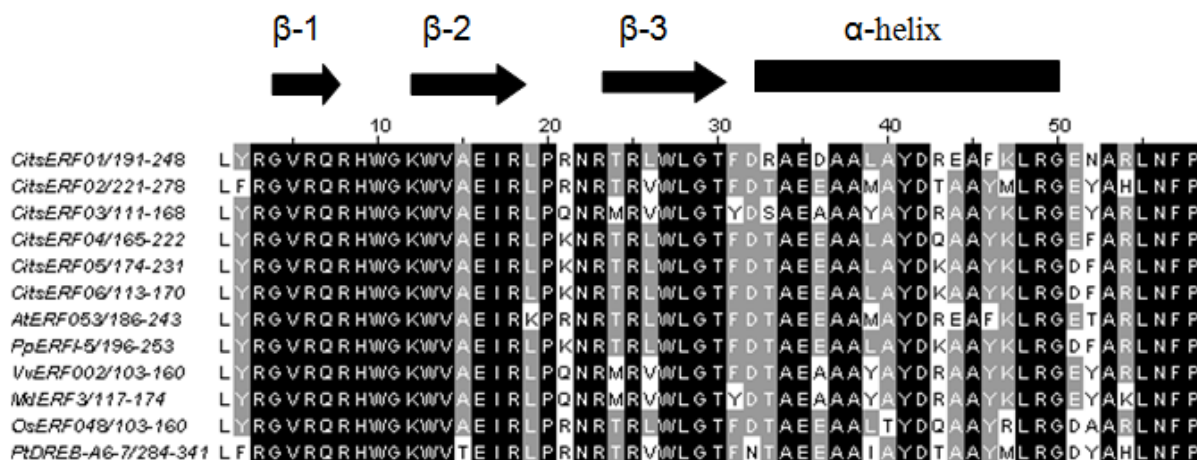


Figure 2. Alignments of AP2/ERF domains from *C. sinensis* proteins with other plant proteins, *A. thaliana* AtERF053 (AT2G20880), *P. persica* PpERFI-5 (ppa007193m), *V. vinifera* VvERF002 (GSVIVP00002438001) and *M. domestica* MdERF3 (MDP0000292965), *O. sativa* OsERF048 (Os08g31580) and *P. trichocarpa* PtDREB-A6-7 (eugene3.00012264). The black and gray columns indicate amino acid residues that are identical and preserved, respectively. The black bar and the arrows represent the α -helix and β -sheet regions, respectively, within AP2/ERF domain (Allen et al., 1998).

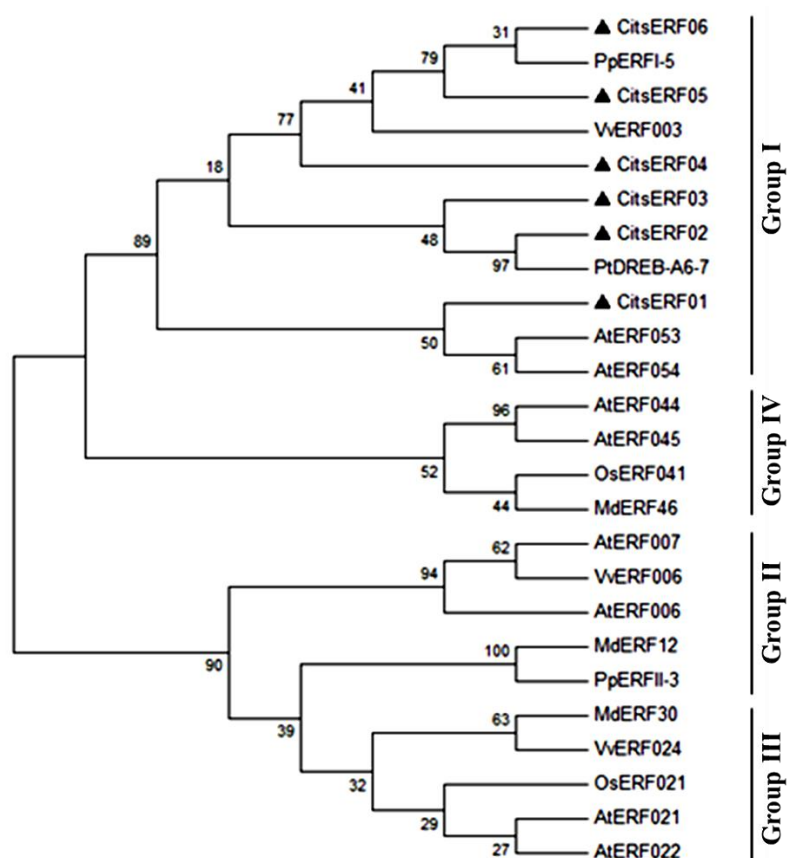


Figure 3. Phylogenetic analysis of DREB subfamily of *C. sinensis* and other plant organisms. The citrus proteins are marked with ▲. The names of the other proteins and their respective access numbers are *Arabidopsis thaliana* AtERF006 (AT1G46768), AtERF007 (AT4G06746), AtERF021 (AT1G71450), AtERF022 (AT1G33760), AtERF044 (AT3G11020), AtERF045 (AT5G05410), AtERF053 (AT2G20880), AtERF054 (AT4G28140); *Vitis vinifera* VvERF003 (GSVIVP00010923001), VvERF006 (GSVIVP00014863001) VvERF024 (GSVIVP00016137001); *Oryza sativa* OsERF021 (Os02g35240), OsERF041 (Os03g07830); *Malus x domestica* MdERF12 (MDP0000923690), MdERF30 (MDP0000652413), MdERF46 (MDP0000139446); *Populus trichocarpa* PtDREB-A6-7 (eugene3.00012264) and *P. persica* PpERFI-5 (ppa007193m), PpERFII-3 (ppa010649m).

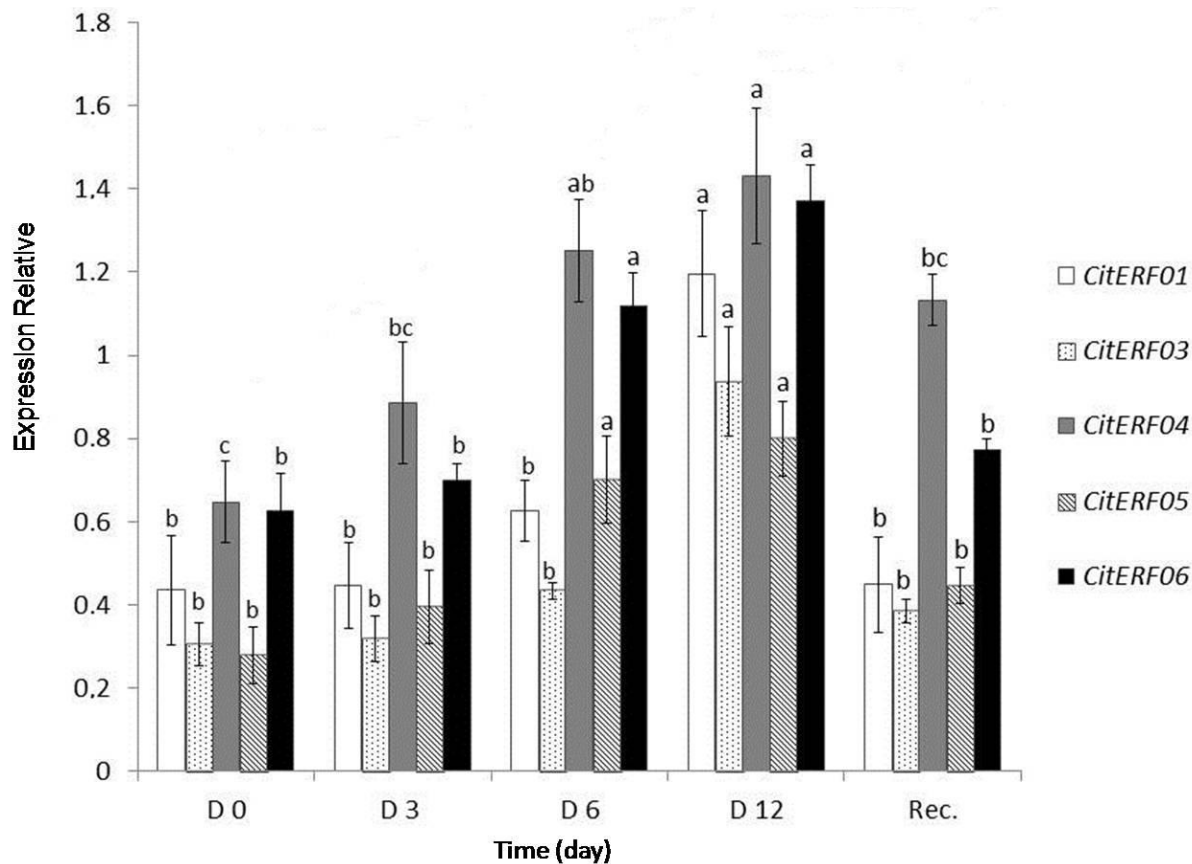


Figure 4. Changes during treatment in mRNA levels of *CitsERF01*, *CitsERF03*, *CitsERF04*, *CitsERF05* and *CitsERF06* in response to drought stress treatment (D0 – control, D3 – day 3, D6 – day 6, D12 – day 12 and Rec – recovery (72 h of irrigation after the plants reached 12 days of stress)). Transcript level of each sample was quantified by densitometry and normalized according to its corresponding *ACTB* density value (*CitsERF/ACTB*). The values represent the averages \pm standard deviation of three biological replications. Averages followed by the same low case letters do not differ among the treatments by Tukey's test at 5%.

at recovery. *CitsERF04* (Figure 4) had a gradual increase of transcripts starting at control, 3 and 6 days after the beginning of the stress and reaching maximum activity 12 days after the beginning of the treatment and decreasing at recovery, but still at a higher level than the control. For *CitsERF05* and *CitsERF06* (Figure 4), it was observed that, between the control day and three days of stress, there was a small increase of expression and, after six days of stress, there was a greater transcriptional activity, reaching the maximum accumulation of transcripts at 12 days without water addition and reducing at recovery, after the stress. Based on the transcriptional profile of ERF genes subjected to drought stress, two expression standards were identified (Figure 5). In the first standard, *CitsERF01* and *03* exhibited expression at basal levels in control and at three and six days, whereas at 12 days, the maximum transcriptional activity was observed. In the second standard, it was verified that *Cits04*, *05* and *06* showed a very similar expression standard among themselves at the beginning of the stress (D0, D3 and D6), reaching the maximum 12 days after the beginning

of the stress and decreasing at recovery.

DISCUSSION

Transcription factors (TF) are regulators that control biological processes and have been considered as an important tool in the complex metabolic ways in plants (Grotewold, 2008). TFs of ERF family are involved in the response to biotic and abiotic stresses and have a highly-preserved element that includes an AP2 domain which is essential for the plant survival (Riechmann and Meyerowitz, 1998; Sharma et al., 2010). In this study, the group I of DREB subfamily in citrus was analyzed and the identity among ERFs was high, varying from 22.76 to 100%, and all six *CitsERFs* presented a highly-preserved element, AP2/ERF domain. The presence of this domain is responsible for the DNA-binding activity (Fujimoto et al., 2000). AP2/ERF domain consists of 58 amino acids and has three anti-parallel β -sheets and one α -helix. This structure has a fundamental role in the recognition of the

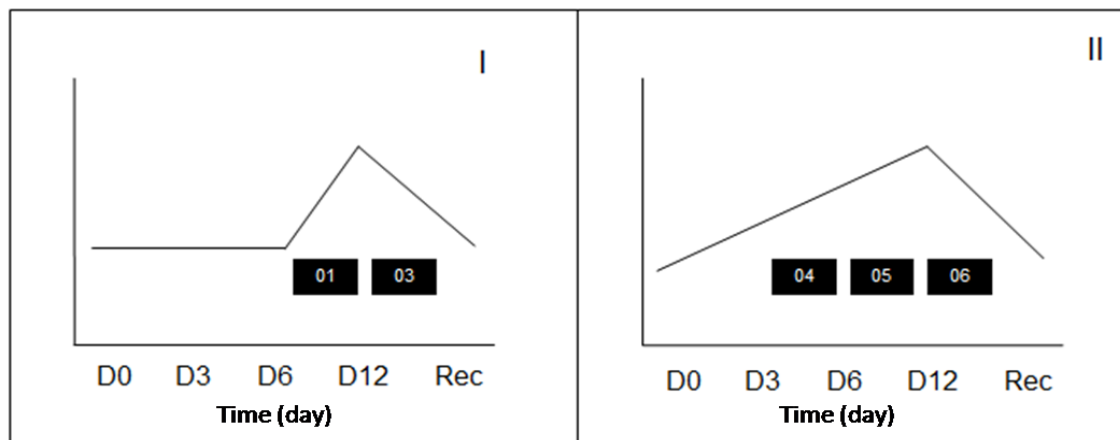


Figure 5. Two patterns expression during drought stress. Axis x represents: D0-control, D3- day 3, D6- day 6, D12- day 12 and Rec – recovery (72 h of irrigation after the plants reached 12 days of stress).

specific binding to cis-element (Allen et al., 1998; Sakuma et al., 2002). This observation is generally consistent with that in *Arabidopsis*, whereby the AP2/ERF domain is reported to contain an N-terminal, which is a three-stranded β -sheet that recognizes a target sequence as well as a C-terminal α -helix (Allen et al., 1998; Zhang et al., 2012). Due to the preserved residues, it was possible to identify that in DREB subfamily, all proteins had valine residue in position 14, but in position 19, most of them presented leucine. In previous studies, it is suggested that valine 14 and glutamic acid 19 in the AP2/ERF domain are essential to the specific binding to the element that is responsive to dehydration (Cao et al., 2001; Sakuma et al., 2002). However, this difference found in citrus was also found in other plants including *Arabidopsis* (Nakano et al., 2006), cotton (Champion et al., 2009), peach (Zhang et al., 2012) and apple (Zhuang et al., 2011). These observations suggest that the function of valine 14 is probably more important than the amino acid in position 19 for the DNA-binding activity (Wang et al., 2011). Alanine in position 37 of AP2/ERF domain found in citrus can be essential to the binding stability of ERF domain or the binding with DRE element or GCC box (Liu et al., 2006).

Overall, the genes of CBF/DREB subfamily have an important role in plant tolerance to abiotic stress, recognizing the dehydration responsive element (DRE), with TACCGACAT sequence, known as a cis-acting element that responds to cold or osmotic stress. DREB domain binds to the DRE element and regulates expression in response to dehydration and low temperature (Shinozaki and Shinozaki, 1994; Sakuma et al., 2002). In a previous study, Shinozaki and Shinozaki (1994) verified that the dehydration responsive element (DRE) is involved in the response of rd29A promoter in *Arabidopsis* and transgenic tobacco under drought or salinity conditions. The activity of most genes of ERF family is related to the increase of plant tolerance to biotic

and abiotic stresses (Park et al., 2001; Guo et al., 2004; Zhang et al., 2009). Based on the phylogenetic analysis and the response expression to abiotic stress, most *CitsERFs* have a possible role as transcription activators that can be part of mechanisms of tolerance increase to abiotic stress in *C. sinensis*. Five analyzed ERFs were differently regulated at mRNA levels. These results suggest that all *CitsERFs* are up-regulated during drought stress, where the maximum level of the transcriptional activity was accumulated on the most severe day of stress, decreasing at recovery. High expression levels of *CitERF* under drought conditions were also observed in citrus plants, showing that it can be involved in the tolerance mechanisms to drought (Yan et al., 2011). High levels of DREBs *ZmDBF1* and *GhDBP2* were found in corn and cotton, respectively, when the plants were subjected to drought (Kizis and Pages, 2002; Huang et al., 2008). The authors showed that *ZmDBF1* as well as *GhDBP2* can be involved in the regulation of some late embryogenesis abundant (LEA) genes. These proteins accumulate during the advanced stages of embryogenesis and also in tissues exposed to stress like dehydration, osmotic stress and low temperature, and have an important role in protein stabilization and membrane structure during cell dehydration (Hong-Bo et al., 2005). Likewise, Bouaziz et al. (2012) attributed tolerance to drought and salinity of potato *StDREB1* to the expression of *StCDPK4* and *StCDPK5* genes induced by stress that activates Ca^{2+} channels of the plasmatic membrane and *P5CS* gene which is responsible for the biosynthesis of proline, an important osmoprotector. The data found in this study are in accordance with other reports found in literature that revealed that the members of ERF family have important roles in response to abiotic stresses through the regulation of numerous stress induced genes (Agarwal et al., 2006). However, future studies must be carried out to identify the possible genes and the multiple ways of regulated transduction by

CitsERFs found in this study.

CitsERF genes of citrus responded differently to stress. It is noteworthy that exposure of orange seedlings to growing periods of drought resulted in two patterns of *CitsERF* gene transcription (Figure 5). In the first pattern, transcription of *CitsERF01* and *CitsERF03* genes was delayed for many days and did not occur until the 12th day. In the second pattern, transcription of *CitsERF04*, *CitsERF05* and *CitsERF06* was immediate, and their relative value increased steadily, also reaching its peak at the 12th day. However, in both cases, transcription of *CitsERF* genes was down regulated with water recovery. In fact, the two different patterns could be explained by the fact that the regulation mechanisms and gene expression due to environmental stress are many times controlled by factors acting in cascade (Riechmann, 2000). Earlier responses are possibly related to genes that encode signaling or regulatory components such as protein kinases and transcript factors. Later responses are often related to genes that encode "effector proteins" such as enzymes in the metabolism (Du and Chen, 2000). Plants may regulate their response to stress with the transcription of genes at early stage to prevent damage, and at later stage to limiting damage-processes. The early response is quickly induced but it is often transient. This induction does not need new protein synthesis because all signaling components are already in place (Zhu, 2002). On the other hand, the late response genes are activated more slowly by stress and their expression is many times kept (Cheong et al., 2002). Thus, probably the two types of responses found in this work are involved in plant tolerance to drought. In several cases, the protein products of early response function to regulate the expression of late response, suggesting a cascade of gene regulation (Cheong et al., 2002). Nevertheless, it is certain that further studies with genetic and biochemical approaches are required to test a model for the *CitsERF* genes pathways.

Conclusion

In this study, it was possible to identify that the genes of DREB subfamily in citrus have a crucial role in the development and regulation of this plant as well as in the responses to environmental stress. Thus, the data obtained in this study provide resources to select the genes for future functional analyses of ERF family in *C. sinensis*, which will help understand the genetic determiners of tolerance to abiotic stresses. This will be a fundamental step to genetic improvement programs of citrus to better the production and quality of fruits in limiting environmental situations.

Conflict of interests

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

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Full Length Research Paper

The impact of aeration on potato (*Solanum tuberosum* L.) minituber production under soilless conditions

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Aeroponic systems are more effective than hydroponics for minituber production, as provided by the optimal system for root oxygenation. The study was conducted to improve conventional hydroponic systems by applying aeration so as to enhance potato minituber production yield via providing adequate oxygen in the root zone. The responses of Agria and Sante cultivars to different levels of aeration [0 as control, 12.5, 25 and 75% ($V_{Air}/V_{media}/min$)] were compared, particularly on tuberization and growth characteristics. In both cultivars examined, increasing the level of aeration led to higher number of stolons and tubers. Agria was more responsive and consisted of more large tubers rather than Sante, which influenced the number, yield and dry matter of tubers. Aerated plants had (a) more dry matter in their haulms because of more leaves and shoots, (b) larger leaf areas, and (c) delay in physiological maturity. Higher levels of tuber growth, longer roots and more stolons led to improvements in root: shoot ratio. Leaf area index duration increased remarkably by increased aeration. These features resulted in a remarkable rise in the total minituber number up to 3600/m² which were 70% higher compared to the non-aerated group. Moreover, the minituber yield was achieved by applying the moderate level of aeration to 23.9 kg/m² in Agria and high level of aeration to 17 kg/m² in Sante rather than 8.73 and 6.51 kg/m² in the control group, respectively.

Key words: Aeroponics, hydroponics, dissolved oxygen, minituber, potato, tuberization.

INTRODUCTION

There is a great potential for the production of a large number of potato tubers, from potatoes grown in nutrient cultures and bioreactors (Akita and Takayama, 1994; Wurr et al., 1997). During the last decade, applying aeroponic systems in potato minituber production showed remarkable increase in the rate of minituber production. It is claimed that, depending on cultivars, the number of minitubers is between 10000 to 13000 /m² with diameters

of 12 to 15 mm and weights of 1.5 g (Technico, 2015) in greenhouses. Applying aeroponic system in comparison with bed culture, Tierno et al. (2014) study showed 60 - 80% increase in tuber numbers and 34- 87% higher tuber yield per plant in three potato cultivars. In a recent study, Roosta et al. (2013) compared aeroponic system with hydroponics in three potato cultivars. They reported higher tuber yield (58%) and number of minitubers(277%)

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per plant in aeroponic system when compared to classic hydroponics system. This fact is a milestone in seed potato production.

Considerable yield improvement, favorable germination capability and tuber uniformity in an aeroponic system can be obtained from root availability to water and nutrients, the most important factor, abundant oxygen in the root zone and around the tubers, in conjunction with harvesting interval of uniform tubers during the growing season by the omission of terminal dominance (Farran and Mingo-Castel, 2006; Lommen, 2008; Soffer and Burger, 1988). However, the (a) high cost of installation, (b) maintenance and management, including infrastructure expenditure, (c) complex technology (d) a specialized organization of growers, (e) the lack of buffering, and (f) root zone high humidity which can result in the opening of lenticels and rapid dissemination of contamination have made this system expensive and sensitive for potato minituber production. Indeed, applying aeroponic systems for minituber production encounters challenges worldwide (Mateus-Rodriguez et al., 2013; Mbiyu et al., 2012; Jones, 2005; Millam and Shama, 2007).

In commercial hydroponic conditions, including container systems, root density must increase with decreasing substrate volume in order to compensate for the canopy water and nutrient requirements. The increased root density contributes to greater oxygen and nutrient consumption per unit volume of root zone. Intense root competition, consequently, occurs for oxygen and available nutrients. Simultaneously, the reduced dissolved oxygen (DO) levels can negatively influence the root function; increase their susceptibility to diseases and eventually death (Jones, 2005). Lack of sufficient DO may inhibit ammonium oxidation, leading to pH decrease, accumulation of toxic levels of ammonium in the liquid and even toxic level of ammonium in the gaseous phase of the root zone. Consumption of oxygen by micro-organisms can aggravate this condition owing to decomposition of organic matter. This will provide competition between roots and micro-organisms in absorbing oxygen and leads to more oxygen deficiency in root zone (Boland et al., 2000; Raviv et al., 2008). Frequent solution replenishment with water highly saturated by DO or supplying fresh air in the root zone is likely to minimize the severity of these problems.

Aerobic respiration of roots requires adequate amount of oxygen. Root zone aeration plays an important role in plant growth. Metabolic processes such as cell division, water gradient into the roots and mineral uptake can be inhibited by root oxygen deficiency and may result in changes in root system morphology, nutritional deficiencies and increased water stress (Morard and Silvestre, 1996; Softer et al., 1991).

The comparison of minituber production between aeroponic and hydroponic systems by Roosta et al. (2013), Mbiyu et al. (2012); Factor et al. (2007), and Ritter et al. (2001) demonstrates the superiority of

aeroponic systems in potato production. Ritter et al. (2001) emphasized that the annual average for minituber production for aeroponics is almost 70% higher in tuber yield per plant and tuber number is approximately 2.5 folds more than average compared to hydroponic production. Adjusting hydroponic systems with aeroponic characteristics seems critical for increasing the cost effectiveness of the system besides obtaining higher yield in potato minituber production. Therefore, the core purpose of this research was to improve existing hydroponic systems by applying adequate oxygen level in the root zone, in order to enhance potato minituber production yield. In this study, the responses of cultivars to different levels of aeration were compared, in terms of tuberization and growth characteristics.

MATERIALS AND METHODS

This study was conducted in a factorial experiment using randomized complete block design with four replications in order to determine the effect of aeration in rooting medium on potato minituber production in soilless conditions. The involving factors in this experiment included four levels of aeration in the root zone 0 as control, 12.5, 25 and 75% ($V_{Air} / V_{media} / \text{min}$) as well as two commercial cultivars (Agria and Sante). This study was carried out in the glasshouse of Horticulture Science Department in Ferdowsi University of Mashhad, Iran from March to July by applying the close container hydroponic system.

At the bottom of each container (10 l volume with 22.5 cm diameter and 25 cm height) including 8 l media [25%:75% (1:3) composition of perlite and coir], a round air stone was installed that injected fresh air by an air pump to the media via separate flow meters. The flow meters were arranged to allow flowing of 1, 2 and 6 L air per minute, in terms of 12.5, 25 and 75% of the volume of media, respectively. The air pump was equipped with a timer which worked from sunlight to sunset (5:30 to 19:30). Aeration with fresh air was done at four levels, 0 (control), 12.5 (low aeration), 25 (medium aeration) and 75% (high aeration) volume of media per minute. Different levels of aeration were estimated based on DO changes after aeration in the hot time of day (that 2 pm). To obtain adequate number of *in vitro* plantlet, tissue culturing of single nodal cuttings in modified Murashige and Skoog medium (Zamora et al., 1994) was conducted by using virus-free plantlet of 'Agria' and 'Sante' cultivars.

The three weeks old *in vitro* plantlets were transplanted after one week acclimatization period with 200 plant density/m² (Lommen and Struik, 1992) in the composition of perlite and coconut coir. Nutrient solution containing N, P, K, Ca, Mg, S, Fe, B, Cu, Mn, Zn and Mo (182, 41, 300, 200, 48, 158, 3, 1, 0.3, 1.3, 0.3 and 0.07 ppm, respectively) was continuously re-circulated four times per day and replaced every three weeks by changing the amount of N, P and Ca to 100, 141, 180 ppm after stolon initiation and to 60, 180, 160 ppm after tuber initiation, respectively. Also, the pH and electrical conductivity of the solution were maintained at 5.8 and 2.2 to 2.5. Using non-destructive monitoring, the growth characteristics including stem number, plant height, leaf area, stem diameter and DO inside the media were measured at 14, 28, 42, 56, 70, 84, 91 and 105 days after transplanting (DATP). The estimated leaf area index (LAI) was calculated by correlation between length, width and number of leaves with surface counted from checkered sheet. Oxygen concentration of media was monitored by a Clark-type oxygen meter (Hana, YSI 55-12 USA). Interval harvesting was done in four periods in 42, 70, 91 and 105 DATP (Lommen and Struik,

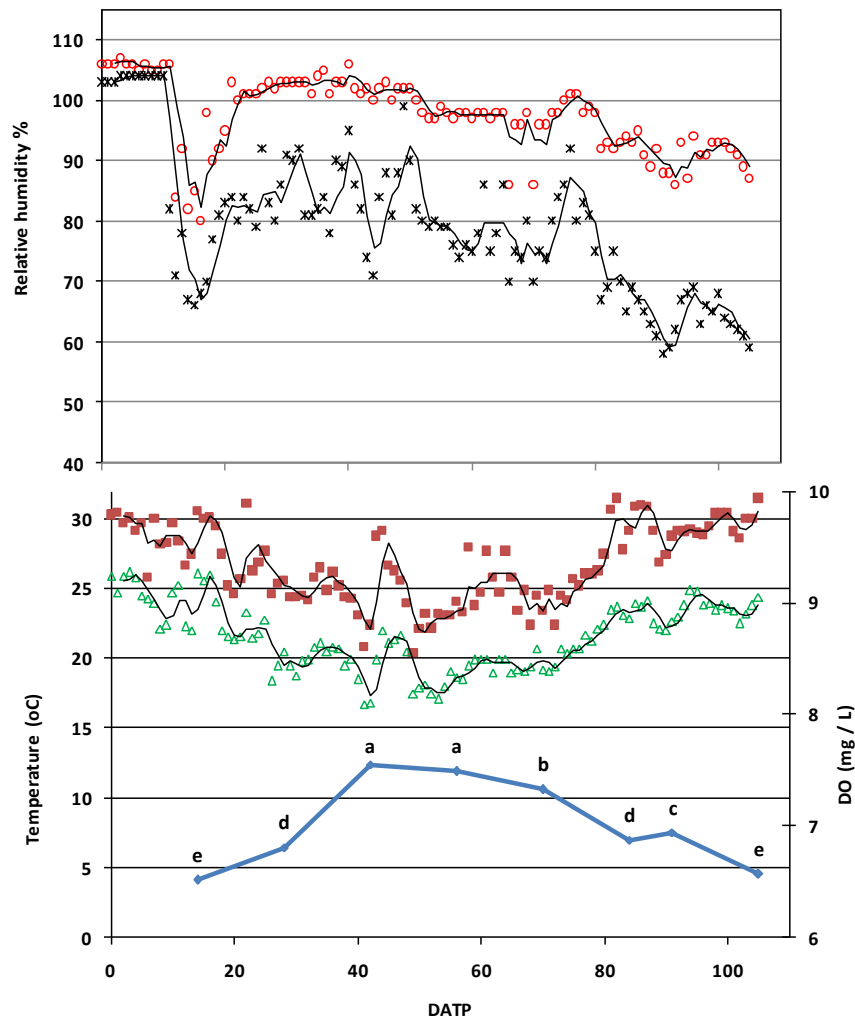


Figure 1. Temperature (■ maximum, △ average), humidity (○ maximum, ✕ average) and average dissolved oxygen (—●—) fluctuation during experiment in the greenhouse, equipped with cooling/heating, and moisture regulation systems. Means of dissolved oxygen are significantly different according to Duncan multiple range test, $p < 0.01$.

1992). Root length, number of root and stolon and dry matter of tubers were measured.

Harvested minitubers were classified into four groups according to C_1 : <5, C_2 : 5 to 10, C_3 : 10 to 15, C_4 : > 15 mm diameter. Fresh weight and dry matter (Oven dried for 72 h at 75°C) of classified minitubers were determined (Simmonds, 1977). The average temperature was maintained between 25°C during day and 17°C at night with 80% relative humidity (RH) in glasshouse under natural light conditions. The data were recorded continually by a computer. Time of tuber initiation was dated when 70% of plants of each plot had at least one tuber (>5 mm diameter). Physiological maturity was assigned when 70% of leaves turned yellow (Kawakami et al., 2004).

To test for field emergence and uniformity of a crop, minitubers (2 minitubers per experimental unit on each class) were dried for two days, cured for 14 days and stored at 4°C in darkness and 80% RH for three weeks. Then they were put in the pre-sprouting condition (15°C, 8 Wm⁻² light and 80% RH) for 10 days, after treating by 2 ppm gibberellic acid for 10 min in order to break its dormancy. Field planting took place on the loamy soil with drip irrigation. Emergence

of three visible leaves was recorded every day. Analysis of variance for all data, orthogonal contrasts and mean comparison was performed by general liner model (SAS Institute Inc., Cary, NC, USA, version 8.2.0).

RESULTS

Environmental condition

This experiment was carried out in a glasshouse equipped with cooling/heating, and moisture regulation systems. However, the temperature and moisture fluctuations during day/night and in the growing season were seen (Figure 1), which usually occurs in the majority of greenhouses. The maximum and minimum temperatures were from 32 to 17°C with the average RH of 80% (59 to 97%). The increment of temperature in the

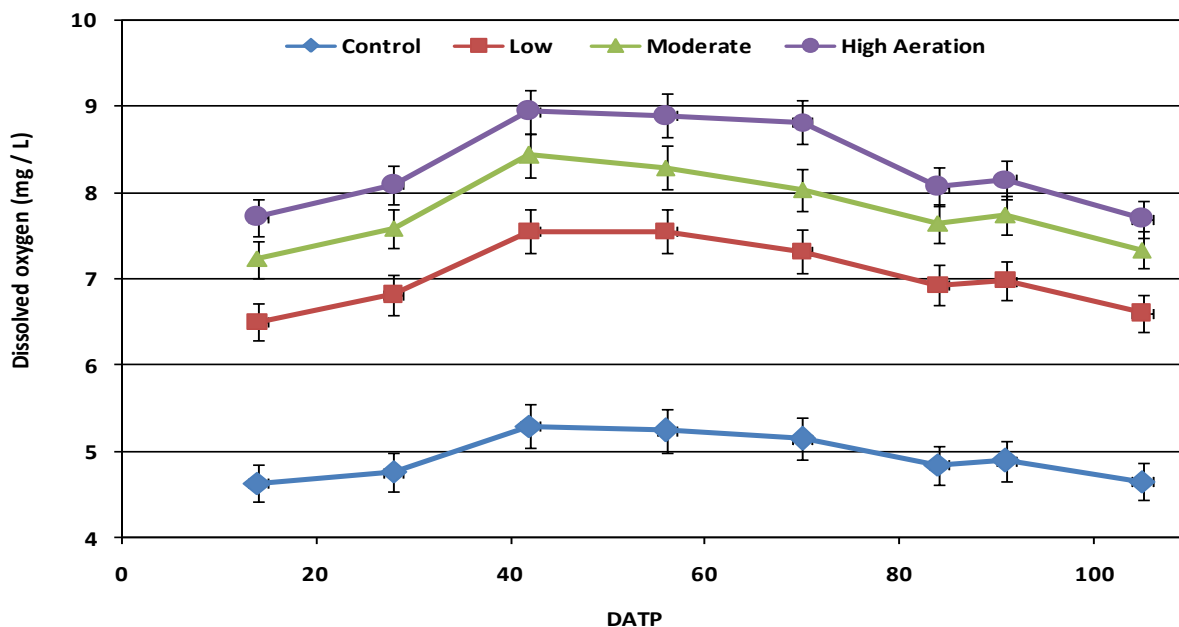


Figure 2. Dissolved oxygen in the media; comparison of different aeration levels (Control = 0, Low = 12.5, Moderate = 25, High = 75% air V/media V min) was significantly different than for the control. Bars indicate standard error, $P < 0.05$.

hot time of day leads to decreasing the level of DO. During sunny days, the radiation rate above the canopy was 15800 lux and during cloudy days, this amount was 7033 lux (27 days) with a photoperiod of 14 h.

Dynamic changes of dissolved oxygen in the root zone

The oxygen concentration of media was measured during the growing season in order to determine the effect of aeration on improvement of DO level in the root zone. Based on Figure 1, the results indicate that the reverse strong correlation between oxygen concentration and the average of temperature fluctuations ($R^2 = 0.89$ $P < 0.001$) were influenced markedly by applying aeration during the growing season. Moreover, dissimilar levels of aeration, low (12.5% V/min) moderate (25% V/min) and high (75% V/min), illustrated different amounts of DO which ranged from minimum of 4.47 mg/l (~ 41%) to maximum of 9.14 mg/l (~ 95%) in the warm hours of the day (Figure 2).

Plant growth characteristics and performance

Applying aeration in both cultivars (Agria and Sante) resulted in higher vigor, dry weight and leaf area compared to the control. The number of shoots and leaves in each bush were considerably higher in high and moderate levels of aeration compared to the low level and control group. The height of the Agria plant was

greater in the high and moderate levels of aeration than the low level and in the control treatment which was the lowest. In *var. Sante*, height of the plant was low in control than aerated levels. The length of the largest root and stolon number were less in the control in both cultivars (Table 1).

Tuberization started simultaneously in all aerated treatments two to three days earlier compared to the control group. The period of tuber induction until the appearance of ripeness, yellowing of leaves, and physiological maturity, increased significantly in treatments with moderate and high levels of aeration. Different levels of aeration influenced significantly stem diameter which is a practical index of vascular diameter and conductivity of sap wood. Higher numbers of leaves and larger leaf area index and the delay in physiological maturity by applying higher levels of aeration were obtained; also, it was found that the leaf area index duration (LAID) increased remarkably by increment in aeration level. All the mentioned characteristics (except starting time for tuberization) were different in both cultivars. This may reflect the different genetic characteristics in these cultivars (Tables 1 and 2).

High level of aeration resulted in 10 and 8 days earlier flowering in Agria and Sante cultivars, respectively in comparison with the control treatment. Physiological maturity occurred 5 to 12 days earlier in the non-aerated treatment. It was revealed that by applying aeration, the growing season was prolonged from 7 to 12 days in *var. Agria* and 5 to 8 days in *var. Sante* compared to the control treatment (Table 2).

Table 1. Effects of different levels of aeration on morphological growth characteristics of potato *var.* Agria and Sante.

Parameter	Stem number per plant		Plant height (cm)		Length of the largest root (cm)		Stolon number per plant		Leaf number per plant		Stem diameter (mm)	
	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante
Control	1.05	0.93	92.1	74.13	23.38	24.11	2.86	3.42	10.6	10.33	4.68	3.8
Low	1.11	1.05	115.92	105.37	41.3	36.56	9.16	6.54	15.27	12.99	6.1	5.13
Moderate	1.34	1.05	127.83	110.58	41.58	33.94	9.7	6.3	18.74	14.96	7.03	5.42
High	1.39	1.12	126.99	105.41	44.7	34.64	9.91	5.68	20.33	14.6	7.58	6.16
LSD 5% (25df)	0.14	0.12	8.77	10.61	8.68	8.96	1.76	1.73	2.21	1.67	0.61	0.5
LSD 1% (25df)	0.2	0.16	11.88	14.36	11.75	12.13	2.38	2.34	3	2.27	0.83	0.67

Aeration levels: Control = 0; low = 12.5; moderate = 25; high = 75 (% air V/media V/ min).

Table 2. Effects of different aeration levels on physiological traits of potato *var.* Agria and *var.* Sante.

Aeration levels	LAI		LAID (m ² /m ² /day)		Root/ shoot ratio		HI		DATP to tuberization		DATP to ripeness symptom		Days to emergence		Minituber emergence (%)	
	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante
Control	1.3	1.3	123.6	118.9	1.89	4.31	57.17	70.25	38.13	39.13	92.88	89	5.88	6	85.94	90.63
Low	2.93	2.47	296.1	236.3	3.38	8.29	67.79	80.23	36.63	36.88	100.13	94.38	5	5.63	96.88	92.19
Moderate	4.07	3.23	424.7	315.1	3.05	6.36	67.03	79.29	37.13	37.63	103.5	96.75	4.38	4.75	96.88	98.44
High	4.48	3.53	474.3	347.8	3.42	9.86	71.18	83.02	36.75	36.25	105.13	97.5	4	4.25	96.88	96.88
LSD 5% (25df)	0.29	0.48	30.3	46.7	1.2	4.15	7.24	10.17	1.23	1.66	1.16	2.02	0.39	0.57	6.58	6.87
LSD 1% (25df)	0.39	0.65	41.1	63.3	1.62	5.62	9.79	13.76	1.67	2.25	1.57	2.74	0.53	0.77	8.9	9.3

Aeration levels: Control = 0; low = 12.5; moderate = 25; high = 75 (% air V/media V/ min).

Dry matter accumulation and partitioning

Aerated plants had more dry matter in their haulms due to more existing leaves and shoots because of aeration. In the case of the aerated treatment, a higher level of root growth (especially longer roots, more stolon and dry matter in produced tubers) led to an improvement in root: shoot ratio. A comparison of harvest index (HI) between aerated treatments was also consistent with these results (Table 2).

Figure 3 shows that by applying low and moderate level of aeration, a considerable increase in dry matter in leaves, stem, root and stolon and dry weight of minitubers was observed in *var.* Agria (with more vegetative growth), while in *var.* Sante (with less growth), root and stolon weight did not change significantly. High level of aeration compared to low and moderate level showed no perceivable decrease in dry matter of leaves, stem, root and stolon, except the root weight in Agria which decreased significantly, whereas dry

matter of minitubers did not demonstrate any difference with other levels of aeration ($p < 0.05$). It is concluded that *var.* Agria was more responsive to higher aeration than *var.* Sante (Figure 3A and C).

Tuberization and minituber formation

Aeration increased the minituber numbers in class C₄ (diameter > 15 mm), specifically, followed by

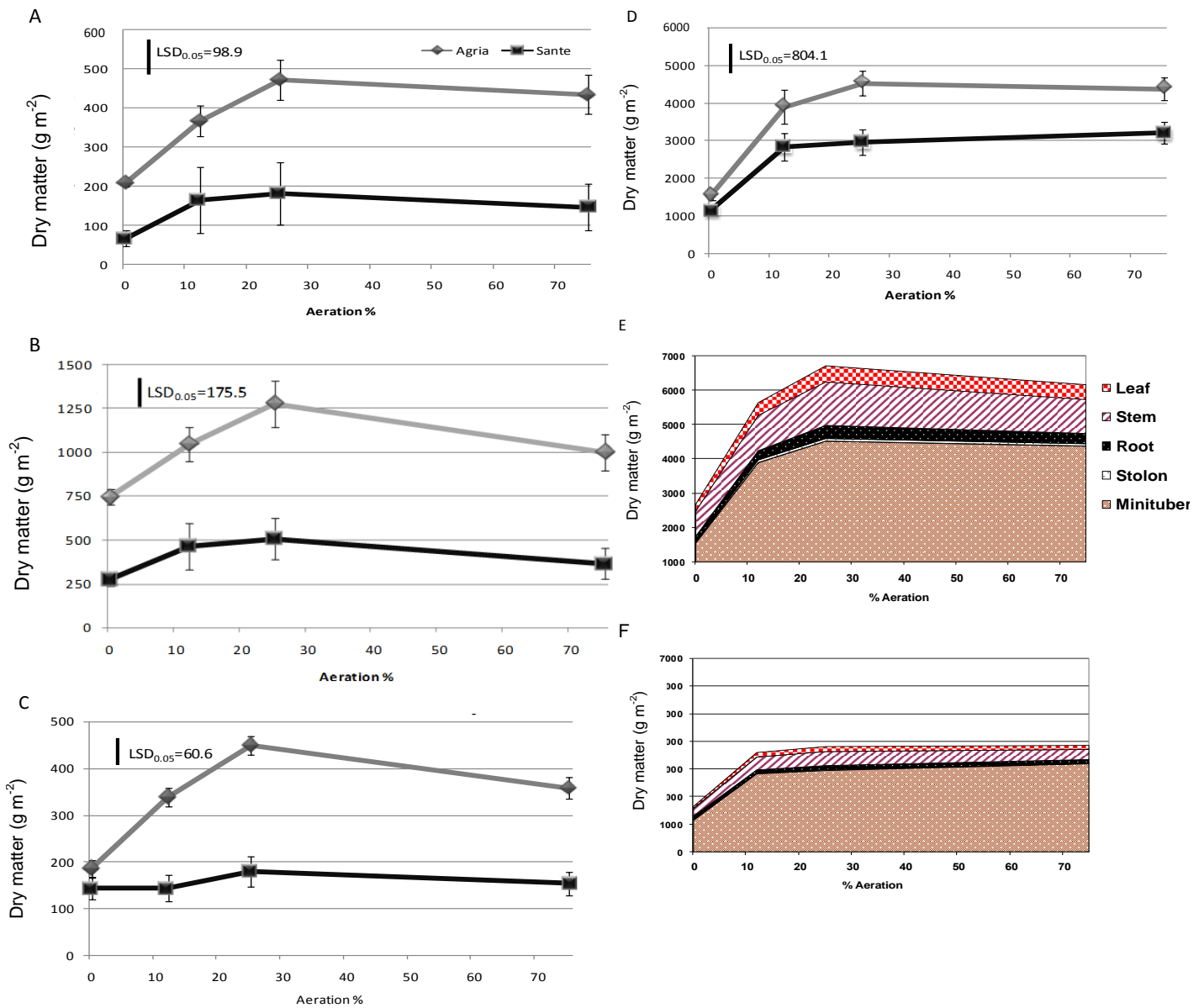


Figure 3. Effect of aeration on dry matter accumulation in different parts of plant, indicating plant biomass partitioning changes in two potato cultivars. **A.** Leaf dry matter. **B.** Stem dry matter. **C.** Root and stolon dry matter. **D.** Minituber dry matter. **E.** Mean of dry matter accumulation in Agria cultivar. **F.** Mean of dry matter accumulation in Sante cultivar. % aeration: % air V/media V/ min. Bars indicate standard error, $P < 0.05$.

class C₃ (diameter 10 to 15 mm). The ratio of C₄ and C₁ to total tuber number was 33 and 15% in control treatment, whereas by applying high/moderate level of aeration this ratio was shifted to 43.5 and 9%, respectively. Class C₃ of tubers showed an interaction effect between different levels of aeration and cultivars. Orthogonal contrast analysis of minituber numbers in two examined cultivars among different sizes of minitubers demonstrated no significant difference in C₁ and C₂ classes (Figure 4A and B). By increasing the level of aeration, class C₃ showed a sharp rise in the number of minitubers

especially in Sante cultivar. The mean of minituber number of both cultivars significantly increased in aerated levels compared to the control group ($p < 0.0001$) and in high and moderate levels of aeration compared with the low level ($p = 0.006$), whereas there were no significant differences between the high and moderate levels of aeration (Figure 4C). Moreover, aeration levels contributed to greater minituber numbers in class C₄ in Agria and Sante which were included 20 and 12 kg/m² of tuber yield respectively compared with the control group ($p < 0.0001$). Nevertheless, the different levels of aeration

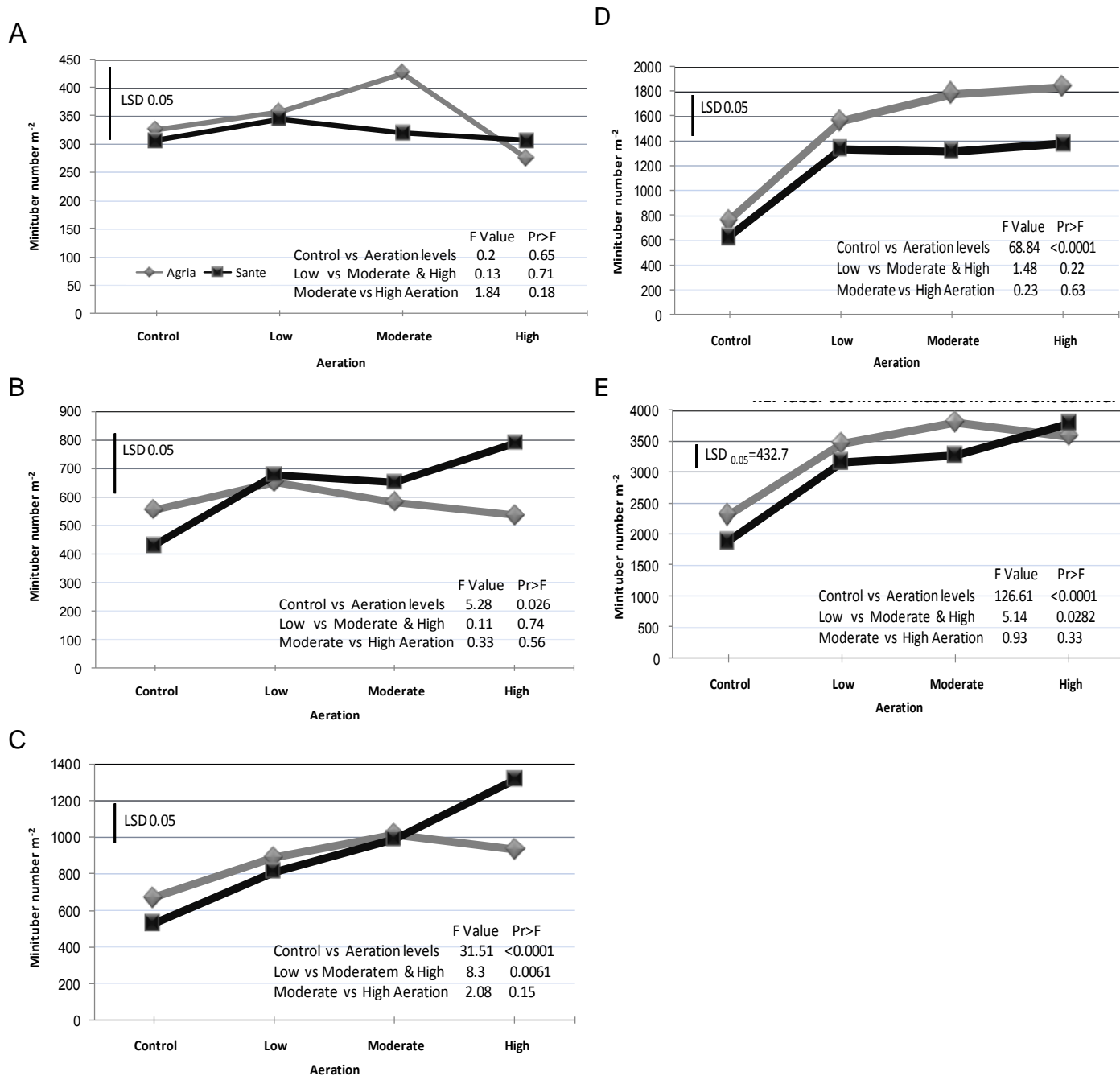


Figure 4. Minituber number of potato plantlet under different levels of aeration and cultivars (Agria Sante) based on tuber size grading. **A.** Class C1 tuber set in different cultivar. **B.** Class C2 tuber set in different cultivar Class C3 tuber set in different cultivar. **C.** Class C4 tuber set in different cultivar. **D.** Class C1 tuber set in different cultivar. **E.** Tuber set in sum classes in different cultivar. There were more tuber number at class C3 and C4 in all aeration levels versus control. Significant differences in orthogonal contrast were shown in each figure (Pr < 0.5). Bar indicates LSD 5 % for cultivars comparison. Classified groups: C1: <5, C2: 5 to 10, C3:10 to 15, C4: > 15 mm tuber diameter. Aeration levels: control = 0, low = 12.5, moderate = 25, high = 75 (% air V/media V min).

did not show any significant difference in this class (Figure 4D). The results demonstrate that the sum of produced minitubers in these two cultivars significantly increased by aeration ($p < 0.0001$). Although there was no difference between the moderate and high level of

aeration, a substantial increase in the number of minitubers was observed in the moderate and high level ($p = 0.028$) compared with the low level of aeration (Figure 4E).

Orthogonal contrast analysis for produced minituber

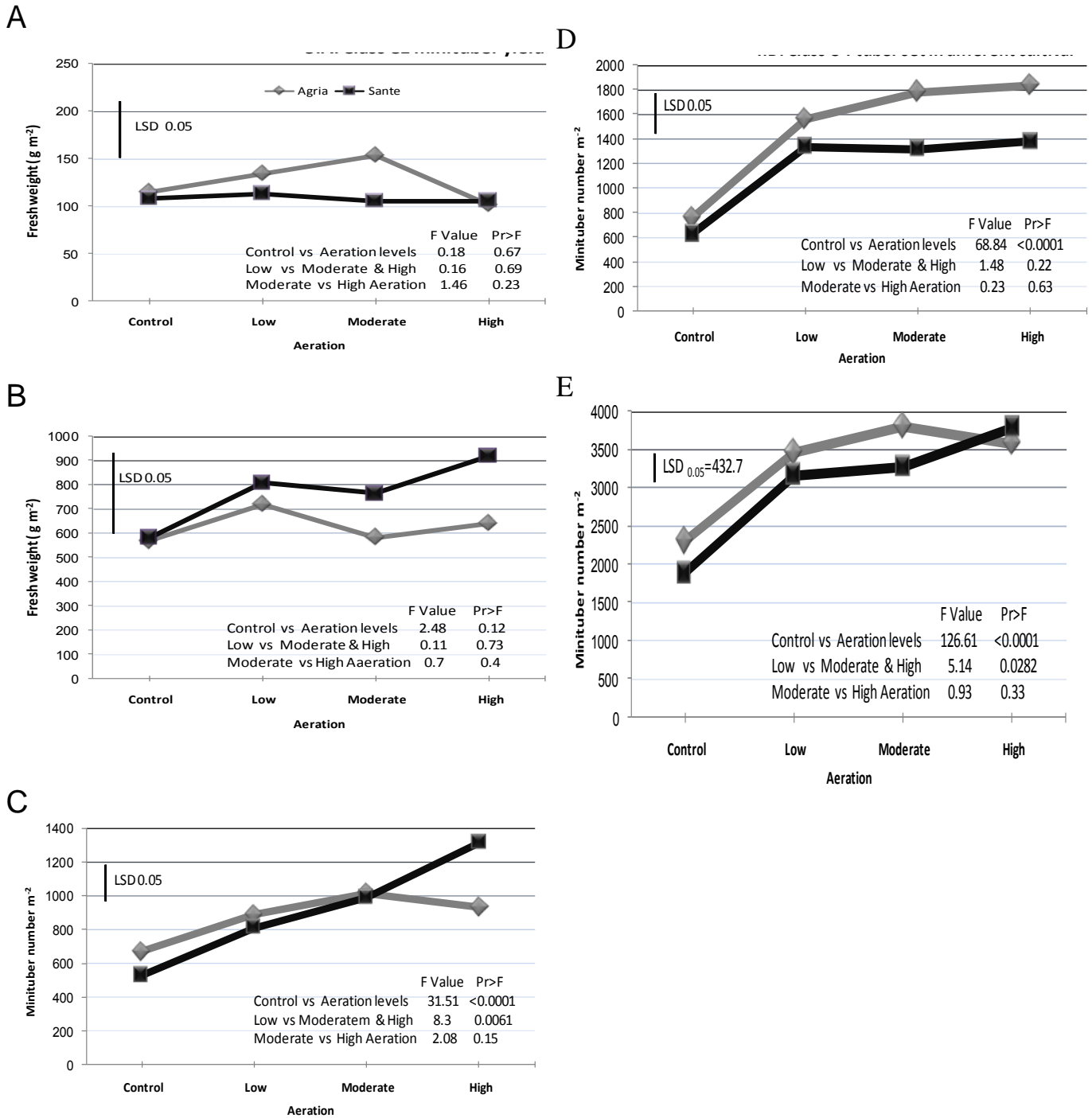


Figure 5. Minituber yield of potato plantlet under different levels of aeration and cultivars (—◆— Agria —■— Sante) based on tuber size grading. **A.** Class C1 minituber yield. **B.** Class C2 minituber yield. **C.** Class C3 minituber yield. **D.** Class C4 minituber yield. **E.** Class C1 minituber yield: Minituber yield in sum classes Larger tuber production (class C3 and C4) occurred by aeration application than control. Significant differences in orthogonal contrast were shown in each figure (Pr < 0.5). Bar indicates LSD 5% for cultivars comparison. Classified groups: C1: <5, C2: 5 to 10, C3:10 to 15, C4: > 15 mm tuber diameter. Aeration levels: control = 0, low = 12.5, moderate = 25, high = 75 (% air V/media V min).

yield indicated that in class C₁ and C₂, minitubers were similar to the number of tubers and were not affected by the aeration (Figure 5A and B), whereas aeration in class C₃, remarkably increased tuber yield compared to the

control group ($p < 0.0001$). This difference was considerable between low versus moderate/high levels of aeration ($p = 0.007$). However, high and moderate level of aeration did not lead to any notable difference (Figure

Table 2. Effects of different aeration levels on physiological traits of potato *var.* Agria and *var.* Sante.

Aeration levels	LAI		LAID (m ² /m ² /day)		Root/ shoot ratio		HI		DATP to tuberization		DATP to ripeness symptom		Days to emergence		Minituber emergence (%)	
	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante	Agria	Sante
Control	1.3	1.3	123.6	118.9	1.89	4.31	57.17	70.25	38.13	39.13	92.88	89	5.88	6	85.94	90.63
Low	2.93	2.47	296.1	236.3	3.38	8.29	67.79	80.23	36.63	36.88	100.13	94.38	5	5.63	96.88	92.19
Moderate	4.07	3.23	424.7	315.1	3.05	6.36	67.03	79.29	37.13	37.63	103.5	96.75	4.38	4.75	96.88	98.44
High	4.48	3.53	474.3	347.8	3.42	9.86	71.18	83.02	36.75	36.25	105.13	97.5	4	4.25	96.88	96.88
LSD 5% (25df)	0.29	0.48	30.3	46.7	1.2	4.15	7.24	10.17	1.23	1.66	1.16	2.02	0.39	0.57	6.58	6.87
LSD 1% (25df)	0.39	0.65	41.1	63.3	1.62	5.62	9.79	13.76	1.67	2.25	1.57	2.74	0.53	0.77	8.9	9.3

Aeration levels: Control = 0; low = 12.5; moderate = 25; high = 75 (% air V/media V/ min).

5C). Class C₄, minitubers of more than 15 mm, were affected by different levels of aeration significantly compared to the control group ($p < 0.0001$), whereas there was no difference between different levels of aeration in this regard (Figure 5D). Collectively, in both cultivars and different classes of minitubers, the minituber yield increased appreciably to 23.9 kg/m² in Agria and 17 kg/m² in Sante by applying aeration ($p < 0.0001$). This increment occurred in high and moderate levels of aeration and was remarkably different ($p = 0.049$) compared to low level of aeration (Figure 5E).

Comparison between the two tested cultivars in this experiment clarified that *var.* Agria, consisted of more class C₄ tubers rather than *var.* Sante due to its special cultivar characteristics, which influenced the number, yield and dry matter of tubers (Figures 4D and 5D) whereas in both cultivars, increasing the level of aeration led to higher number of stolons and tubers especially in the second and third harvest compared to first and fourth harvest. Moreover the minituber yield was achieved by applying the moderate and high level of aeration to 23.9 kg/m² in Agria and 17 kg/m² in Sante. As a result, their large minitubers (>15

mm) included 20 and 12 kg/m² of tuber yield, respectively.

Field emergence test

To ascertain the emergence ability of tubers which were produced under different levels of aeration, pre-sprouted minitubers were planted in a loamy soil. This emergence test proved that all minitubers (86 to 98 %) germinated after four to six days. They were superior in emergence rate for aerated plants than the control, due to higher quality of seeds and the larger tubers which have higher and faster emergence rate (Table 2).

DISCUSSION

Nowadays, container systems are developed commercially for economic and other benefits. Although such systems provide adequate water and nutrients in root zone, they are not so efficient for plants with higher level of oxygen demand in the root zone. These types of plants, due to higher leaf surface and growth velocity, need more active

roots to provide water and nutrients for leaves. Desirable performance of aerated plant in this study could be related to higher root oxygen demand of potato that is not affordable by normal ventilation in media. Appropriate oxygen level in root zone promotes the production of larger tubers. On the other hand, potato plant would have more efficient and longer roots with higher number of stolon.

No direct measurement is proven for oxygen deficiency in potato zone. However, it is defined as the concentration of oxygen which inhibits metabolic activities, including cell division, mineral uptake and water movement into roots (Softer et al., 1991). Tuber crops such as potato have a large leaf surface and high production potential consisting of a large biomass underground which has high respiration rate. Oxygen deficiency on hydroponics system is more probable in potato due to the higher demand for oxygen in the root zone (Özkaynak and Samanci, 2006).

In this experiment, temperature fluctuations influenced significantly on the oxygen concentration in the root zone, even in aerated treatments (Figures 1 and 2). Dissolved oxygen decrease was detected (minimum DO = 4.47 mg/l)

in the control treatment which led to predictable oxygen deficiency in the root zone.

Consistent with the current experiment, previous studies have depicted the role of aeration in plant yield improvement such as tomato, lettuce, cucumber and melons (Chun and Takakura, 1994; Goto et al., 1996; Bhattaria et al., 2006; Zheng et al., 2007). This role of aeration is facilitated by improving DO concentration. Furthermore, aeration not only offsets hypoxic conditions but also satisfies an unmet demand for oxygen in the root zone (Bhattarai et al., 2006).

According to Zheng et al. (2007), oxygen supersaturation of tomato root zone improves plant performance by oxygen enrichment in nutrition solution, using hydroponic system. Root respiration rates, based on fresh or dry weight, showed a positive linear regression with DO level in the nutrient solution. No changes in dry and fresh weight of plant, stem diameter and leaf area were detected among different treatments from DO = 8.5 to 30 mg/l, due to the short period of their experience (four weeks). Also, Nichols et al. (2002), by growing tomato and cucumber seedlings in an aeroponics system with root zone oxygen ranged from 5 (2 mg/l) to 80% (32 mg/l), clarified that plants' relative growth rates are significantly lower in the treatments with root zone oxygen levels of 5 and 10% than with ambient DO (20%). In lettuce, root browning occurred when DO concentration was as low as 12 mg/l (Chun and Takakura, 1994; Goto et al., 1996). Holtman et al. (2005) showed decreased plant development and reduced root mass, when cucumbers were subjected to the lowest DO (0.5 mg/l). Applying different oxygen levels, escalating differences in leaf area were observed, showing the largest leaf area at 10 mg/l oxygen.

Oxygen in nutrient solution which is seen in the substrates can be completely consumed within 30 min, indicating that roots are very susceptible suffering from anoxia. Oxyfertiligation, the injection of pure pressurized oxygen gas to the nutrient solution above saturation levels, has been commercially adapted for use in horticultural greenhouses. Applying oxyfertiligation on melon crop, Acuna et al. (2008) claimed higher final yield (quality and quantity) of marketable fruits for oxygen-enriched crops. The oxyfertiligation was also described by Marfa et al. (2005) to guarantee root convenience to improve rhizosphere oxygen availability in soilless culture under Mediterranean coastal conditions.

The average tuber numbers of various potato cultivars are 6.2 to 7.9 in plantlets grown in polyethylene bags (Kaur et al., 2000). Lommen and Struik (1992) reported the tuber yield from 16.9 to 23 g and the average tuber number from 8 to 8.8 in two potato cultivars in a glasshouse state (200 plant/m²). Control group of current research showed similar results. At same plant density, tuber number changed from 1.85 to 2.52 and average tuber weight from 9.8 to 10.9 g, in hydroponics glasshouse conditions (Grigoriadou and Leventakis, 1999).

In another study, the tuber yield was 22.95 to 31.23 g, tuber number was from 6.39 to 9.7 and average tuber weight was from 3.36 to 3.63 g in potato plantlets grown under hydroponics glasshouse conditions (Vosátka and Gryndler, 2000). In the result of the present experiment, the maximum yield reached up to 19 tubers per plant weighing 6.3 g, in Agria, by applying the moderate level of aeration. The high level aeration produced 18.9 tubers per plant, weighing 4.5 g, in Sante (Figures 4E and 5E) which explains that aeration requirement might be cultivar dependent.

The results of the current study, regarding yield, biomass production and potato vigor proved that the yield of minituber production was considerably high. Also plants had a longer vegetative period with higher plant height and root length, using a model of aeroponic system. These were consistent with the results of Ritter et al. (2001) and Factor et al. (2007) comparison of hydroponic and aeroponic systems (Table 2).

Diengdoh et al. (2012) point to the size of minituber seed as an important factor for growth and total yield aspects in potato production system. Compared to Lommen (1995) study with 800 plant/m² plant density, in the present experiment similar minituber number was produced with plant density 200 plant/m² (Figure 4E). Also, applying aeration, the weight and size of tubers increased significantly. This increment resulted in superiority in average minituber number up to 3600/m² weighting 5.4 g which was 70% higher compared to non-aeration group. Mateus-Rodríguez et al., (2011) showed that using aeroponics system, the number of minitubers per plant was in the range of 30.6 - 71.7 in three potato cultivars. Tuber yield per plant ranged from 197.6 to 860.2 g per plant within cultivars. Average tuber weight ranged from 6.3 to 12.1 g per minituber. This high variability between cultivars exists as regards their response and production in an aeroponics system under uniform conditions.

The reaction of plant tuberization to aeration which led to increment in size and weight of tubers can be related not only to higher LAID and the preference of aerated plant vigor but also to better root efficiency in water and nutrient absorption (data not shown) due to removal of oxygen deficiency and more efficient respiration inside the tubers in aerobic condition confirming that potato tubers might not tolerate low O₂ concentration. It is concluded that applying aeration leads to a decrease in metabolism and an increase in tuber assimilate (Lipton, 1967).

In another research carried out by Schroeder and Engwicht (2005) on the rose, the growth and productivity was not affected by aeration. It seems that in such plants slighter root biomass, small leaf area and lower optimal growth temperature lead to more resistance to oxygen deficiency. It is concluded that in tuber crops, due to higher oxygen demand especially in tropical area and warm hours of the day, in which the temperature of root

zone increases, applying aeration in the root zone can play an important role in improving root requirement and its efficacy. Also aeration and media ventilation results in decreasing harmful gases such as ethylene and ammonium which can affect root growth and activity (Raviv et al., 2008; Weathers and Zobel, 1992).

Relative high temperature in Kang et al. (1996) experiment in tropic area for minituber production systems decreased oxygen solubility, which is related to temperature reversely (Zhang and Shao, 2006). More oxygen in nutrition solution, consequently improved its absorption by the root. In the middle hours of the day when radiation is maximum and a favorable condition for photosynthesis is available, increase in temperature results in a remarkable decline in DO in the root zone (Moore and Townsend, 1998). Therefore, in tropical conditions, oxygen deficiency might be more visible in hydroponic systems. Aeroponic system helps enhancing the solubility of oxygen, via scattering mist in the air. It is suggested that aeroponic system replace hydroponic system if they are equipped with technologies such as aeration or DO super-saturation of nutrition solution that can enhance dissolved oxygen level in the root zone.

Since the present experiment was run at 200 plant/m² density, the examined cultivars accomplished up to 3793 minituber/m² for *Agria* and 3781/m² for *Sante* in just 105 days interval that all minitubers grew perfectly in field conditions. For commercial purposes, it can be repeated three times per year; therefore, the annual yield by applying aeration will be more than 10000/m² minitubers with 5.4 g weight. This fact is consistent with the claim of professionally minituber production in aeroponic systems. Shortly, it is concluded that applying aeration with suitable level, higher than 25% air V per media V/ min, can improve hydroponic systems toward aeroponics with lower expenditure and much fewer problems.

The overall outcome evidently indicates that hydroponics system with supplementary aeration equipment can be used effectively to amplify the good quantity and quality of minituber production of potato and applying minitubers seems to be a suitable method for large scale use in a seed production program. Yet, minituber production will only be successful if they are economic and in quality superior to other tubers by the existing technologies. Installing aeration system for commercial production needs more investigation. These researches should be related to physical condition of substrate, the place of root dense inside the container and management of root temperature by aeration. By appropriately designing irrigation systems, the pump and tubes can be used alternatively for irrigation and aeration. The greenhouse environment always requires fresh air which is the cheapest and the most vital substance.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Desiccation tolerance of embryos of *Syagrus oleracea*, a cerrado native bitter palm heart

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There is great interest in seedling production of plant species with ornamental and food potential; however, there is great difficulty in propagating this species. A method of fruit drying was proposed with the goal of evaluating the desiccation tolerance of embryos of guariroba palm *Syagrus oleracea*. Tissue culture was used to test the effect of different fruit drying times (0, 4, 8 and 12 days) on embryo viability and *in vitro* germination. Desiccation tolerance of guariroba palm embryos was determined, and a methodology for their *in vitro* establishment was proposed. The seed water content was estimated from the fruit water content without extracting the seed. Drying at 37±2°C did not negatively affect embryo viability or germination. In addition, contamination of zygotic embryos decreased with increasing drying time. Fruit drying at 37±2°C for 8 days is recommended because it made the embryo extraction easier without embryo viability loss or contamination during the *in vitro* establishment of guariroba palm.

Key words: Desiccation, guariroba, propagation, ornamentation, physiological quality, Arecaceae.

INTRODUCTION

The guariroba palm (*Syagrus oleracea* (Mart.) Becc.), which belongs to the family Arecaceae, is commonly known as palmito-amargo (biter palm heart) and gueroba. The nut and palm heart of the guariroba palm are eaten fresh, and they are rich in peroxidase and

polyphenoloxidase enzymes, which together with the phenols cause the darkening of the palm heart, giving it a characteristic taste much appreciated in cooking, especially in the central region of Brazil. Due to its beauty, this plant has become a popular ornamentation in

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urban and rural landscapes. The fruits of the guariroba palm are the basis of wildlife diets (Matteucci et al., 1995; Silva et al., 2001; Jaime et al., 2007).

To improve the process of seed extraction, some authors have recommended decreasing the seed water content in a forced air circulation oven at temperatures that result in the release of the seed from the fruit's endocarp, keeping the seed intact when the endocarp is broken (Ferreira and Gentil, 2006; Rubio Neto et al., 2012). However, the loss of seed water can decrease the seed viability and germination in some species, as reported for açai seeds (*Euterpe oleracea* Mart.) (Nascimento et al., 2007) and red palm heart seeds (*Euterpe espirotosantensis* Fernandes) (Martins et al., 1999). For *S. oleracea*, there are no reports relating the loss of fruit and seed water content with germination, but there are reports on diaspores (endocarp+seed) that are collected with approximately 20% water content.

Because the drying process can make the embryos unviable, several quick tests to determine viability, such as the tetrazolium test and electrical conductivity, have been proposed for crops with high agricultural importance. These studies are scarce for plants of the family Arecaceae, but adaptations of the tetrazolium test have been performed using salt concentrations ranging from 0.075 to 1% and imbibition times in the tetrazolium solution for enzyme activation between 2 and 6 h, with preference for a 0.5% salt concentration and 4 h of imbibition at 30°C. Under these conditions, tetrazolium confers different colours to the embryos, allowing their classification into different vigour classes according to lesion proportion, quantity and localisation (Ferreira and Sader, 1987; Reis et al., 1999; Spera et al., 2001; Ribeiro et al., 2010).

Plants from the family Arecaceae often have difficulties in germination due to morphological characteristics of the seed and peculiarities of the germination process. It is common for some species to not germinate even under adequate germination conditions, which can result from dormancy mechanisms. For *S. oleracea*, the highest percentages of emergence have been obtained from seeds kept in vermiculite reaching 53.8% after 82 days of culture (Batista et al., 2011). The culture of embryos can therefore be advantageous because extracting the embryo from the seed removes all mechanical resistance and a large part of the germination inhibitors, thereby accelerating the process of seedling production (Hu and Ferreira, 1998; Lorenzi et al., 2004).

Embryo culture studies have focused on *in vitro* establishment, growth medium composition, embryo oxidation and maturation. Different disinfection times as well as alcohol and sodium hypochlorite concentrations have been recommended to minimise embryo contamination of species from the family Arecaceae (Sugii, 2011). Another important problem of embryo culture is the oxidation rate, which can be controlled by ascorbic acid in the case of *S. oleracea* (Melo et al.,

2001).

The goal of the study was to evaluate the effect of fruit drying on seed vigour and viability, enabling *in vitro* establishment of plantlets of guariroba palm. This study is warranted by the fact that *S. oleracea* has high social, environmental and economic importance, but its propagation using conventional methods is difficult. This difficulty can be overcome using *in vitro* embryo culture as highlighted by Melo et al. (2001).

MATERIALS AND METHODS

This study was performed at the Seed Laboratory (Laboratório de Sementes) and Plant Tissue Culture Laboratory (Laboratório de Cultura de Tecidos Vegetais) of the Goiás Federal Institute (Instituto Federal Goiano), Rio Verde Campus, Goiás (GO), Brazil. Ripe fruits of *S. oleracea* collected in October 2012 at the Gameleira farm in the Montes Claros de Goiás municipality, GO (16° 07' S and 51° 18' W; 592 m altitude) were used. Damaged fruits were discarded, and due to their high heterogeneity, fruits were classified into three classes according to their total mass (Figure 1A). The number of fruits per class followed the frequency distribution found at the field, that is, 30 small fruits (≤ 27.8 g), 35 medium fruits (28.0 to 34.9 g) and 15 large fruits (≥ 35.0 g). For this study, 80 fruits in total were used, from which 80 embryos were extracted.

To evaluate the effect of the drying temperature, whole fruits were dried in a forced air circulation oven at $37 \pm 2^\circ\text{C}$ for 0, 4, 8 or 12 days (Figure 1B). For all tested drying times, water contents of fruits and seeds were measured using an oven at 105°C until constant weight was reached.

For all tested times, a fruit lot was removed from the oven and broken using a 1.5 kg mallet and a concrete plate to evaluate the physiological quality of the embryos. Immediately following extraction, the embryos were covered in gauze and placed in running water for 3 h according to Melo et al. (2001). The embryos were then disinfected with 70% alcohol for 30 s, 20% commercial sodium hypochlorite for 20 min and rinsed three times in autoclaved distilled water (Figure 1C). Following inoculation in half-strength MS (Murashige and Skoog, 1962) growth medium, the embryos were kept in a growth chamber at $25 \pm 3^\circ\text{C}$ in the absence of light for 15 days. Following this period, the embryos were kept under a 16 h light/8 h dark photoperiod at $25 \pm 3^\circ\text{C}$ and $40\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$ active photosynthetic radiation supplied by fluorescent lights.

At the same time that embryos were extracted for *in vitro* germination, embryos were also removed for viability evaluation. The tetrazolium test was adapted from Ribeiro et al. (2010) using four replicates of 10 embryos for each drying time (Figure 1C).

The experimental design was completely randomised with four drying times and four replicates of 20 fruits and/or seeds. Analysis of variance was performed, and regression analysis was performed when necessary. The Pearson product-moment correlation coefficient was used to evaluate correlations at $p < 0.05$.

RESULTS AND DISCUSSION

Regression analysis revealed pronounced fruit water loss, which increased exponentially with increasing drying time. The model fitted to the drying data was significant, and it showed that fruits exhibited 34.6% water content at the time of harvest and that a water content of 8.84% was reached after 12 days at 37°C (Figure 2A). Diaspores of

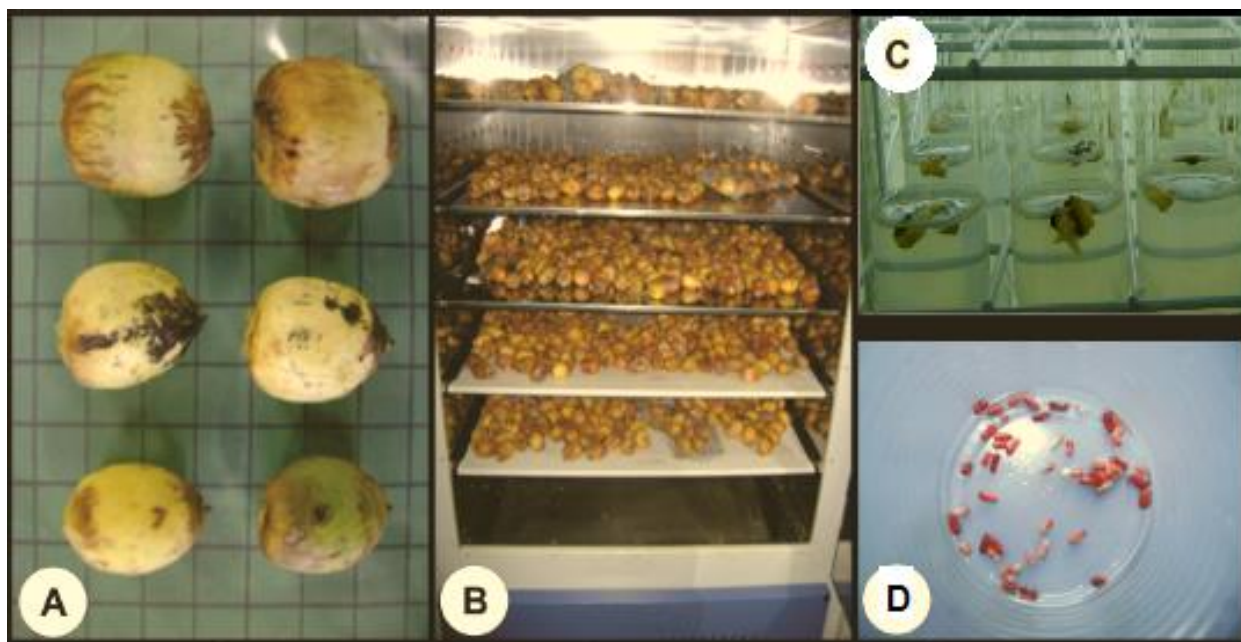


Figure 1. Method for *in vitro* establishment and tetrazolium test. A) Fruit classification according to their mass. B) Drying in a forced air circulation oven at 37°C. C) Embryo *in vitro* germination. D) Tetrazolium test following drying for 4 days.

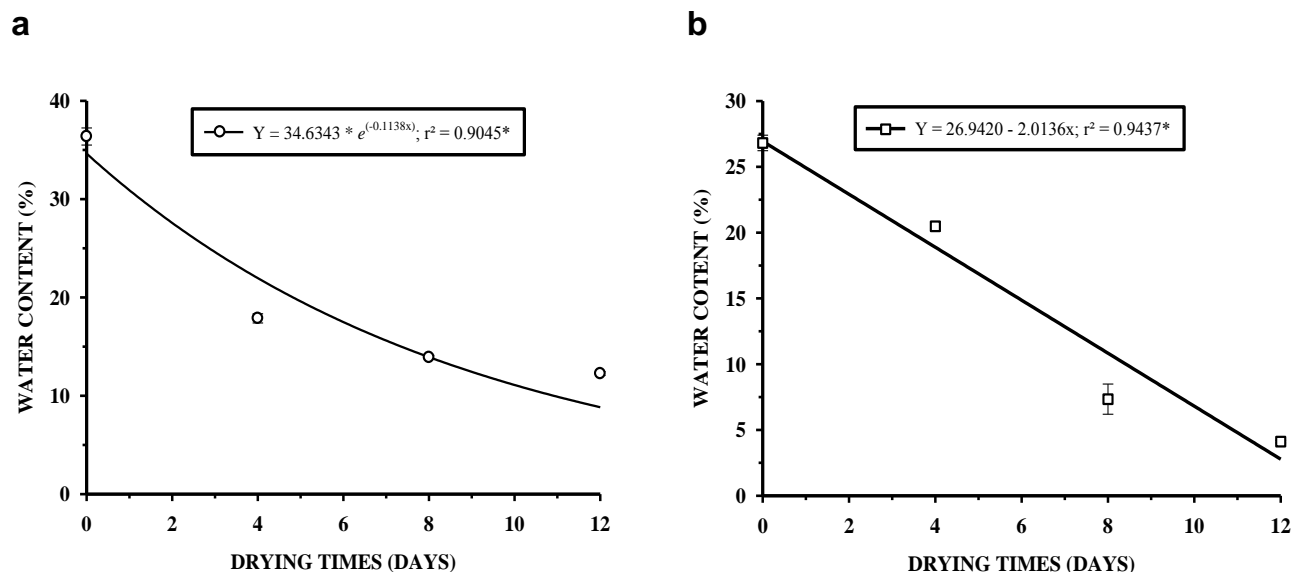


Figure 2. Water content of fruits (A) and seeds (B) of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] dried in a forced air circulation oven at 37°C for different times. *Significant at $p < 0.05$.

S. oleracea collected in 2009 have been shown to reach a 19.9% water content (Batista et al., 2011), indicating that the epicarp and mesocarp contain a large part of the whole fruit water content.

The seed water content was 26.9% at the time of harvest. The seed water content linearly decreased with increasing drying time at a rate of 2.01% per day, and it reached 2.79% after 12 days of drying. The exponential

fruit water loss may have been due to the morphological characteristics of the fruits, which allow the easy exit of water from the epicarp and mesocarp. In contrast, the seed water loss was slower, which was most likely due to the presence of the rigid fruit endocarp that made the water loss linear with increasing drying time (Figure 2B).

Water loss at 37°C in a forced air circulation oven has been found to be efficient for the drying of macaúba palm

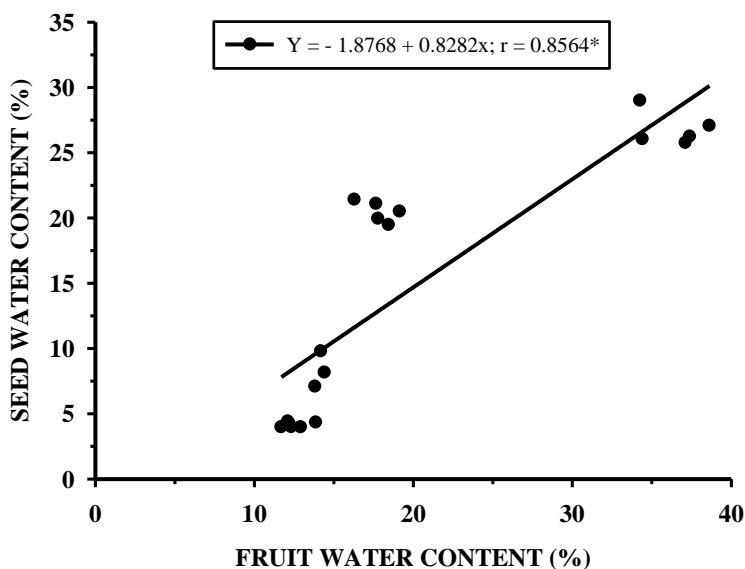


Figure 3. Correlation between water contents of fruits and seeds of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] dried for 12 days in an oven at 37°C. *Significant at $p < 0.05$.

[*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.] and babassu palm (*Orbignya phalerata* Mart.) fruits, thereby reducing fruit water content and facilitating the extraction of intact seeds and zygotic embryos similarly to what has been observed for *S. oleracea* (Rubio Neto et al., 2012; Silva et al., 2012).

A positive correlation was observed between the water content of fruits and seeds ($r=0.8564^*$) during drying at $37 \pm 2^\circ\text{C}$. Therefore, the seed water content was correlated with the fruit water loss. In future studies, it will be possible to estimate seed water loss based on fruit water loss at 37°C without the need to break the fruits for seed extraction. The water loss of fruits and seeds dried at $37 \pm 2^\circ\text{C}$ occurred at a 1:0.8282% ratio, thereby showing that the fruits lose water in a higher proportion than the seeds (Figure 3).

The importance of studying this effect for each species is reinforced by the fact that some species, such as palm heart (*Euterpe edulis* Mart.), açai palm (*Euterpe oleracea* Mart.), red palm heart (*Euterpe espirosantensis* Fernandes), bacaba (*Oenocarpus bacaba* Mart.) and Alexander palm (*Archontophoenix alexandrae* Wendl. and Drude), are considered recalcitrant because they lose germination capacity with increasing water loss. In contrast, other species, such as babassu palm (*Orbignya phalerata* Mart.), macaúba palm [*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.] and carandá (*Copernicia alba* Morong.), are considered orthodox. In the present work, *S. oleracea* was observed to present orthodox characteristics because the decrease in seed water content did not affect embryo *in vitro* germination and viability. However, further studies should be performed to confirm

these results, namely testing different times and storage environments (Martins et al., 1999; Reis et al., 1999; Nascimento et al., 2007; Ribeiro et al., 2010; José et al., 2012; Masetto et al., 2012; Silva et al., 2012).

At the beginning of the drying process, the *S. oleracea* embryos exhibited an intense red colour, thereby being classified as vigorous (Figure 4A and B). With the increase in drying time, the embryos became light pink at the haustorium and at some lesions at the petiole, thereby being classified as viable (class two of vigour) (Figure 4C and D).

The modification of the tetrazolium test proposed by Ribeiro et al. (2010) resulted in adequate colouring of *S. oleracea* embryos, enabling the identification of their vigour. With the increase in drying time, a decrease in class one (vigorous) embryos and an increase in class two (viable) embryos (which also germinated *in vitro*) were observed, thereby confirming the results of the vigour tests.

Tetrazolium salt concentrations between 0.075 and 1% in addition to imbibition times varying between 2 and 6 h have been tested for the family Arecaceae. The tetrazolium test has been evaluated for several different species. According to Ferreira and Sader (1987), the efficiency of the tetrazolium test should be confirmed by correlation with germination tests. The concentration of and imbibition time in tetrazolium solution most often used is 0.5% and 4 h, respectively, for enzyme activation at 30°C (Ferreira and Sader, 1987; Reis et al., 1999; Spera et al., 2001; Ribeiro et al., 2010; Rubio Neto et al., 2012).

The number of viable embryos increased quadratically,

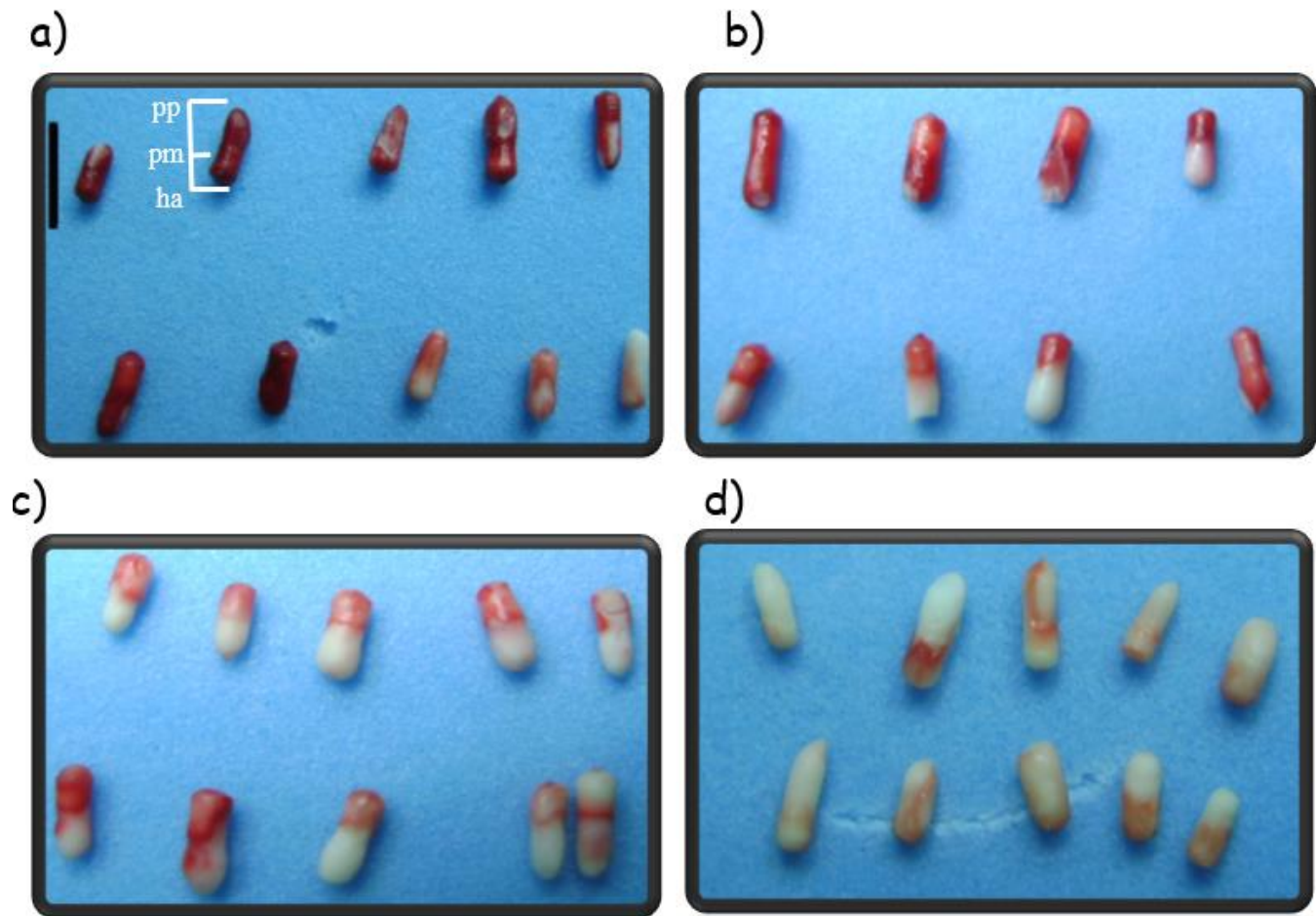


Figure 4. Zygotic embryos of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] in the tetrazolium test (Bar = 1 cm). ha, Haustorium; pm, median; pp, proximal petiole regions. **a)** Embryos obtained from fruits without drying. **(b, c and d)** Embryos obtained from fruits dried at $37\pm 2^{\circ}\text{C}$ for 4, 8 and 12 days, respectively.

with increasing drying time reaching its maximum (75.2%) following 7.5 days of drying (Figure 5A and B). The percentages of unviable and dead embryos were low at all drying times, with averages of 12.0 and 3.5%, respectively (Figure 5C and D). However, the average percentage of vigorous embryos (vigour class one) linearly decreased with increasing drying time, with values decreasing from 67.2% at the time of harvest to 0% following 12 days of drying. This trend was accompanied by an increase in the average percentage of viable embryos (vigour class two), which also germinate *in vitro*.

The present study demonstrated that drying for up to 12 days in a forced air circulation oven does not negatively affect germination and plantlet initial growth (Figure 6). Using *S. oleracea* diaspores (seed + endocarp), however, Matteucci et al. (1995) obtained higher germination percentages in seeds of depulped freshly harvested fruits than in fruits dried for 30 days and not depulped. Thus, it can be concluded that different results can be obtained for the same species depending

on the form of drying because the water removal can damage cell walls and result in loss of vigour.

The average germination percentage remained stable with the increase in drying time, with an average of 71.1% (Figure 7). In contrast, contamination by microorganisms was high in zygotic embryos extracted from fruits that were not dried at 37°C . This contamination decreased with increasing drying time in an oven over 7.5 days of drying, reaching an average of 3.33% contamination, which is considered satisfactory for *in vitro* establishment.

A positive correlation was observed between *in vitro* contamination and seed water content. As the seed water content decreased, the average percentage of *in vitro* contamination also decreased in a 1:0.82 ratio, which indicated that drying at this temperature improves seed and embryo extraction but also results in lower *in vitro* embryo contamination (Figure 8). Similar results have been observed for macaw palm [*Acrocomia aculeata* (Jacq.) Lood. ex Mart.] (Rubio Neto et al., 2012) {Rubio

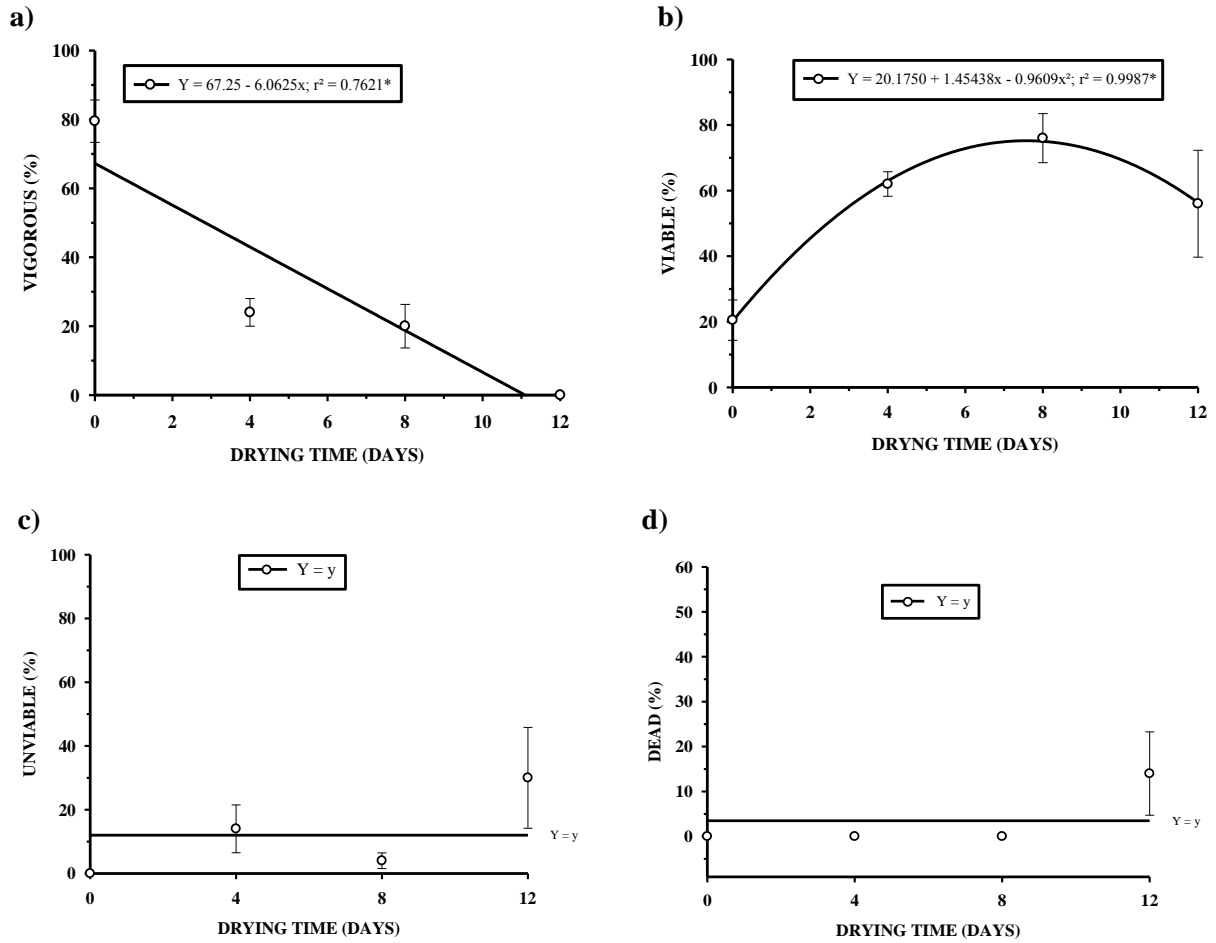


Figure 5. Percentage of vigorous (A), viable (B), unviable (C) and dead (D) zygotic embryos of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] dried for 12 days in an oven at 37°C. *Significant at $p < 0.05$.

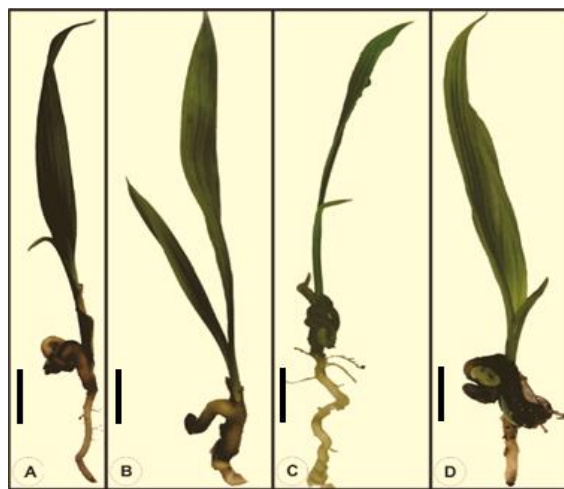


Figure 6. Plantlets of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] grown for 90 days obtained from zygotic embryos extracted from fruits dried superficially A) without drying, B) with 4 days of drying, C) with 8 days of drying and D) with 12 days of drying at 37°C. Bar = 2 cm.

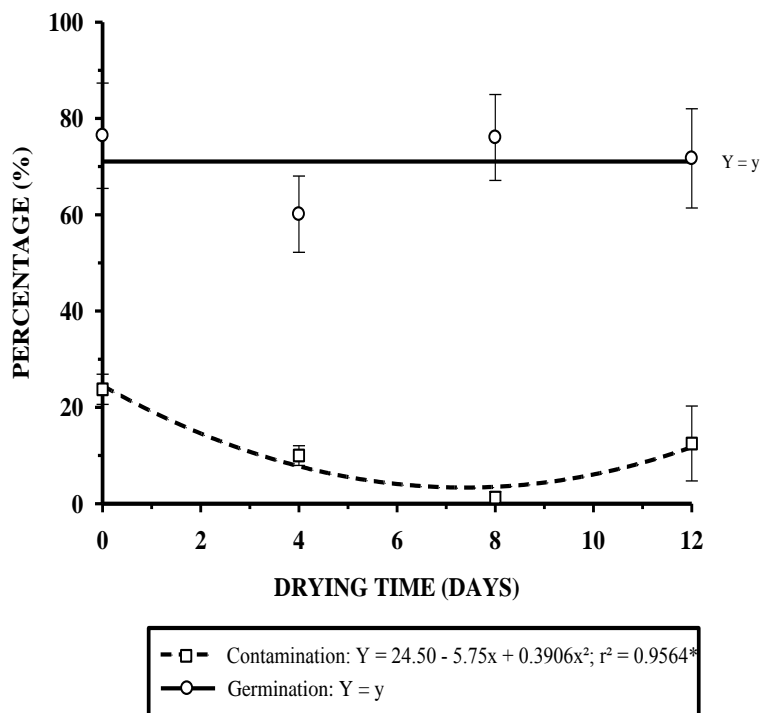


Figure 7. Germination and contamination by microorganisms (%) in zygotic embryos of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] extracted from fruits dried for different times at 37±2°C.

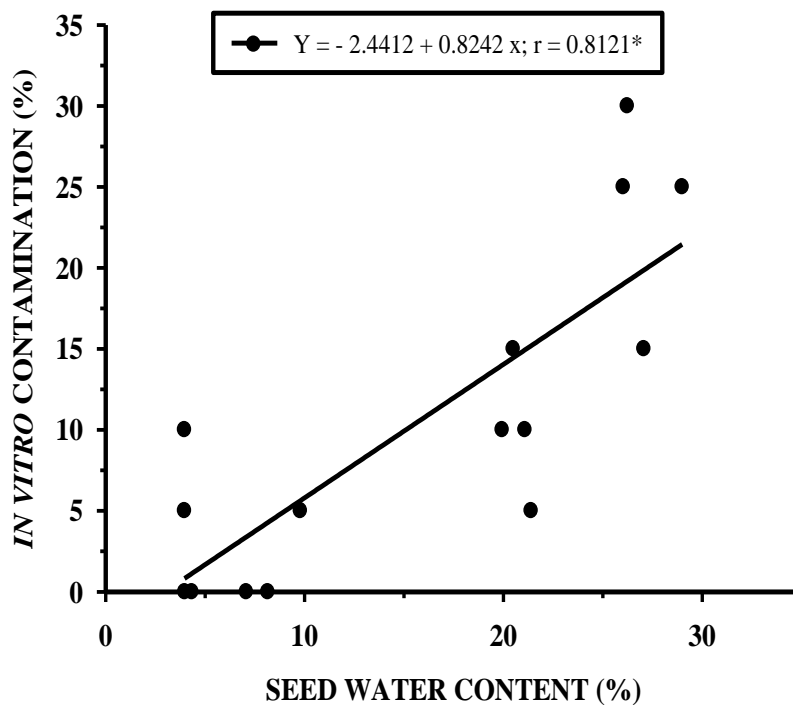


Figure 8. Correlation between *in vitro* contamination and water content of seeds of guariroba palm [*Syagrus oleracea* (Mart.) Becc.] extracted from fruits dried at 37±2°C for different times.

Neto, 2012 #50}.

Conclusions

Drying at $37\pm 2^{\circ}\text{C}$ for 8 days was effective for the extraction of *S. oleracea* embryos without being detrimental to embryo viability and *in vitro* germination, thereby indicating a possible orthodox behaviour of this species. *In vitro* establishment of *S. oleracea* via zygotic embryo culture can be optimised by drying the fruits at 37°C , as there was a decrease in contamination without decreasing embryo vigour. It was possible to estimate the seed water content without seed extraction by determining the fruit water content. The tetrazolium test, as modified for this species, in association with the germination test allows the evaluation of *S. oleracea* embryo viability.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Biological treatment of drinking water by chitosan based nanocomposites

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Nanotechnology is a promising interdisciplinary area that is likely to have wide ranging implications in all fields of science and technology. The rapid growth in nanotechnology has significant interest in the environmental application of nanomaterials. One of the latent applications of antimicrobial nanomaterials is their use in decentralized or point- of- use water treatment. The present study focuses on chitosan loaded nanoparticles and secondary metabolites (*Streptomyces* sp.) loaded chitosan nanoparticles which were synthesized by ionotropic gelation method. The synthesized nanoparticles were proven by antimicrobial activity test. Then the nanoparticles were coated on 4 micron membrane by dipping method. A membrane filtration technique is used for the treatment of water to remove or kill the bacteria from drinking water sample. The characterization of synthesized nanoparticles was done by dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR). The size of the chitosan loaded nanoparticles and secondary metabolites loaded chitosan nanoparticles were 164 and 177 nm, respectively and the zeta potential was highly stable and found to be 35 and 47 mV, respectively. The synthesized nanoparticles have a lot of surface areas contrasted to macro particles. They can be improved with a variety of reactor groups to raise their affinity to target compounds for removal of organic and inorganic pollutants from contaminated water. The quality of water is confirmed by membrane filtration method and multiple tube fermentation techniques.

Key words: Nanoparticles coated membrane, membrane filtration method, MPN techniques.

INTRODUCTION

Nanotechnology is an interesting field of science contributing materials showed designed features between atoms and large materials in the nano range (Patil et al., 2012; Ali, 2012). There is a need to develop new and powerful antibacterial agents because of increasing

concerns of bacterial infections. Predominantly, nanoparticles were used in different fields of human life like food preservation, burn dressings, safe cosmetics, medical devices and water treatment (Pant et al., 2011) and other range of products.

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The wide range of bio-application of nanoparticles is due to their tremendous antibacterial activity on a number of both Gram positive and negative bacteria (Li et al., 2011). The bactericidal effect of nanoparticles was confirmed by means of their size, shape, size distribution, morphology, surface functionalization and stability. Water is essential for the existence of life on earth. About 75% of earth is covered by water beyond which 97% is salt water and just 3% is available for drinking, agriculture, domestic and industrial consumption, at the same time as the rest is confined in oceans and underground reservoirs like salt water, polar ice caps, glaciers and ground water. Due to increase in industrialization and human population, demands for water supply have also been increased (Dara, 1998).

Water pollution is caused by toxic metals and microbial contamination that has a serious environmental and public health issue. Bacterial contamination of water persists to be an extensive problem across the country and is a major source of illness and deaths among 37.7 million affected by waterborne diseases annually in India. The most important pathogenic organisms responsible for water borne diseases in India are bacteria (*E. coli*, *Shigella*, and *V. cholera*), viruses (*Hepatitis A*, *Polio Virus*, *Rota Virus*) and parasites (*E histolytica*, *Giardia*, *Hook worm*). Water treatment is the exclusion of suspended and colloidal particles, organic matter, and microorganisms rather than other material that are harmful to health, looking for the lowest cost of consumption, process and maintenance, and reduced environmental impact to the surrounding region (Ali and Gupta, 2007; Libanius, 2008; Ali, 2010; Ali et al., 2012 and Ali, 2014).

Nanoscaled chitosan has latent drinking water disinfection application as an antimicrobial agent in membranes, sponges or outside coating of water storage tanks. It has greater reward than other disinfections since it has a higher antibacterial activity, a broad range of activity against bacteria, viruses and fungi and a lower toxicity to higher order animals and humans. On the other hand, the efficacy of microbial control depends upon the material preparation method and presence of organics. Chitosan act as an effective disinfectant only at acidic pH because of its solubility and the accessibility of charged amino groups (Kavanagh, 1972; Rabea et al., 2003). The preparation of water- soluble derivatives of chitosan could ultimately conquer this limitation.

Nanofiltration (NF) is a relatively modern membrane process used most frequently with low total dissolved solids (TDS) waters such as surface water and fresh groundwater, with the purpose of softening (polyvalent cation removal) and elimination of disinfection by-product precursors such as natural organic matter and synthetic organic matter. Along with the surface charged character, NF membranes can be separated into charged and neutral NF membranes, respectively. A number of researches recommended that have better permeation, separation and fouling resistant properties in charged NF membranes

than neutral NF membranes as they reject a solute not only by the steric hindrance effect, but as well as by the electrostatic repulsive effect. The present study focuses on the removal of bacteria from drinking water sample by using chitosan with bioactive compounds loaded (from *Streptomyces* sp.) nanocomposites coated membrane.

MATERIALS AND METHODS

Isolation of *Streptomyces* sp

The soil samples were collected from various agricultural areas which have been serially diluted up to 10^{-5} . The spread plate technique was followed and incubated at 28°C for seven days. The *Streptomyces* sp. isolates were maintained on starch casein agar slants.

Screening for antimicrobial activity by well diffusion method

The *Streptomyces* isolates were grown in starch casein broth at 28°C for 10 days and the activity were observed by well diffusion method. The culture filtrate was loaded into the wells which had been inoculated with test organism, *S. aureus*, *K. pneumonia*, *P. aeruginosa* and *E. coli* and incubated for overnight and measured the zone of inhibition (No et al., 2002).

Extraction of the secondary metabolites from culture supernatant using different solvents

The secondary metabolites from crude culture broth were extracted with different solvents like n-hexane, ethyl acetate, petroleum ether and chloroform. The solvents were proportionally mixed in the ratio of 1:1 and tested for the presence of secondary metabolites using well diffusion method (Augustine et al., 2005).

Thin layer chromatography

The secondary metabolites obtained after solvent extraction was spotted on silica gel. Thin-layer chromatography (TLC) plates were developed by using ethanol: water: chloroform (40: 40: 20) solvent system. The spotted TLC plates were exposed to iodine vapors for the development of spots concerning the presence of secondary metabolites.

Synthesis of chitosan loaded nanoparticles

Chitosan loaded nanoparticles were synthesized by ionotropic gelation method. Chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.3% (w/v) chitosan solution. Tripolyphosphate (TPP) was dissolved in Millipore water to a concentration of 1%. Chitosan loaded nanoparticles (CNP) were obtained by the 1 ml of TPP solution which was poured drop wise to the 5 ml chitosan solution. The solution was stirred for 15 min and then sonicated. The nanoparticles were collected by centrifugation at 9000 rpm for 40 min and freeze dried at -70°C for further analysis (Ali et al., 2010).

The secondary metabolites loaded chitosan nanoparticles (SCNP) were attained by adding a extract of secondary metabolites (obtained from *Streptomyces* spp.) to the nanoparticle suspension during chitosan nanoparticle synthesis earlier than centrifugation and stirred for 1 h. Then it was further purified as described above.

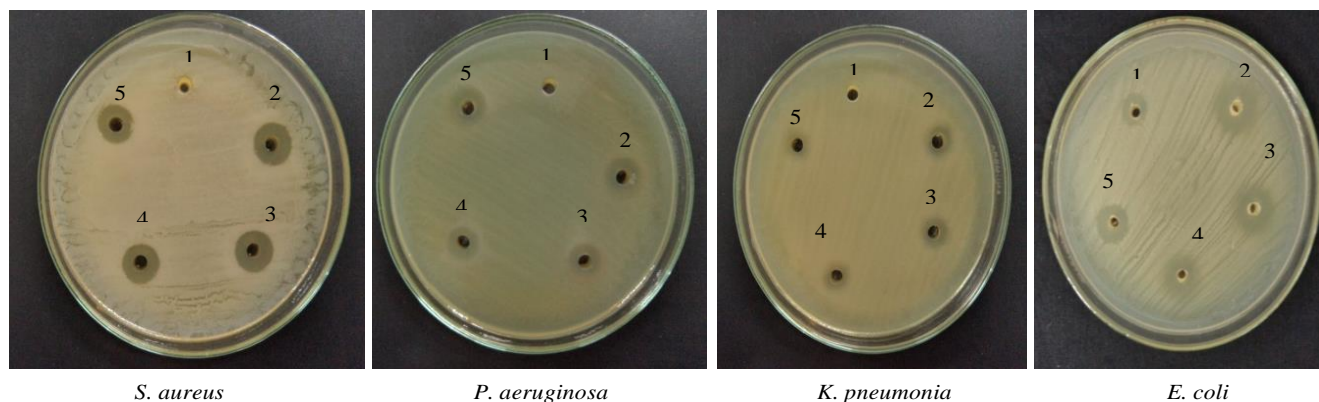


Figure 1. Antibacterial activity of *Streptomyces* sp. (1-10 µl, 2-20 µl, 3-30 µl, 4-50 µl and 5- control).

Table 1. Antibacterial activity of *Streptomyces* sp against various microorganisms by well diffusion method

Test organism	Zone of inhibition (mm)				
	10 µl	20 µl	30 µl	50 µl	Control
<i>S. aureus</i>	-	9	11	14	15
<i>P. aeruginosa</i>	-	8	10	12	13
<i>K. pneumonia</i>	-	9	11	13	10
<i>E. coli</i>	8	11	13	14	13

Characterization of nanoparticles

Dynamic light scattering (DLS)

The particle size and zeta potential for chitosan loaded nanoparticles (CNP) and secondary metabolites loaded chitosan nanoparticles (SCNP) were identified by using Zetasizer ver.620 (Malvern Instruments). The nanoparticles were performed on a scattering angle at 90° at a temperature of 25°C.

FTIR spectroscopy

FTIR spectroscopy is used to study the chemical characterization of the material. FTIR analyses were observed in the range of 400-4000 cm⁻¹ with the help of KBr pellets. The diverse mode of vibrations were identified and consigned to conclude the functional groups in the sample.

Nanoparticles coated on membrane

The synthesized chitosan loaded nanoparticles (CNP) and secondary metabolites loaded chitosan nanoparticles (SCNP) were coated on 4 micron membrane by dipping method. The membrane was placed in synthesized nanoparticles and kept in a rotary shaker at 110 rpm for 12 h. Then the coated membrane was kept in a sterile closed container for drying at room temperature.

Membrane filtration technique

The membrane filtration techniques provide a direct count of total coliforms and faecal coliforms in the water sample. This test was carried out in a flow through membrane filtration system on a CNP

coated membrane, SCNP coated membrane and nanoparticles non-coated membrane in a sterile condition. The water sample was collected in a sterile container for further analysis. It is clear that the permeability of nanoparticles coated membrane has slightly reduced contrast to non coated membrane. It is also observed that permeability reduced and removal of compound increased (Stafie, 2004).

Multiple tube fermentation (MPN) technique

The multiple tube fermentation technique had presumptive test, confirmed test and completed test was used to identify the presence of coliform in the filtered water sample (APHA, 1975).

RESULTS AND DISCUSSION

Isolation and screening of *Streptomyces* sp.

The study which was undertaken to "isolate and screen the *Streptomyces* sp. for secondary metabolites from various agricultural soil samples resulted in the following observations: the *Streptomyces* colonies were typically identified in the (10⁻³) dilution in starch casein agar (SCA). Out of the 20 *Streptomyces* sp. that were subjected to screening process, some of them showed antimicrobial activity against test organisms like *S. aureus*, *K. pneumonia*, *P. aeruginosa* and *E. coli* (Figure 1 and Table 1). The best activity showed *Streptomyces* sp. was chosen for extraction of secondary metabolites using different solvents.

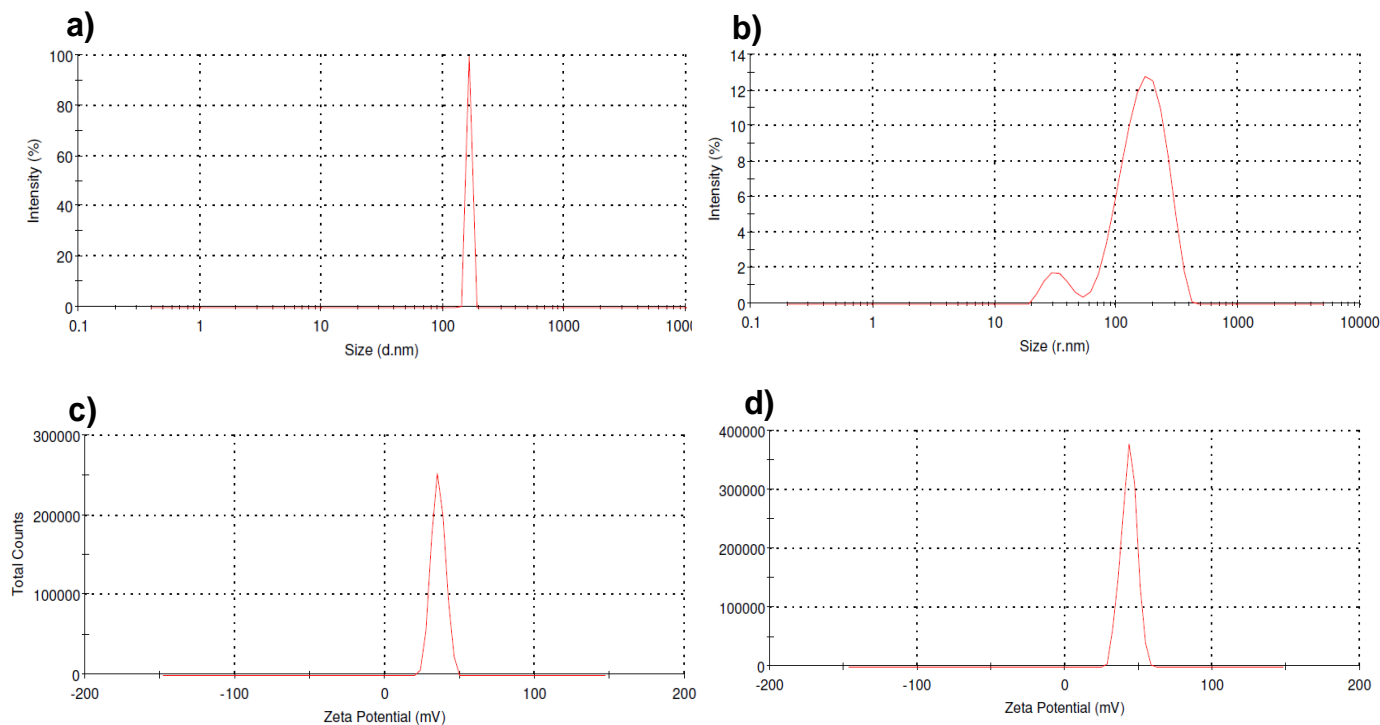


Figure 2. Size and zeta potential distribution of chitosan loaded nanoparticles (a & c) and secondary metabolites loaded chitosan nanoparticles (b & d).

Extraction of the secondary metabolites

The different solvents like chloroform, n-butanol, ethyl acetate and petroleum ether were used for the extraction of the secondary metabolites. The ethyl acetate extract showed higher antibacterial activity by well diffusion method and it was loaded into chitosan nanoparticles.

Thin layer chromatography

The partially purified secondary metabolites obtained after solvent extraction analyzed using pre-coated silica gel plates. The different solvent extracts were spotted on the TLC plate and exposed to iodine vapors for the development different bands. The different yellow color bands appeared at various R_f values of 0.68, 0.65, 0.72, 0.63, 0.52 of the partially purified product. The diverse range of R_f values were analytical of the association of secondary metabolites in the different solvent systems because of the variation in the solubility (Boer et al., 2005).

Characterization of nanoparticles

Dynamic light scattering

The size and zeta potential are the important features for

nanoparticles. Chitosan nanoparticles (CNP) and secondary metabolites loaded chitosan nanoparticles (SCNP) size were 164 and 177 nm, respectively (Figure 2a and 2b). The zeta potential of chitosan nanoparticles (CNP) was 35 mV and secondary metabolites loaded chitosan nanoparticles (SCNP) was 43 mV (Figure 2c and 2d). The zeta potential was improved radically as a result of loading the secondary metabolites. The zeta potential is an essential parameter for the identification of stability in nanoparticles. The nanoparticles was physically stabilized by electrostatic repulsion, the minimum value of zeta potential has ± 30 mV (Muller et al., 2001).

The zeta potentials were improved extensively due to the loading of secondary metabolites because the positive charge of amino groups in chitosan interacted with it. As a result of both chitosan loaded nanoparticles and secondary metabolites loaded, chitosan nanoparticles were very stable.

FTIR Spectroscopic analysis

The infrared spectra were recorded on Fourier Transform Spectrometer within the range ($400-4000\text{ cm}^{-1}$). The FTIR spectrums for chitosan loaded nanoparticles and secondary metabolites loaded nanoparticles are presented in Figure 3. The data presents a strong wide peak about $3600-3200\text{ cm}^{-1}$ that is related to hydrogen bounded O-H stretching vibration of alcohol and phenols at 3441 and

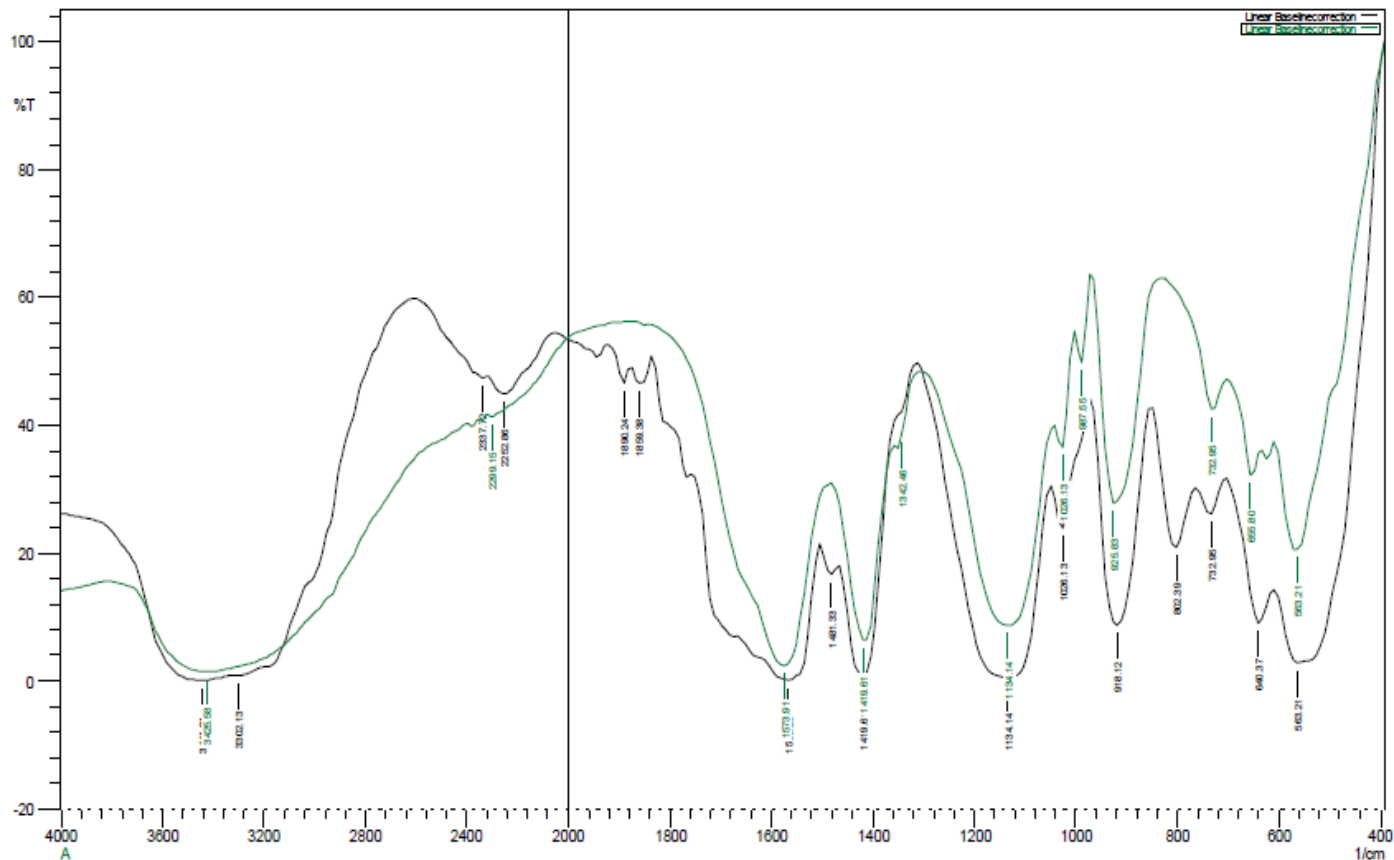


Figure 3. FTIR spectrum for chitosan loaded nanoparticles (black line) and secondary metabolites loaded nanoparticles (blue line). Black line: Chitosan loaded nanoparticles; Green line: Bioactive compound loaded nanocomposites.

3302 cm^{-1} shifted to 3425 cm^{-1} . Primary amine N-H stretching vibration has the same absorption in this region that overlap with O-H stretching vibration peak. The peak of stretching vibration for C-N in primary amine is observed in 1481 cm^{-1} . The peaks at 1566 cm^{-1} shifted to 1573 cm^{-1} belong to N-H bending vibration in primary amine and carbonyl group stretching vibration in amide type II, respectively. An asymmetric stretching vibration peak of C-O-C is observed in 1134 cm^{-1} on CNP and SCNP. The peaks in N-H bending vibration and carbonyl stretch in amide type II have 1419 and 1566 cm^{-1} shifted to 1419 and 1573 cm^{-1} , respectively. The bending of O-H bond was shifted from 918 to 925 cm^{-1} of carboxylic acid. The formation of nitro compounds with N-O stretching was observed at 1342 cm^{-1} and C-H bending of alkenes observed at 987 cm^{-1} in secondary metabolites loaded nanoparticles whereas, chitosan loaded nanoparticles contain no peak. The peak of 640 cm^{-1} was shifted to 655 cm^{-1} due to C-Br stretching of alkyl halides of secondary metabolites (Balozet, 1971; Radmanesh, 1990). Therefore, we converse that the ammonium groups of chitosan are linked with tripolyphosphoric groups of TPP. The intra and inter- molecular actions are improved in chitosan nanoparticles. In addition to this, the interaction

of chitosan and TPP to secondary metabolites to form a different some peak is also observed.

Membrane filtration technique

The filtered membrane was placed overturned on nutrient agar medium and incubated at 37°C for 24 h and observed the growth of colonies and it was counted directly. The chitosan loaded nanoparticles coated membrane hold the moderate amount of bacterial growth and secondary metabolites loaded chitosan nanoparticles has the highest amount of the bacterial growth and non coated membrane holds the less amount of bacterial growth. Mille et al. (2002) confirmed that the increase in external osmotic pressures to a bacterial cell, decline in cytoplasmic volume and as a result decrease in the cell volume can be seen.

Multiple tube fermentation (MPN) technique

The occurrence of coliforms was identified by MPN techniques. A large amount of positive combination and MPN index were attained from the non coated membrane

Table 2. MPN index for different combination of positive results while five tubes were used per dilution (10 ml, 1.0 ml, 0.1ml sample).

Types of membrane filtered water sample	5 /10 ml	5/1 ml	5/0.1 ml	No. of positive combination	MPN index
Non coated membrane filtered water sample	3	2	2	3-2-2	17
CNP coated membrane filtered water sample	2	1	1	2-1-1	9
SCNP coated membrane filtered water sample	1	0	1	0-1-1	4

filtered water sample. The SCNP coated membrane and CNP coated membrane showed maximum removal of coliforms identified by MPN techniques than non-coated membrane. The MPN index of the filtered water sample is shown in Table 2. The nanoparticles coated membrane has slightly charged surface because the pore size are less than individual sort of magnitude higher than the size of ions, charge interaction plays a dominant role (Rautenbach and Groschl, 1990). This effect can be used to remove the molecules.

Conclusion

Nanofiltration membranes (NF) are used in drinking water treatment or wastewater treatment. It was a low pressure membrane development with the intention of separate materials in micrometer range. It was a pressure-driven membranes with properties between those of reverse osmosis and ultra filtration membranes. It has the ability to remove turbidity, microorganisms and inorganic ions. They proved that they were very effective for removing bacteria from contaminated water. The *Streptomyces* sp. was isolated and screened for secondary metabolites by well diffusion method. The chitosan loaded nanoparticles and secondary metabolites loaded chitosan nanoparticles were successfully synthesized. They were characterized by DLS and FTIR and they proved the size and Zeta potential (stability) of the synthesized nanoparticles. Then the synthesized nanoparticles were coated on membrane by dipping method. The water sample is filtered through membrane filtration technique of nanoparticles coated membrane and non coated membrane and analysis by multiple tube fermentation (MPN) technique for the comparison of nanoparticles coated membrane and non coated membrane for the maximum removal of bacteria in the water sample.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

A biocoagulant slow sand filtration for disinfection of *Toxoplasma gondii* oocysts from Mezam River in Bamenda, Cameroon

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An integrated low-tech biocoagulant-sand filter drum for disinfection of oocysts of *Toxoplasma gondii* targeted for developing countries was evaluated. Dirty and turbid water (130.3 NTU) from Mezam River and leachates from dump sites and stagnant water in Bamenda, Cameroon, was analyzed microscopically after centrifugation for oocyst of *T. gondii*. Leachates from dump sites and stagnant water in Bamenda city had a very high concentration of oocyst of *T. gondii* too numerous to count per 10 ml while the major Mezam River had 50 oocysts per 10 ml. Considering that is widely used for various domestic chores; filtration disinfection of *T. gondii* was considered. A bench scale disinfection of oocysts of *T. gondii* with 0.2 g of powdered *Moringa oleifera* seeds per 10 ml of contaminated water from Mezam River showed a reduction of 50 oocysts per 10 ml to 10 oocysts in 15 min retention time. To optimize this, a pilot scale up was carried out using 120 L (120,000 ml) of water from Mezam River pretreated with 2400 g of powdered *M. oleifera* seeds for 15 min retention time and filtered through a sand filter drum made of fine sand, coarse sand, charcoal and gravel for 1 h filtration time. The total mean values of oocysts counts for 120 L of water to be filtered were 600,000 per 120,000 ml. The oocysts counts reduced to 10,000 after pretreatment with 2400 g of powdered seeds of *M. oleifera* and after a final filtration through a sand filter drum, no oocysts of *T. gondii* was detected in the final treated water. The findings from this study suggests strongly that the application of natural coagulants and sand filtration systems could serve a simple low cost disinfection for oocysts of *T. gondii* from water systems in resource limited countries.

Key words: *Toxoplasma gondii*, oocysts, disinfection, water, biocoagulant, moringa, turbidity, sand filter, drum, Cameroon.

INTRODUCTION

Toxoplasmosis is a widely distributed protozoan disease in Sub Saharan Africa, caused by *Toxoplasma gondii*. According to Dubey, the infective stages of *T. gondii* are capable of infecting a variety of vertebrates including humans (Dubey and Joes, 2008). Domestic and wild felids are capable of serving as definitive host and *T.*

gondii oocysts are excreted in their faeces (Ortis and Pinon, 2004). Toxoplasmosis is caused by ingesting *T. gondii* oocysts from contaminated water or foodstuff or by consuming *T. gondii* tissue cysts from infected hosts (Wallon et al., 1999). In Cameroon, and in most of Africa, many domestic animals are reared close to homes and

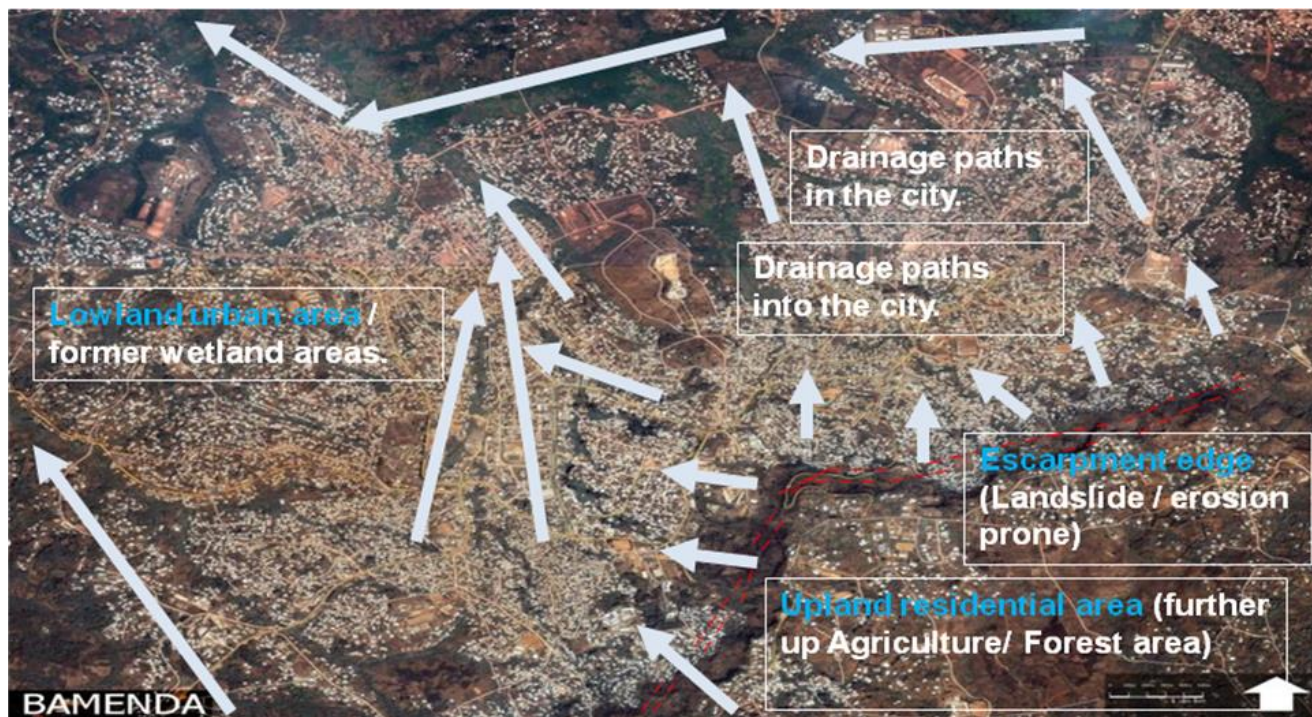


Figure 1. The map of Bamenda.

many cats stray in the wild, feed on and excrete on garbage as well as drink from surface water that are used by humans. Consequently, the impact of oocysts on toxoplasmosis epidemiology in Cameroon needs to be carefully studied because they are suspected to be associated with *T. gondii* seroprevalence in some emerging outbreaks of acute toxoplasmosis in humans and patients with compromised immune systems (De Moura et al., 2006; Yongabi, 2013). *T. gondii* oocysts are probably responsible for a significant part of infections in animals that are later consumed by humans (Sroka et al., 2006). The incidence of zoonotic toxoplasmosis in the Cameroonian population is high (Yongabi, 2013). Toxoplasma epidemiology in Cameroon has not been well reported. Less than 40% of the 22 million Cameroonians can afford clean and safe drinking water. Treated water still remains unaffordable for many Cameroonians. The treatment systems are expensive and not well implemented with poor plant management. The level of water and environmental pollution is increasing with increasing population amidst poor pollution management (Yongabi et al., 2011). The need to adopt simple sand filtration for water treatment is exigent. Slow sand filters have been observed as effective and lowcost (Yongabi et al., 2011). Furthermore, *Moringa oleifera*, a vegetable plant found across Africa

have been noted to possess coagulant activity and recommended for water purification (Yongabi et al., 2011). In routine microbial analyses of water in Sub Saharan Africa, the indicator organisms such as faecal coliforms have been applied. Such indicator organisms may not correlate well with the presence of oocysts in water bodies, thus rendering the reliance on this suspicious (WHO, 1984). Detection of *T. gondii* oocysts in environmental samples such as water and leachates in Cameroon and possible disinfection is crucial, as this coccidian parasite can be responsible for severe infections in humans and animals via ingestion of a single oocyst from contaminated water. In this paper, we report the potential use of *Moringa oleifera* and sand filter system to disinfect oocysts of *T. gondii* from surface water in Cameroon.

MATERIAL AND METHODS

Study site: Bamenda City Council

The study area was Bamenda metropolis and its environs in the North West Region of Cameroon. The map of the study area and description of the area is shown in Figure 1. Bamenda has three local councils under its municipal jurisdiction. Bamenda I, Bamenda II and Bamenda III. Approximately 85% of the inhabitants are

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Figure 2. River with garbage dumped into it.

subsistence farmers. The major development trends fall under, rehabilitating existing road network and creating new ones, reclaiming and developing wetlands and daily collection and disposal of garbage. Some of the greatest short-comings are the dilapidated state of roads and other networks; the poor state of habitations, especially in urban slums; the problem of waste management and drainage. Additionally, water and sanitation crisis, many homes do not have proper toilets. There is the haphazard digging of latrines and burials near habitations, with potentials to pollute the little underground water available. About 1,500 people are farming and grazing on the different watersheds of Age for and Bamendankwe and Akum, constituting the biggest threat to the forest, soil and water conservation in the area. The people at the foothills, plains and valleys (Mankon, Nkwen, Bamendankwe, Njah, Mbatu, Nsongwa, Chomba), estimated at more than 500,000, and the population of the Tubah Sub Division (Bambui, Kedjom, Sabga, Kedjom, Bamessing), estimated at more than 350,000, need the water from the watershed uphill. These areas constitute the slums of the city, with streams choked by forest and agricultural plants, and sanitary conditions are very poor, with malaria epidemics and periodic cases of cholera (Figures 2 and 3)

Collection of water and leachate samples and processing

Ten water sample from different points of the Mezam River, and ten leachate samples from waste dumps within the city of Bamenda were collected at different points located in the city. The samples were taken from places where cats, often excrete *T. gondii* oocysts. Samples were obtained according to the following procedure

described by Burns and Otterloo (1974); Ellis (1988); HACH (1990); APHA (1995) and Yongabi (2013a). A litre of the water and leachate were taken from the surface. Ten ml of each sample were placed in 10 ml test tubes for centrifugation.

Concentration technique and centrifugation

Concentration technique was used to detect *T. gondii* oocysts. This procedure included the concentration of 50 to 1000 oocysts per litre by flocculation or filtration, purification and detection of oocysts as described by Dubey and Jones (1988) and Pekzar et al, (1993). 10 ml of the water and leachate samples each were centrifuged at 300 rpm for 5 min; the supernatant were discarded and the deposit were examined directly under the microscope at x10 and x 40 magnifications after which stained films with mythelene blue were also examined microscopically (Villena et al., 2004)

Microscopic detection

The light microscopy was used. For the detection of unsporulated and sporulated oocysts, epifluorescence technique with UV light was used (excitation filter 330 to 385 nm, dichroic mirror 400 nm, barrier filter 420 nm). This facilitated the detection because both unsporulated and sporulated oocysts exhibit typical blue auto fluorescence (Yongabi, 2013; Yongabi, 2013b). The materials used in the construction of the sand filter were locally gotten at a river bed and included; 150 L carrying capacity drum (plastic), 1½ yards of hose, four clips, three nipples, strainer or sieve, sharp river sand



Figure 3. Subsurbs of Bamenda, a foot bridge with stray pig feeding close to a water fetching point.

(coarse and fine), charcoal and gravel. All these materials (sand, gravel and charcoal) were carefully washed and rinsed repeatedly in clean water (Yongabi, 2010). The laying of the materials in the drum was done in the order: laying of perforated hose connected to the collector tank, then a layer of gravel, followed by a layer of charcoal, then coarse sand (2 mm in size) and two layers of fine sand (0.15 to 0.30 mm size) on top. Ten litres of each of these materials were filled into the filtration drum. A test trial was carried out by flushing the set up repeated with clean water (Yongabi et al., 2011). The moringa pretreated water was then passed through the system. A hundred (100) gram of Moringa powder was sprinkled into 100 L of turbid water at a residence time of 25 min filtered using sack muslin cloths before pouring the filtrate into the sand packed drum. The final filtered water was collected in drum 2 and samples taken for analyses (Figure 4). Three water samples from Mezam River and leachates were collected and subjected to all these treatments. Mature seeds of *M. oleifera* were obtained from Maroua in the far north region of Cameroon. A stock of the powder was prepared and kept for use. A 2000 seeds were deshelled and pulverized in clean mortar using a pestle. The powder (from 2400 g) was sprinkled onto 120 L of the dirty pond water in a 150 L capacity drum (see picture) and stirred using a clean wood stirrer and the set up allowed to sit for 15 min retention time. It was then filtered off using a muslin sack cloth and the filtered water was then passed through a sand filter drum.

pH

The pH of the water samples before and after treatment with biocoagulants and sand filter was measured using a pH meter

model pH 1 to 125. The electrodes of the pH meter were standardized by calibrating in acidic and basic buffers raised on distilled water. The pH was taken by inserting the electrodes into test tubes containing wastewater samples and pH read off from the meter screen. The values obtained were consistent with values from HACH DR 2000.

RESULTS AND DISCUSSION

The result of the initial oocysts of *T. gondii* counts from Mezam River per 10 ml was 50 (Table 1). These counts per 10 ml suggest that *T. gondii* oocysts are very prevalent in environmental samples in Cameroon. It was also observed that the leachates from wastes dumped in and around Bamenda had very high oocysts counts per 10 ml, much more than in water sample from Mezam River. In previous studies, Dubey and Jones (2008) reported the presence of *T. gondii* oocyst from environmental samples in the United States. In this study, *T. gondii* oocysts were detected in Mezam River. Sroka et al. (2006) reported the occurrence of *T. gondii* in water from wells located on farms. In a related study in 2004, Ortis and Pinon also detected oocysts of *T. gondii* in water samples. Waste management in Cameroon and in Bamenda in particular is poorly managed with many domestic animals that stray around on the major street.



Figure 4. Sand filter drum. Drum 1 (left), sand filter media; drum 2 (right), collection /storage of filtered water.

Table 1. Physico-chemical and detection of Oocysts of *T. gondii* before filtration.

Parameter	Mezam River sample 1	Mezam River sample 2	Mezam River samples 3	Mean values (X)
Temperature (O°C)	27.0	26.0	27.1	25.0
PH	7.6	7.5	7.6	7.1
Turbidity (NTU)	130.3	130.1	130.3	130.2
Total solids mg/dm ³	466.0	466.0	466.0	466.0
<i>Toxoplasma gondii</i> , Oocyst counts (cfu/10 ml)	50	50	50	50

TNTC – Too Numerous To Count, * Water source, people use the water for other domestic chore, - Stray animals, cats, dogs and pigs drink from the source sporadic, - Analysis was done in the peak of rainy season (July to August, 2013 and 2014)

Cats in Cameroon are hardly confined and very common to find cat faeces as well as other faces of other animals on wastes dumped on the major streets. In Cameroon, rivers and streams are generally used for recreation as well as water fetched for household chores. The detection of *T. gondii* oocysts in Mezam River suggests that surface water must be checked for *T. gondii*. This pathogen poses a serious risk to pregnant women and

potential fetal transmission (Wallon et al., 1999; Cook et al., 2002). Slow sand filtration has been reported to be more than 99.99% efficient in removing pathogens from water. However, certain groups of organisms may still leached through the filter bad especially when poorly constructed (Yongabi et al., 2011). The efficacy of the slow sand filter systems on removal of *T. gondii* oocysts has not been previously reported.

Table 2. Physico-chemical and detection of *Toxoplasma gondii* oocyst after pretreatment with 0.2 g of *Moringa oleifera* (Lam) seed powder.

Parameter	Mezam River sample 1	Mezam River sample 2	Mezam River sample 3	Mean values (X)
Temperature	27.0	27.0	27.0	27.0
PH	7.0	7.0	7.0	7.0
Turbidity (NTU)	29.5	29.5	29.5	29.5
Total solids mg/ml	350	350	350.1	352.025
<i>Toxoplasma gondii</i> , Oocyst counts (cfu/10 ml)	10	10	10	10

Table 3. Physico-chemical and detection of *T. gondii* after purification with sand filter drum.

Parameter	Mezam River sample 1	Mezam River sample 2	Mezam River sample 3	Mean values (X)
Temperature (0°C)	25.1	25.1	25.1	25.1
PH	7.4	7.4	7.4	7.4
Turbidity (NTU)	12	13	13	12.5
<i>Toxoplasma gondii</i> , Oocyst counts (cfu/120,000 ml)120 L 600,000 Oocysts detected	nil	nil	nil	nil

The results from this study also showed conclusively that combining *M. oleifera* seeds powder on to slow sand filter system potentially reduced drastically oocysts of *T. gondii* from the contaminated water (Tables 2 and 3). Yongabi et al. (2011) reported the beneficial effects of *M. oleifera* extracts in disinfection of bacterial contamination of water. The toxic effects of plant extracts such as *M. oleifera* on *T. gondii* have not been previously reported. The efficacy of *M. oleifera* and slow sand filter systems in the removal of other pathogens has been observed (Eilert, 1978; Pollard et al., 1995; Yongabi, 2010). With the sand filter system, very dirty water for consumption can be recovered. Although, coliforms may be absent after treatment, a few oocyst of *T. gondii* may be present. The combined effects of a *M. oleifera* hybrid sand filter drum demonstrated 100% disinfection of oocyst of *T. gondii*. This observation is very important in that the materials are usually low cost and readily available in Africa. Additionally, information available on the prevalence of *T. gondii* oocysts in the environment in most African countries are lacking. This observation has been reported elsewhere. Very little information is available on the presence of *T. gondii* oocysts in naturally contaminated water (Villena et al., 2004; De Moura et al., 2006; Sroka et al., 2006; Villena et al., 2004; Sroka et al., 2006; Vaudaux et al., 2011). However, soil is also an environmental source of Toxoplasmosis in humans (Teutsch et al., 1979; Stagno et al., 1980; Weigel et al., 1999). It was indicated that pregnant women mostly get infection from soil (Cook et al., 2002). The conclusion made is that *T. gondii* infection can be prevented in Cameroon and in Africa at large through appropriate filtration methods using low cost materials.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Isolation and characterization of culturable bacteria from bulk soil samples and the rhizosphere of arid-adapted *Tylosema esculentum* (Burchell). A. Schreiber (Marama bean) in Namibia

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Plant growth promoting (PGP) bacteria are microorganisms living in association with plants. PGP bacteria have various physiological activities that they perform which are beneficial to plants. For example, phosphate solubilisation, nitrogen fixation, phytostimulation and formation of siderophores. The aim of this investigation was to determine the diversity of PGP bacteria and to characterise them for possible promotion of plant growth from the rhizosphere of *Tylosema esculentum*, a nutritious arid adapted legume. For that purpose, in this study, bacteria were isolated from marama bean rhizosphere and bulk soil. The bacteria were screened for their ability to solubilise phosphates, for aminocyclopropane-1-carboxylate (ACC) deaminase activity, production of catalase, hydrogen cyanide, ammonia and protease activity. Efficiency of phosphate solubilising activity by bacteria was determined by phosphate solubilisation index. DNA was extracted from bacterial cultures and used to obtain 16S rDNA amplicons for bacterial molecular identification. A total of eight bacterial strains were isolated from the rhizosphere and 19 strains from bulk soil with potential plant growth promoting traits. The 27 bacterial isolates showed phosphate solubilising activity and five of the isolates had a solubilisation index of at least 6. A total of 23 isolates showed ACC deaminase activity. Hydrogen cyanide was produced by 16 isolates; 26 isolates had catalase activity, 23 isolates showed protease activity and all the isolates produced ammonia. The identified genera include *Bacillus*, *Raoultella*, *Klebsiella*, *Acinetobacter*, *Arthrobacter*, *Kosakonia* and *Burkholderia*. From this study, we conclude that there is a community of bacteria living in *T. esculentum* rhizosphere and proposed that in future these native bacterial strains can be used as biofertilisers for this arid agro-ecological area after verifying their suitability for inoculum development.

Key words: Rhizobacteria, plant growth promoting bacteria, *Kosakonia*, *Burkholderia*.

INTRODUCTION

Tylosema esculentum (marama bean) is a long-lived perennial, non-nodulation and non-nitrogen fixing legume native to arid areas of Southern Africa in the Kalahari sandy regions. It produces a raceme up to 25 mm long,

containing many yellow-orange flowers and it has circular pods with large brownish-black oil and protein-rich seeds (Holse et al., 2010). It has an underground water storage tuberous root that can grow very large to at least 20 kg.

The tuberous root enables it to thrive in environments with high temperatures (typical daily maximum of 37°C in the growing season), low rainfall (50 to 500 mm) and long periods of drought (Jackson et al., 2011). The edible and nutritious seeds from marama bean are underutilized as food, but constitute a part of the traditional diet for the San people and other indigenous groups in southern Africa (Jackson et al., 2011). The marama beans are gathered from the wild and are mostly eaten as a snack after roasting in hot sand (Jackson et al., 2011). The seeds have a high lipid and protein content (Holse et al., 2010) which gives them a socio-economic value. Besides the high nutritional value of the roasted seeds, the marama bean also has potential as a source of oil production and other healthy food products such as marama milk and defatted marama flour (Jackson et al., 2011). Hence, this neglected legume may be applied in food systems and has a potential to improve both human nutrition and increase food availability in arid ecological zones.

It is intriguing to imagine where this plant could be getting nutrients to survive and thrive in a soil environment which is nutrient deficient especially nitrogen-poor, phosphorous-poor and also dry. For a long time, plant growth promoting (PGP) bacteria have been proposed to provide these nutrients to plants in a symbiotic relationship (Bulgarelli et al., 2013). However, such studies have been neglected for the nutritionally-rich marama bean. PGP bacteria are a group of bacteria that live in association with plants, while enhancing and stimulating plant growth and development using various mechanisms (Banik and Dey, 1983; Bulgarelli et al., 2013; Ali et al., 2014). Microorganisms can solubilise insoluble phosphates while maintaining a high quality and healthy soil (Richardson, 2001). They use several direct and indirect mechanisms of action to improve plant growth and health. These mechanisms can be active simultaneously or independently at different stages of plant growth. Direct mechanisms such as phosphate solubilisation (Kim et al., 1998) and indirect methods include hydrogen cyanide, catalase production and aminocyclopropane-1-carboxylate (ACC) deaminase activity and formation of siderophores (Penrose and Glick, 2002; Grönemeyer et al., 2012; Bulgarelli et al., 2013; Ali et al., 2014). Phosphate solubilisation provides phosphorus which is an essential nutrient to plants. It is involved in several key plant functions, including energy transfer, photosynthesis, transformation of sugars and starch, nutrient movement within the plant and transfer of genetic characteristics from one generation to the next (Kim et al., 1998). Similarly, ACC deaminase is an enzyme

that enhances seed emergence, promotes root elongation, lowers ethylene levels and enhances plant growth (Leidi and Rodriguez-Navarro, 2000; Penrose and Glick, 2002; Bulgarelli et al., 2013). Phosphorous deficiency is a major limiting factor to plant growth, as well as crop production. Plants can only absorb inorganic phosphorus, provided that it is soluble. Most phosphorus added as fertiliser becomes insoluble and thus unavailable to plants. It accumulates due to excessive use of chemical fertilisers, resulting in soil contamination with lethal consequences to beneficial microbes, poor growth rate and low crop production. Large proportions of added fertilisers are converted to the insoluble form, becoming unavailable to plant uptake and accumulates in the soil (Rodriguez and Fraga, 1999; Borch et al., 1999).

The phosphorus content in soil is usually much higher than plant requirements, however, bioavailability of phosphorus to plant is one of the major plant growth limiting constrains. Thus, there is direct need to mobilize this big pool of soil phosphorus to improve crop yields on a sustainable basis and one of the strategies useful for this purpose is the use of specific microorganisms applied in biofertiliser inoculants. Some PGB bacteria are known to promote growth of plants by solubilizing these unavailable/insoluble phosphates in soil while others enhance phosphorus acquisition by plants indirectly through promoting extensive root growth because of their ACC-deaminase activity (Higa and Wididana, 1991; Arshad and Frankenberger, 2002; Dudeja and Giri, 2014).

Bacterial ACC deaminase plays a significant role in the regulation of a plant hormone, ethylene, and thus, enhances the growth and development of plants. Low levels of ethylene like 10 µg l⁻¹ have been found to enhance root initiation and growth while the higher levels like 25 µg l⁻¹ may lead to inhibition of root growth (Mattoo and Suttle, 1991). Bacterial strains with ACC-deaminase activity can at least partially eliminate the stress-induced ethylene-mediated negative impact on plants by converting the germinating seed/roots ACC into α-ketobutyrate and ammonia (Glick et al., 1998; Dudeja and Giri, 2014). Plants grown under natural soil conditions are generally exposed to environmental stresses and more ethylene is produced by the plant in response to various kinds of stress.

Microbial inoculants as biofertilisers promote plant growth, crop production, increase the nutrient status of the plants, maintain a healthy environment and have been accepted worldwide as an alternative source for chemical fertilizers (Vessey, 2003; Dudeja and Giri, 2014). The use of microorganisms to improve plant growth is increasing every year in various parts of the

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Abbreviation: PGP, Plant growth promoting.

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world. Plant growth promoting bacteria affect both growth and development of plants by direct and indirect mechanisms. Indirectly, the bacteria may exert positive influence on plant growth by lessening certain deleterious effects of pathogenic organisms by inducing host resistance to the pathogen or by knocking out the pathogen from root surfaces or producing chitinases or other pathogen suppressing substances. Although, scientists have reported both direct and indirect ways of growth stimulation by plant growth promoting bacteria, there is no clear distinction. A bacterium influencing plant growth by regulating synthesis of plant hormones can also play a role in controlling plant pathogens and diseases and vice versa (Gray and Smith, 2005). The aim of this study was to examine the diversity of rhizospheric bacteria in the arid-adapted and non-nitrogen fixing legume, *T. esculentum* and to screen for potential phosphate solubilizing bacteria and/or ACC deaminase activity associated with marama bean.

MATERIALS AND METHODS

Sample collection

The rhizospheric soils from five uprooted 2-year old plants and five bulk soil samples randomly from marama growing field were collected from the Omaheke and Otjozondjupa regions which are the native lands for *T. esculentum* in Namibia. Samples were randomly collected and carefully labelled from sites located at Okatumba, Ehungiro, African Wild Dog Conservancy and Okahamupurunga. For rhizospheric soils, the roots were carefully washed in distilled water to collect all the soil tightly attached to the roots, and for the bulk soil samples, a 500 g sample was carefully dug out from top soil of random locations of marama growing fields. The samples were put in zip lock bags, wrapped in foil paper and transported to the laboratory in liquid nitrogen. In the laboratory, samples were immediately processed for culturing bacteria.

Isolation of bacteria

Each bulk soil sample, 1 g was suspended in 9 ml of double distilled water containing 0.1 M phosphate buffered saline (PBS) buffer and vortexed for 2 min, and for rhizospheric soil samples a similar treatment was done but to 1 ml of soil solution. The resulting suspensions were serially diluted to 10^{-10} . Each dilution (0.1 ml) was spread on pre-solidified tryptone soy agar (TSA) plates. Growth was monitored on a daily basis and a single colony was sub-cultured onto TSA plates. Sub-culturing was done until pure colonies were obtained. All the subsequent *in vitro* plate assay analyses were done in triplicate with positive and negative controls.

Screening of phosphate solubilising bacteria

All the isolated pure culture strains were screened for their ability to solubilise insoluble phosphates by an agar assay. The National Botanical Research Institute's phosphate (NBRIP) medium, supplemented with 15 g of bacteriological agar was used (Nautiyal, 1999). The NBRIP growth medium contained in grams per litre: glucose (10 g), $\text{Ca}_3(\text{PO}_4)_2$ (5 g), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g), KCl (0.2 g) and $(\text{NH}_4)_2\text{SO}_4$ (0.1 g) (Nautiyal, 1999). One strain was stabbed four times on a plate using sterile loops. The

plates were incubated at 29°C for 14 days. The colony and halo diameters were measured. Solubilisation index was used to determine the strains abilities to solubilise insoluble phosphates. Solubilisation index is the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi Premono et al., 1996).

Production of plant growth promoting enzymes and hormones

Aminocyclopropane-1-carboxylate (ACC) deaminase

The ACC deaminase activity was determined using Dworkin and Foster (DF) minimal salts medium containing ACC as sole nitrogen source (Dworkin and Foster, 1958). The composition of the solidified salt minimal media containing ACC as sole nitrogen source in grams per litre (sterile distilled water) was as follows: agar (10 g), KH_2PO_2 (1.36 g), Na_2HPO_4 (2.13 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.7 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.04 g), $\text{MnSO}_2 \cdot \text{H}_2\text{O}$ (0.02 g), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02 g), H_3BO_3 (0.003 g), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.007 g), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.004 g), substrate ACC (5 mM), and glucose (10 g). Strains were inoculated using the streaking method. The plates were incubated at 29°C and growth was monitored daily for three days.

Catalase and protease activity

The catalase test was performed to study the presence of the catalase enzyme in the phosphate solubilising bacteria. A drop of 3% hydrogen peroxide was added to a colony on a sterile glass slide and mixed well using a sterile loop. The effervescence indicated catalase activity. The protease activity was determined using skim milk agar medium, which contained per litre: pancreatic digest of casein (5 g), yeast extract (2.5 g), glucose (1 g), 7% skim milk solution and bacteriological agar (15 g). Bacterial cultures were spot inoculated and incubated for 48 h at 29°C. Clear zones around the cultures indicated protease activity (Smibert and Krieg, 1994).

HCN and ammonia production

The bacterial isolates were screened for hydrogen cyanide (HCN) production (Castric, 1975). The bacterial cell cultures were streaked on nutrient agar medium, which contained 4.4 g per litre of glycine. A Whatman filter paper was soaked in 0.5% picric acid solution and it was placed inside the lid of the plate. The plates were sealed with parafilm and incubated at 29°C for four days. The appearance of a light brown to dark brown colour of the filter paper indicated HCN production. The bacterial isolates were tested for the production of ammonia (Cappuccino and Sherman, 1992). Bacterial cultures were inoculated in 10 ml peptone broth and incubated at 29°C for 48 h. Shaking using a shaker at 1000 rpm for 1 h was done daily. After incubation, 0.5 ml of Nessler's reagent (0.09 mol l^{-1} solution of potassium tetraiodomercurate (II) ($\text{K}_2[\text{HgI}_4]$) in 2.5 mol l^{-1} potassium hydroxide) was added. The development of faint yellow to dark brown colour indicated the production of ammonia.

Molecular identification of bacterial strains

DNA extraction 16S rRNA gene amplification and sequencing analysis

The DNA of each isolate was extracted from fresh broth cultures grown for 24 h using the Zymo Research ZR Soil Microbe DNA MiniPrep™ Catalog No. D6001 extraction kit. The protocol followed was enclosed in the instruction manual of the manufacturer (Zymo Research, California, USA) <http://www.zymoresearch.com>. Purified

Table 1. Description of the bacterial isolates.

Sample	Type of soil	No. of isolates	Isolate designation code
1	Bulk*	Three	BP1, BP2, BP3
2	Bulk	Two	BP4, BP5
3	Bulk	Eight	BP6, BP7, BP8, BP9, BP10, BP11, BP12, BP13
4	Bulk	Three	BP14, BP15, BP16
5	Bulk	Three	BP17, BP18, BP19
6	Rhizosphere	Two	RP1, RP2
7	Rhizosphere	Two	RP3, RP4
8	Rhizosphere	Two	RP5, RP6
9	Rhizosphere	One	RP7
10	Rhizosphere	One	RP8

*soil obtained in the marama bean growing fields. BP means bulk soil portion, RP means rhizosphere soil portion.

DNA quality was confirmed by electrophoresis on a 1% agarose gel stained with 5 µl of 10 mg/ml ethidium bromide and visualised under ultraviolet light. Amplification of the 16S rDNA was carried out using the universal primers Bac8uf (5'-AGAGTTTGTATNHTGGYTACG-3') and Univ1492r (5'-GGNTCCTTGTTACGACTT-3') as reported in Grönemeyer et al. (2012). The 50 µl amplification mixture consisted of: water (33.5 µl), 10xDream Taq buffer (5 µl), 500 mM dNTPs (10 µl), 500 nM of each primer, DNA template (1 µl) and 1 unit of MolTaq polymerase (Molzym, Germany). The amplification profile was carried out with an initial denaturation at 95°C (4 min) followed by 35 cycles of denaturation at 95°C (1 min), annealing at 50°C (30 s), extension at 72°C (1 min) and a final extension at 72°C (10 min) in a thermocycler (BioRad, England). Sequencing of amplicons was done at Inqaba Biotech (Pretoria, South Africa) with the primers Bac8uf and Univ1492r. The quality of the sequences obtained was assessed by eye using BioEdit. The sequences were used to do similarity searches with BLAST in the National Centre of Biotechnology Information (NCBI) genbank (<http://blast.ncbi.nlm.nih.gov>).

RESULTS

A total of 27 bacterial strains were isolated from a total of five rhizospheric soil samples associated with the marama bean plants and five bulk soil samples (Table 1). All the isolates were given designation codes as shown in Table 1. The characterisation assays revealed that these strains had various plant growth promoting activities including, phosphate solubilisation, production of ACC deaminase and protease activity. The partial 16S rDNA sequencing managed to determine the identity of the isolated bacteria. The isolates were identified as belonging to seven genera namely *Bacillus*, *Raoultella*, *Klebsiella*, *Acinetobacter*, *Arthrobacter*, *Kosakonia* and *Burkholderia*. Some of the well-known PGP bacterial genera like *Rhizobium*, *Azospirillum* and *Herbaspirillum* could not be isolated, suggesting the possible need to use alternative media to isolate them.

Isolation and screening of phosphate solubilizing bacteria on agar assay

All the isolates formed halozones (data not shown)

around the colonies which was a characteristic signature of phosphate solubilising activity. The halos indicated the solubilisation of the phosphate source used in the media, which was tri calcium phosphate (Gaur, 1990). Isolates BP18 (*A. calcoaceticus*), BP6 (*Acinetobacter calcoaceticus*) and BP7 (*Klebsiella oxytoca*) showed the highest phosphate solubilising activity with solubilisation indices of 10, 11 and 12, respectively (Table 2). Isolates BP16 and BP17 (both *A. calcoaceticus*) showed good phosphate solubilising activity with a solubilisation index of 6 each. Isolate BP13 (*Kosakonia ludwigii*) had the lowest solubilisation activity with a solubilisation index of 2.07. The solubilisation index is directly proportional to the solubilisation activity. Isolates with high solubilisation indices are good candidates to be included in the design of biofertilizer inoculants.

Production of plant growth promoting enzymes

ACC deaminase activity

The ACC deaminase activity of the isolates was observed in 23 isolates (85% of the total isolates) (Table 3). Only four isolates were negative for the production of ACC deaminase. These were identified to be closest to *Arthrobacter mysorens*, 2 *K. oxytoca* isolates and *Burkholderia ferrariae*. It was, however, observed that some isolates of *K. oxytoca* were ACC deaminase positive. It was observed that most of the isolates of *Klebsiella* were positive for protease activity. The PGP characteristics displayed by isolates closest to *K. oxytoca* are clearly indicative for the need to use another gene region to decipher conclusively the species identity of the 10 isolates since 16S rDNA may not have conclusively resolved their identity. The possibility of new species cannot be ruled out.

Catalase and protease activity

It was observed that 96% of the isolates were catalase

Table 2. Phosphate solubilisation activity of the bacterial isolates.

Isolate	*Name of bacterial species closest to the isolate	Colony diameter (cm)	Halozone diameter (cm)	Solubilisation index
BP1	<i>Bacillus megaterium</i>	0.4	0.6	2.5
BP2	<i>Raoultella ornithinolytica</i>	0.7	1.2	2.7
BP3	<i>Klebsiella oxytoca</i>	0.6	1.2	3
BP4	<i>Klebsiella oxytoca</i>	0.6	1.0	2.67
BP5	<i>Klebsiella oxytoca</i>	0.6	1.0	2.67
BP6	<i>Acinetobacter calcoaceticus</i>	0.1	1.0	11
BP7	<i>Klebsiella oxytoca</i>	0.1	1.1	12
BP8	<i>Acinetobacter oleivorans</i>	0.7	1.1	2.57
BP9	<i>Arthrobacter mysorens</i>	0.4	0.5	2.25
BP10	<i>Kosakonia cloacae</i>	0.6	1.2	3
BP11	<i>Bacillus anthracis</i>	0.3	0.5	2.67
BP12	<i>Klebsiella oxytoca</i>	0.6	0.8	2.33
BP13	<i>Kosakonia ludwigii</i>	1.4	1.5	2.07
BP14	<i>Acinetobacter oleivorans</i>	0.3	1.0	4.33
BP15	<i>Klebsiella oxytoca</i>	0.2	0.5	3.5
BP16	<i>Acinetobacter calcoaceticus</i>	0.2	1.0	6
BP17	<i>Acinetobacter calcoaceticus</i>	0.2	1.0	6
BP18	<i>Acinetobacter calcoaceticus</i>	0.1	0.9	10
BP19	<i>Kosakonia ludwigii</i>	0.4	1.0	3.5
RP1	<i>Klebsiella oxytoca</i>	0.9	1.3	2.44
RP2	<i>Klebsiella oxytoca</i>	0.3	0.6	3
RP3	<i>Raoultella ornithinolytica</i>	0.8	1.0	2.25
RP4	<i>Bacillus megaterium</i>	0.8	1.1	2.38
RP5	<i>Bacillus pumilus</i>	1.0	1.1	2.1
RP6	<i>Klebsiella oxytoca</i>	0.4	0.6	2.5
RP7	<i>Burkholderia ferrariae</i>	0.7	1.2	2.71
RP8	<i>Klebsiella oxytoca</i>	0.1	0.3	4

*Some of the isolates were designated the same names by 16S rDNA sequence since the initial selection was done based on colony morphology.

Table 3. Plant growth promoting activity of the bacterial isolates.

Isolate	Closest species assigned by 16SrDNA analysis	ACC deaminase activity	Catalase test	Hydrogen cyanide test	Ammonia test	Protease test
BP1	<i>Bacillus megaterium</i>	+	+	-	+	-
BP2	<i>Raoultella ornithinolytica</i>	+	+	-	+	+
BP3	<i>Klebsiella oxytoca</i>	+	+	-	+	-
BP4	<i>Klebsiella oxytoca</i>	+	+	+	+	+
BP5	<i>Klebsiella oxytoca</i>	+	+	+	+	+
BP6	<i>Acinetobacter calcoaceticus</i>	+	+	-	+	+
BP7	<i>Klebsiella oxytoca</i>	+	+	+	+	+
BP8	<i>Acinetobacter oleivorans</i>	+	+	-	+	+
BP9	<i>Arthrobacter mysorens</i>	-	+	+	+	+
BP10	<i>Kosakonia cloacae</i>	+	+	+	+	+
BP11	<i>Bacillus anthracis</i>	+	+	-	+	+
BP12	<i>Klebsiella oxytoca</i>	+	+	-	+	+
BP13	<i>Kosakonia ludwigii</i>	+	+	+	+	-
BP14	<i>Acinetobacter oleivorans</i>	+	+	+	+	+
BP15	<i>Klebsiella oxytoca</i>	+	+	+	+	+

Table 3. Contd

BP16	<i>Acinetobacter calcoaceticus</i>	+	+	+	+	+
BP17	<i>Acinetobacter calcoaceticus</i>	+	+	-	+	+
BP18	<i>Acinetobacter calcoaceticus</i>	+	+	-	+	+
BP19	<i>Kosakonia ludwigii</i>	+	+	+	+	+
RP1	<i>Klebsiella oxytoca</i>	+	+	-	+	+
RP2	<i>Klebsiella oxytoca</i>	-	-	+	+	+
RP3	<i>Raoultella ornithinolytica</i>	+	+	+	+	+
RP4	<i>Bacillus megaterium</i>	+	+	+	+	+
RP5	<i>Bacillus pumilus</i>	+	+	+	+	+
RP6	<i>Klebsiella oxytoca</i>	-	+	+	+	+
RP7	<i>Burkholderia ferrariae</i>	-	+	-	+	-
RP8	<i>Klebsiella oxytoca</i>	+	+	+	+	+
Total (+)		23 (85%)	26 (96%)	16 (59%)	27 (100%)	23 (85%)

- Stands for negative test result and + stands for positive test result.

positive (Table 3). Only *K. oxytoca* isolate RP2 was catalase negative but some other *K. oxytoca* were verified to be catalase positive. Of the 27 isolates, 23(85%) of the isolates showed proteolytic activity. The four isolates that were negative for protease activity were identified as closest to: *Bacillus megaterium*, *K. oxytoca*, *K. ludwigii* and *B. ferrariae*.

HCN and ammonia production

It was observed that 59% of the isolates produced hydrogen cyanide. The 11 isolates did not have HCN production activity included isolates of: *B. megaterium*, *Raoultella ornithinolytica*, *K. oxytoca*, *Bacillus anthracis*, *A. calcoaceticus* and *B. ferrariae*. Again, some isolates of *Klebsiella* were observed to be negative for HCN (Table 3). All the isolates tested positive for ammonia production (Table 3).

Molecular identification of bacterial isolates using 16S rRNA gene sequence

A total of 27 16S rRNA gene amplicons were sequenced. The sequences were inspected by eye and edited using BioEdit, and then using the BLAST on the NCBI genbank at nucleotide level. The identity (percentages of similarity) of the isolates closest to the known species in the rDNA database is presented in Table 4. Some of the isolates were repeated because the initial selection was based on colony morphology observation, which was not specific. When considering the proportion of the isolates, *K. oxytoca* isolates had the highest frequency (37%) followed by the *Acinetobacter oleivorans* (15%) (Table 4). The least abundant species were *R. ornithinolytica*, *A. calcoaceticus*, *A. mysorens*, *Kosakonia cloacae*, *B. anthracis*, *Bacillus pumilus* and *B. ferrariae* at 3.7% each

(Table 4). Some of the well-known PGP genera such as *Burkholderia* and *Kosakonia* were isolated.

DISCUSSION

In this paper, microbial analysis of the *T. esculentum* rhizosphere and also bulk soil revealed a considerable diversity of bacterial community with plant growth promoting characteristics. The study is cautious to note that this is only a portion of the actual diversity present as it is excluding the unculturable bacteria as well as those that could be cultured with different complex and enrichment media or altered atmospheric conditions. In this study, 27 strains were identified using colony morphology and characterized to species level by 16S rDNA sequencing. This paper serves as basis for future prospects and acts as a point of reference for future research in understanding plant –microbe interactions of this non-nodule forming marama bean. Regarding ACC deaminase activity, 23 isolates showed ACC deaminase activity. The observation is consistent with the report of Glick et al. (1998) and Glick (2005) who demonstrated that ACC deaminase activity enhances seed emergence, promotes root elongation, lowers ethylene levels and enhance plant growth a characteristic that could be of benefit to marama bean evolutionarily. In this study, 23 isolates showed proteases activity. Protease is fungal cell wall degrading enzymes, which can offer protection of marama bean against mycological pathogens that may encroach its proximity. In this study it was observed that *K. oxytoca*, *A. calcoaceticus*, *K. cloacae*, *K. ludwigii*, *R. ornithinolytica*, *B. megaterium* and *B. pumilus* showed more than one plant growth promoting characteristic. They solubilized insoluble phosphates, displayed ACC deaminase activity, produced ammonia, hydrogen cyanide, catalase and protease. With these results, PGP bacteria possessing simultaneously both phosphate

Table 4. Molecular identification of isolates to closest strain in the NCBI genbank based on sequence similarity.

Isolate	Strain identification*	NCBI Accession number	Percentage similarity (%)
BP1	<i>Bacillus megaterium</i>	NR_043401.1	99
BP2	<i>Raoultella ornithinolytica</i>	NR_102983.1	99
BP3	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP4	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP5	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP6	<i>Acinetobacter calcoaceticus</i>	NR_042387.1	99
BP7	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP8	<i>Acinetobacter oleivorans</i>	NR_102814.1	100
BP9	<i>Arthrobacter mysorens</i>	NR_025613.1	99
BP10	<i>Kosakonia cloacae</i>	NR_028912.1	99
BP11	<i>Bacillus anthracis</i>	NR_074453.1	100
BP12	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP13	<i>Kosakonia ludwigii</i>	NR_042349.1	99
BP14	<i>Acinetobacter oleivorans</i>	NR_102814.1	99
BP15	<i>Klebsiella oxytoca</i>	NR_041749.1	99
BP16	<i>Acinetobacter calcoaceticus</i>	NR_042387.1	99
BP17	<i>Acinetobacter calcoaceticus</i>	NR_042387.1	99
B18	<i>Acinetobacter calcoaceticus</i>	NR_042387.1	99
B19	<i>Kosakonia ludwigii</i>	NR_042349.1	99
RP1	<i>Klebsiella oxytoca</i>	NR_041749.1	99
RP2	<i>Klebsiella oxytoca</i>	NR_102982.1	99
RP3	<i>Raoultella ornithinolytica</i>	NR_102983.1	99
RP4	<i>Bacillus megaterium</i>	NR_074290.1	100
RP5	<i>Bacillus pumilus</i>	NR_074977.1	100
RP6	<i>Klebsiella oxytoca</i>	NR_041749.1	99
RP7	<i>Burkholderia ferrariae</i>	NR_043890.1	99
RP8	<i>Klebsiella oxytoca</i>	NR_102982.1	98

*Species identified based on best score on % similarity to the 16S rDNA sequence.

solubilizing plus ACC deaminase activities may improve phosphate nutrition and plant growth more effectively than those carrying either one of these two growth promoting traits. It is easy to imagine that using bacteria with at least dual plant growth promoting activities as biofertiliser inoculants will be a desirable and sustainable option to replace eco-unfriendly chemical fertilizers. The catalase activity that was observed in the isolated bacteria can be interpreted to be of evolutionary and beneficial value to the *T. esculentum*-bacteria association. Bacterial strains with catalase activity are highly resistant to environmental, mechanical and chemical stress (Glick et al., 1998). The environment where *T. esculentum* grows is stressful with high day's temperatures of above 37°C, low pH and poor nutrient content in the soil (Chimwamurombe, 2010).

Of all the species isolated, 59% were positive for HCN production, including the *Kosakonia* species and some *K. oxytoca* isolates. Hydrogen cyanide production by bacteria has been reported to have an important role in

the biological control of pathogens and as an inducer of plant resistance (Voisard, 1989; Bulgarelli et al., 2013). These bacteria may be playing a role in protection of marama bean since very low disease incidence has been observed in its natural habitat. All the isolates were able to produce ammonia in plate assays. This was an interesting observation since the production of ammonia is an important feature of plant growth promoting bacteria that influences plant growth by making nitrogen (N) available to the plant. This is of particular importance to marama bean that has high protein containing seeds yet it grows in N-poor soils of the Kalahari sandy ecological zone (Dakora et al., 1999).

In general, the mechanisms of action by which plant growth promoting bacteria enhance and promote plant growth are not clearly known and understood, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilisation and promotion of the mineral nutrient uptake are usually believed to be

involved in plant growth promotion (Glick, 1995; Bulgarelli et al., 2013; Ali et al., 2014). Many papers have been published related to the screening of plant growth promoting bacteria from crop plants such as rice, maize and sugar cane but rare from marama bean. It was observed in this study that two bacterial isolates showed the highest solubilisation index of more than 11. Isolates BP6 (*A. calcoaceticus*), and BP7 (*K. cloacae*) showed the highest phosphate solubilising activity. In previous studies, it has been reported that phosphate solubilizing bacteria enhanced the growth and yield of inoculated plants by giving higher yields up to 20% in case of maize and lettuce (Chabot et al., 1993). Similarly, Gai and Gaur (2004) found that the use of rock phosphate, coupled with phosphate solubilizing bacteria, produced results comparable to superphosphate + phosphate solubilizing bacterial inoculants. Thus, phosphate biofertilisers in the form of microorganisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization. PGP bacteria have been reported to produce significant increases in growth and yield of agriculturally important crops in direct response to inoculation (Amara and Dahdoh, 1997; Grönemeyer et al., 2012). Furthermore, inoculation with rhizobacteria containing ACC-deaminase activity has been shown to alter the endogenous levels of ethylene, which subsequently leads to changes in plant growth. The bacterium actually prevents ethylene caused inhibition of root elongation. ACC accumulation is stimulated by several stress factors. If the ethylene concentration remains high after germination, root elongation is inhibited (Bulgarelli et al., 2013). The inhibitory effect of ethylene on plant root elongation can be reduced by the activity of ACC-deaminase; an enzyme produced by some soil microorganisms (Bulgarelli et al., 2013; Ali et al., 2014).

It was anticipated that most of the bacterial genera were isolated from both the bulk soil could also be isolated from the root rhizosphere parts of the experimental plants. However, it was strikingly remarkable to observe that two of the bacterial isolates (*B. ferrariae* and *B. pumilus*) were only retrieved from the rhizosphere. The explanation for this observation remains to be investigated. Likewise, *B. anthracis*, *A. mysorens*, *K. cloacae*, and *A. oleivorans* were only isolated from the bulk soil samples. Similarly, the explanation for this striking observation still needs further investigation.

Conclusion

We conclude that in the *T. esculentum* (marama bean) rhizosphere, there are numerous plant associated bacteria, some of which have potential to be used as plant growth promoting inoculants for arid agro ecological zones since they displayed phytobeneficial traits under *in vitro* conditions. Furthermore, with the exception of well-known PGP bacterial genera like *Rhizobium*, *Bradyrhizobium*,

Azospirillum and *Herbaspirillum*, several novel putative PGP species were detected in our screening procedure. We recommend that the identified isolates may have potential as plant beneficial inoculants for sandy, nutrient deficient agro-ecologies like the Kalahari areas where application of chemical fertilisers will be an economically demanding endeavour for the communal farmers. Similar studies on edible but neglected plants regarding presence of beneficial microbial communities should be enhanced as climate change effects begin to manifest in susceptible areas like the Kalahari region.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Synthesis of copolymer from lactic acid-polyethylene terephthalate (LA-PET) copolymerization

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Bio-plastic has been a need of the hour for the past few decades and the usage of lactic acid (LA) in the production of bio plastic opens a new window to the field. Polyethylene terephthalate (PET) thermoplastic polyester with excellent tensile and impact strength, chemical resistance, clarity, process ability, and transparency has become a bane to the society. The co polymerization increases the mechanical strength of LA. In this study, the production and optimization of the copolymer of LA –PET was done. The blend of sugarcane bagasse and corn stover was used for the production of LA by incubating with *Lactobacillus* sp. for 24 h. *Lactobacillus* sp. is a potent producer of lactic acid and crude curd was used as the source. The optimization in lignin breakdown by 10% NH₄OH and 2% NaOH and acid hydrolysis by 10% H₂SO₄ of the blend was done. The process parameters in the copolymerization LA-PET using p-toluene sulfonic acid, ethylene glycol and stannous chloride were also analyzed. The products formed were confirmed by the FTIR analysis at various transmittance peaks at 1681, 3008 and 3610 cm⁻¹. The process of production of the copolymer proves to be a green technology by decreasing the PET contamination of the environment and also helping in the production of bio-degradable plastics in the run.

Key words: Lactic acid, copolymer, bioplastic, polyethylene terephthalate.

INTRODUCTION

Lactic acid is one of the most important organic acid today, due to its recently gained attention of its biodegradable nature and of its potential polymer application. Conventionally, it is produced by the fermentation of the cellulosic materials with the help of microbes (Sharon and Sharon, 2003; Ahmed and Discher, 2004) and extraction of primary metabolite lactic acid. Previous reports have indicated the sole usage of corn stover or bagasse as a substrate for lactic acid production but the results

indicated lower yield and extraction difficulties. Polyethylene terephthalate (PET) is a thermoplastic resin of the polyester family and is used in synthetic fibers. PET is produced by the polymerization of ethylene glycol and terephthalic acid (Chetna and Madhuri, 2003). Ethylene glycol and terephthalic acid, when heated together under the influence of chemical catalysts, produces PET in the form of a molten, viscous mass that can be spun directly to fibers or solidified for later processing as a plastic

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(Abdelaal et al., 2008). It is synthesized by the tranesterification reaction between ethylene glycol and dimethyl terephthalate (Zulfiqar et al., 2006). The presence of a large aromatic ring in the PET repeating units, gives the polymer, notable stiffness and strength, especially when the polymer chains are aligned with one another in an orderly arrangement (Fariyal et al., 2004). The substance is not classified as dangerous according to Directives 67/548/EEC and 1999/45/EC.

Bio-plastic has been the need of the hour since plastics started showing their true nature. Bioplastics have proven advantageous over their relative plastics, by their easy degradation in the environment without reverse effects. There have been various approaches in producing these plastics, but only few found success. Natural polymers from plants such as cellulose and starch, have been functionally modified and crafted to thermoplastic or synthetic polymers which in few cases, have added monomers like lactic acid (Achani et al., 2011). The material produced represents a high-molecular polymer which is practically insoluble in water (Magdy et al., 2008).

In this study lactic acid was produced from bagasse and corn stover blend which was delignified using 2% NaOH and 10% NH₄OH (Dennis et al., 2004) and sequentially it was acid hydrolyzed using 10% H₂SO₄ resulting in cellulose breakdown to glucose. This serves as the carbon source for the microbe to act on and produce lactic acid (Zulfiqar et al., 2006). The study also employs the usage of copolymerization technique to produce bioplastic consisting of LA and PET. The copolymerization is carried out, after the glycolysis of PET to BHET using ethylene glycol and further grafting to lactic acid (Chetna and Madhuri, 2003) to finally yield the product polymer.

MATERIALS AND METHODS

Raw material

Bagasse obtained from Bannari Amman Sugars Private Ltd., were dried at 70°C for 15 h or until dry weight of the bagasse is measured as constant. Then, it was milled and homogenized to obtain small particles of approximately 5 mm in size and stored at cool temperature. Corn stover which was purchased locally from Sathyamangalam market was also dried at 80°C for 4 h and ground and homogenized to smaller particles of size 2 mm and stored at cool environment. All the chemicals used in the study were purchased from Himedia Laboratories, Mumbai, India and of analytical grade.

Lignin extraction

10% NH₄OH and 2% NaOH was used in order to separate the lignin from bagasse and stover. The experiment was carried out at 37°C for 24 h. Total amount of sample used was 1 g and the composition used in the subsequent trials of the experiments were: a) 1 g of bagasse, b) 0.5 g of bagasse and 0.5 g of corn stover and c) 1 g of corn stover. In the second case, the concentrations of

NaOH and NH₄OH were maintained constant along with the amount of sample. Here the compositions used were: a) 0.2 g of bagasse and 0.8 g of corn, b) 0.4 g of bagasse and 0.6 g of corn, c) 0.6 g of bagasse and 0.4 g of corn, and d) 0.8 g of bagasse and 0.2 g of corn. After a period of 24 h, the samples were filtered cautiously using Whatmann No.1 filter paper. The residues collected in the filter paper were dried in a hot air oven at 190°C and filtrates were taken for analysis. The lignin content present in the soluble fraction was measured in visible spectrophotometer at an absorbance of 549 nm (Zulfiqar et al., 2006).

Acid hydrolysis

After lignin extraction, the dried residue was subjected to treatment with 10% HCl/H₂SO₄ for hydrolysis (Watthana et al., 2012). The entire process was carried out in a controlled environment of temperature of 120°C and for a time period of 60 min (Pattana et al., 2009; Ziyu et al., 2009).

Lactic acid production

Lactobacillus sp. was isolated from the butter milk by centrifuging 15 mL of the sample at 5000 rpm for 5 min at 4°C after which the supernatant was discarded (Bridget et al., 2011). The debris or the pellet left, was thoroughly mixed with PBS and made up to a volume of 10 mL. The cultured strains in PBS were then, added to the acid hydrolyzed samples of bagasse and corn stover. The setup with mixture was placed in an orbital shaker for a time period of 24 h and at 100 rpm and 37°C. After 24 h, the samples were taken and again subjected to centrifugation at 8000 rpm for 3 min and following which the supernatant was extracted for the estimation process (Bertrand et al., 2011; Woranart et al., 2012).

Lactic acid estimation

For the evaluation and estimation of lactic acid, the lactic acid produced has to be extracted from the supernatant by using n-butanol as a solvent for its extraction (Kanungnit and Panarat, 2011). So for, the extraction purpose, n-butanol and the samples were taken at a ratio of 4:1 in a separating funnel. Both n-butanol and the supernatant were taken in the separating funnel and mixed thoroughly and allowed to stand for about 1 h resulting in complete phase separation. Here, the aqueous phase consists of the lactic acid and thereby requires simple removal of bottom phase. The extracted lactic acid was then taken for titration process, where NaOH was taken as the titrant and phenolphthalein as the indicator for the titration purpose. The presence was estimated based on the color produced. Its presence can be confirmed by using p-phenyl phenol reagent, where 0.7 mL of supernatant was added with 3 mL of 96% sulphuric acid, followed by heating for 10 min in boiling water bath and then cooling it to room temperature for about 30 min. The cold solution was added with 50 µl 4% copper sulphate and 100 µl p- phenyl phenol (prepared by dissolving 1.5% of the reagent in 95% ethyl alcohol) which provided a chromogenic complex. The absorbance for lactic acid is measured in UV-VIS double beam spectrophotometer at 570 nm (Ghosh and Ghosh, 2012). This method is otherwise called Kimberly Taylor method.

Glycolysis of PET

40 g of PET was taken and cut into very thin films. The cut pieces were taken in a 500 mL two necked round bottom flask with a reflux condenser attached to it. To it, 70.5 mL of ethylene glycol was added which was dried before (that is completely free from moisture) followed by the maintenance of a constant temperature of

Table 1. Weight of lignin produced by the samples using two reagents NaOH & NH₄OH.

Sample	reagent	Composition	weight of lignin
a	NH ₄ OH (24 h)	Bagasse	0.018
b		Corn	0.03
c		Blend	0.082
a	NH ₄ OH (48 h)	Bagasse	0.036
b		Corn	0.031
c		Blend	0.13
a	NaOH	Bagasse	0.029
b		Corn	0.04
c		Blend	0.142

180°C in an oil bath for about 5 h (Abdelaal et al., 2008). In this, zinc acetate was used as the catalyst and the amount taken is 0.2% by weight of PET used. The reaction, occurring in the round bottom flask was basically glycolysis which will finally result in the production of BHET.

PET-LA copolymerization

35 g of BHET was made to react with lactic acid in the presence of stannous chloride (SnCl₂.2H₂O) which was taken to be around 0.5 to 0.8 weight % along with p-toluene sulfonic acid (0.91 mol/mol of SnCl₂) (Achanai et al., 2011). This reaction mixture was again taken in a 500 mL round bottom flask which was to be immersed in an oil bath at a temperature of 180 to 210°C kept for 6 to 8 h. As the process continues, water and glycol was removed from the reaction simultaneously and the remaining mixture was dissolved in chloroform, which was later precipitated in methanol and filtered and dried at 90°C (Jin et al., 2001).

RESULTS AND DISCUSSION

Weight of lignin

Weight of lignin extracted was calculated after evaluating the dry weight (Table 1) of the residue containing the delignified mixture of bagasse, corn and their blend. The process included two reagents for delignifying, that is NaOH and NH₄OH. After evaluation, it was found that the amount of lignin separated by using NaOH was high compared to NH₄OH (Table 1, Figure 1).

Efficiency of lignin removal

To check the efficiency of lignin separation performed on different substrates like bagasse, corn stover and also the blend of both bagasse and corn, percentage efficiency was calculated (Table 2). By comparing the observations, it was found that the amount of lignin removed was high when bagasse and corn stover were

taken up as a blend rather than individual delignification (Figure 2).

Blend composition

From the previous trials, the higher lignin separation in blend was further optimized by measuring various compositions of blend of bagasse and corn (Table 3). The process of delignification with 10% NaOH was repeated and it was found that blend with a composition of bagasse taken at 0.8 g and corn at 0.2 g showed high lignin content removal. The results were conformed and evaluated using visible spectrometer at 549 nm (Zulfiqar et al., 2006) (Figure 3). This blend provided the best composition for producing lactic acid and further for the production of bioplastic.

Lactic acid production and extraction

The production of lactic acid involved *Lactobacillus* sp., and its production during the bacterial growth curve was tracked for the efficient removal of lactic acid and extraction. It was determined that the lactic acid production reached a peak at the end of 3 h. This also in turn decreased the pH of the media to the acidic level of 4.5 (Ghosh and Ghosh, 2012). The graphical representation of the same is given below (Figure 4).

Extraction of lactic acid

The separation of lactic acid from the media was a strenuous task and the extraction was done using n-butanol. The extraction process was carried out in a 4:1 ratio of butanol with respect to sample taken (Kanungnit and Panarat, 2011). The extraction showed high efficiency (Figure 5a) and this was attributed to the direct solvation between n butanol and lactic acid. The further increase of the organic phase was not performed since n -butanol is partially miscible in water. It was also noted that the extraction process when varied with respect to pH, showed maximum degree of extraction at pH 1 (Figure 5b). Thus, the decrease in the pH of the media due to lactic acid production contributed significantly to its extraction process using n-butanol. The mechanism behind this being solvating extraction method of n-butanol. The higher distribution co-efficient at low pH is mainly due to the availability of unassociated lactic acid form, which at low pH presents itself in a large manner.

Bis(2-hydroxyethyl) terephthalate (BHET) formation

BHET was formed by the glycolysis process, where PET was reduced to BHET by distilling it with proportions of ethylene glycol (Sharon and Sharon, 2003). The

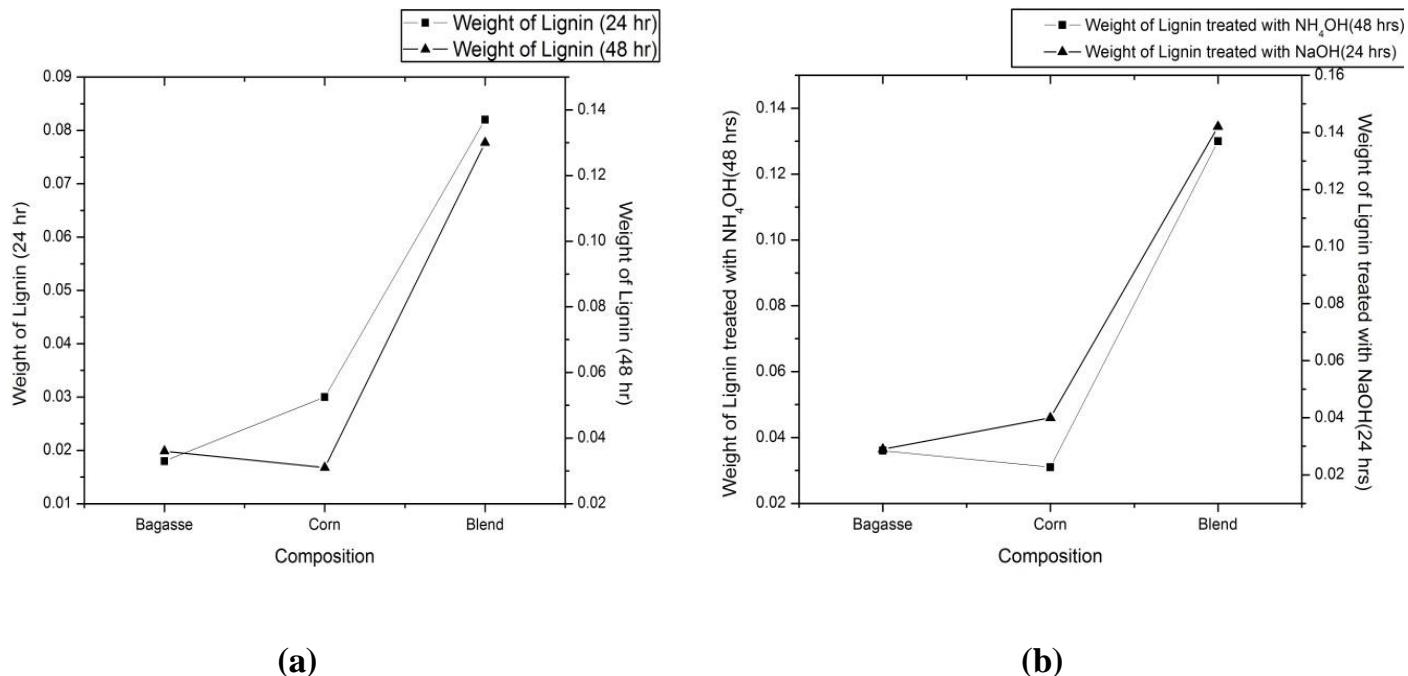


Figure 1. a) Amount of lignin removed on treatment with 10% NH₄OH at 24 and 48 h. The amount of lignin removed was determined spectrophotometrically at 549 nm. **b)** This graph depicts the amount of lignin removed from bagasse, corn and the blend on treatment with NH₄OH for 48 h and NaOH for 24 h.

Table 2. The efficiency of lignin removal by the two reagents NaOH & NH₄OH.

Sample	Reagent	Composition	Percentage efficiency of lignin removal	OD (549 nm)
1	NH ₄ OH (24 h)	Bagasse	18	0.012
2		Corn	28.30	0.022
3		Blend	40.19	0.079
4	NH ₄ OH (48 h)	Bagasse	33.96	0.052
5		Corn	30.097	0.029
6		Blend	63.41	0.091
7	NaOH	Bagasse	29	0.023
8		Corn	38.83	0.068
9		Blend	69.26	0.1

formation of a needle like white crystals on the inner walls of round bottom flask at 180°C during the glycolysis process of PET was visually confirmed and they were separated manually

Copolymerization

During the copolymerization process, the temperature was maintained at 180 to 200°C throughout the process. After completion of the copolymerization, both chloroform

and methanol were added resulting in the formation of two layers with a white precipitate at the bottom part and in the upper part, all the non-copolymerized waste residues were obtained. After filtering and drying of the precipitate, a white powder was obtained which had a melting point of 170°C confirming the formation of copolymer and on melting it at the characteristic melting point, thin films were obtained. The thin films were rigid and tensile in nature even after 2 weeks and the characteristic reaction taking place is depicted in Figure 6. The thin films formed were sent for FTIR analysis and the results

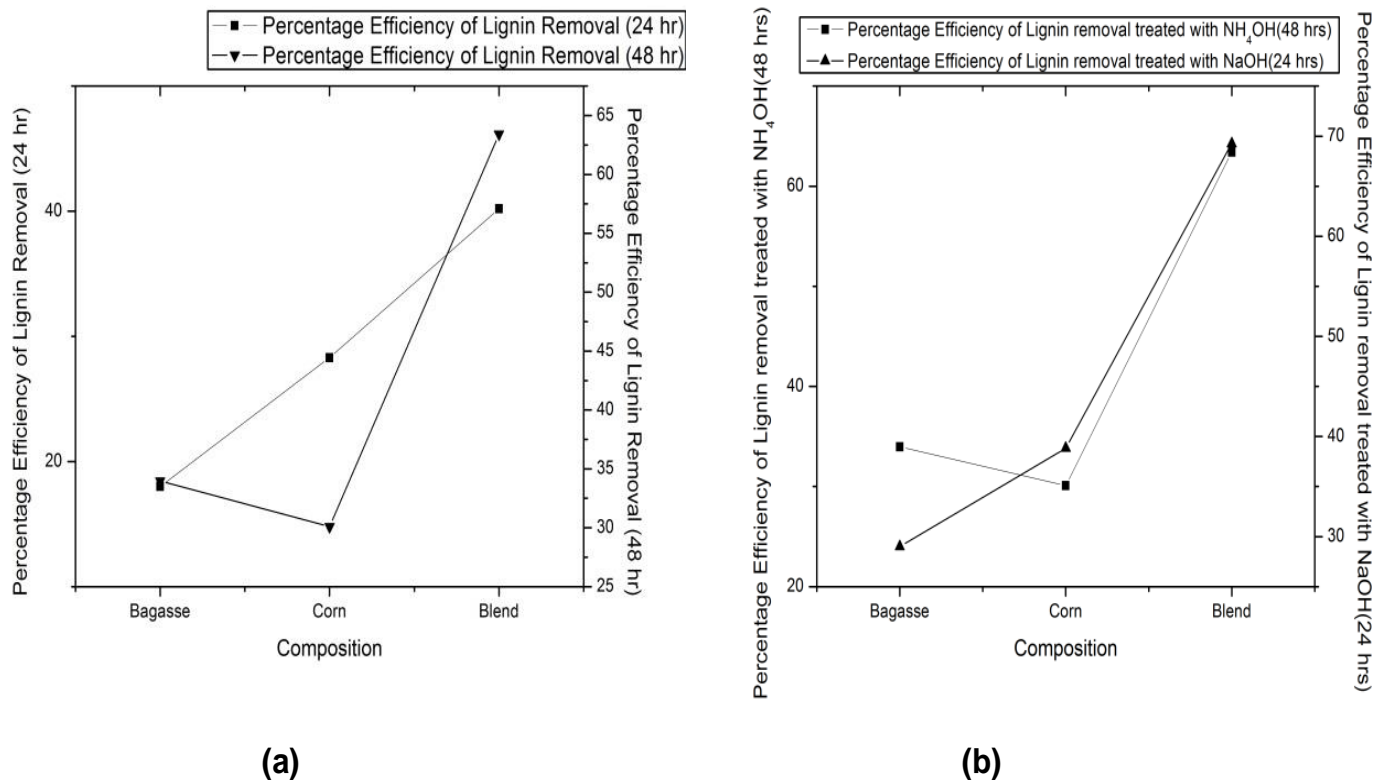


Figure 2. **a)** Percentage efficiency of lignin removal on treatment with NH₄OH at 24 and 48 h. The percentage efficiency of lignin removal was comparatively higher for the blend on treatment with NH₄OH for 48 h. **b)** Percentage efficiency of lignin removal on treatment with NaOH and NH₄OH for 24 and 48 h, respectively. Amount of lignin removed and its percentage efficiency were higher for blend on treatment with NaOH for 24 h

Table 3. The OD (549 nm) value of varying amount of bagasse and corn taken.

Sample	Bagasse	Corn	OD (549 nm)
1	0.2	0.8	0.047
2	0.4	0.6	0.053
3	0.5	0.5	0.063
4	0.6	0.4	0.071
5	0.8	0.2	0.086

obtained are shown in Figure 7 and Table 4. The lignin from the blend concentration (0.8 bagasse + 0.2 corn stover) using NaOH reagent and acid hydrolysis at 180°C was found to be the best optimized conditions for the production of lactic acid. The glycolysis of PET and the copolymerization process of BHET and LA were confirmed using FTIR results. The copolymerized product from lactic acid and polyethylene terephthalate has been reported before to be a biodegradable plastic. The plastic film produced after melting the powder product showed increased stability. The melting point of the powder depends on the rate to which the process of polymerization has taken place. So, based on the product

needed, the melting point of the polymer can be set approximately.

Conclusion

This material however has biodegradability, and does not render any harmful influence on the environment. They contain a large ratio of carbon from the carbon dioxide they have drawn from the air and fixed, due to the photosynthetic process. Thus, the bioplastic produced will be an opening to the near future in providing a successful sustainable environment for coming future generations.

Conflict of interests

The authors did not declare any conflict of interest.

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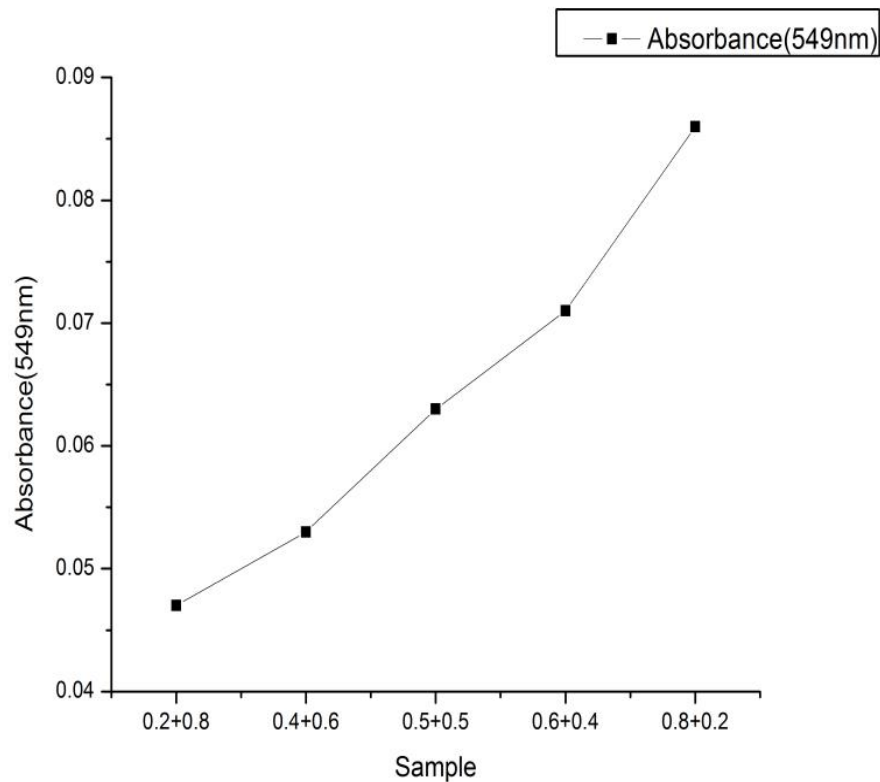


Figure 3. Blend optimization was done for various compositions of bagasse and corn and treatment with NaOH for 24 h was carried out to determine the maximum amount of lignin removal. Blend composition of 0.8 g of bagasse and 0.2 g of corn showed high absorbance at 549 nm depicting the maximum amount of lignin removal.

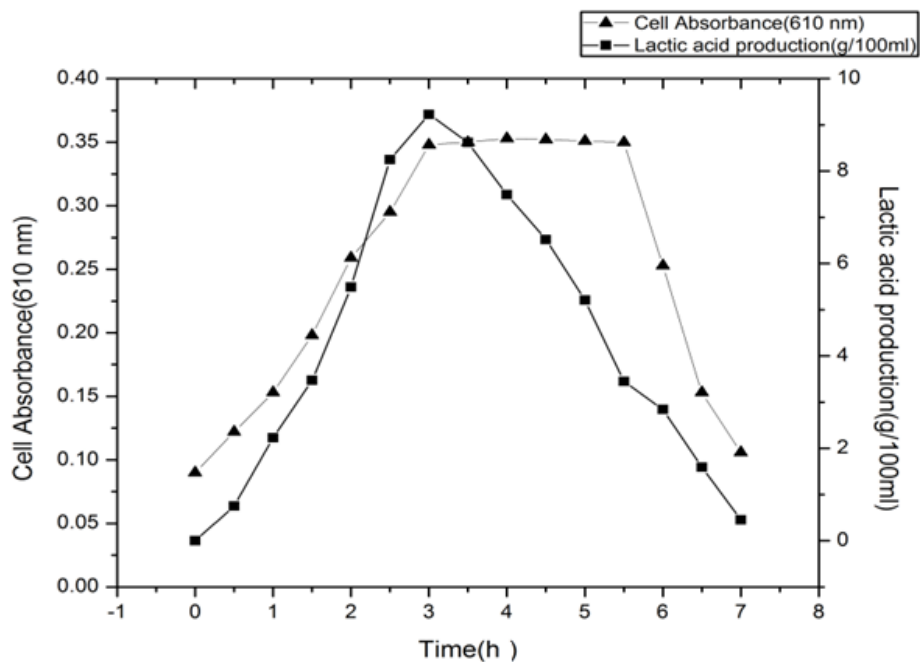


Figure 4. Comparison of Bacterial growth curve of *Lactobacillus sp.* and lactic acid production (g/100 mL) by bacteria.

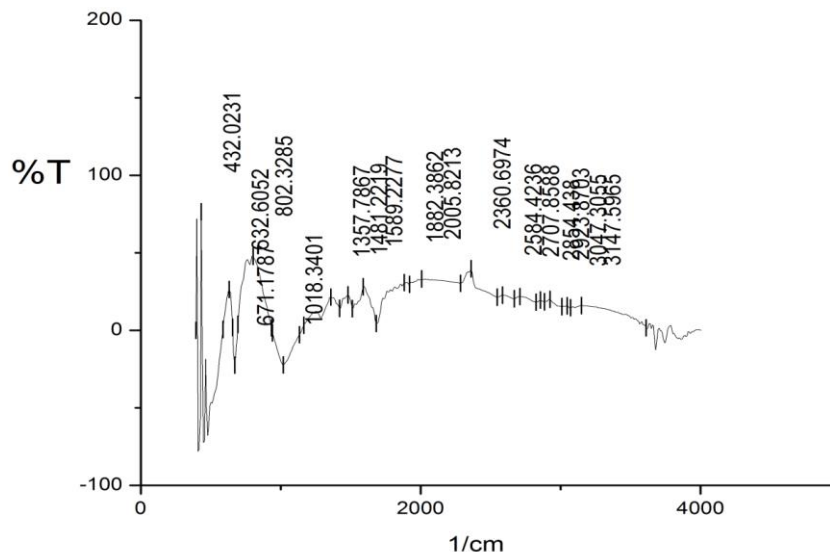


Figure 7. FTIR studies were performed to study the extent of copolymerization of lactate monomers with BHET. Absorption peaks were absorbed at the wave numbers of 1681.804 and 1134.0605 cm^{-1} representing the stretches of C=O and C-O bonds which confirms the presence of an ester group between the BHET and Lactate monomers.

Table 4. Different wave numbers showing the bond between different molecules [FTIR study].

Absorption (cm^{-1})	%T	Functional group
586.31	0.56	
655.75	1.86	Ring plane bending
694.32	3.84	C-C in plane bending
933.48	1.67	C-H in plane bending phenyl ring
1134.06	-2.83	C-O-C
1419.50	14.20	COO-
1512.08	14.01	C=C symmetric stretch
1681.80	4.40	Ar-C=O
1920.96	29.70	C-H out of plane bending
2283.55	30.34	H bonding
2545.85	21.40	C-O stretch in carboxylic group
2669.28	20.40	C-O stretch in carboxylic group
2823.58	18.17	C-C stretch
2885.30	18.44	C-H stretch
3008.73	15.30	CH aromatic
3070.45	14.60	CH ₂ symmetric stretch
3610.48	1.60	OH

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Full Length Research Paper

Effects of submerged and anaerobic fermentations on cassava flour (*Lafun*)

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Cassava tubers for processing into cassava flour, *Lafun* a Nigerian locally fermented product was subjected to two different types of fermentations: submerged and anaerobic fermentation for 72 h. Physicochemical changes that occurred during fermentation and their influence on the functional, rheological and sensory properties of the resulting flour were investigated. There was no significant difference in rate of decrease of pH and hydrocyanide under both fermentation conditions but titratable acidity differed significantly ($p < 0.05$). Crude fibre, crude protein, ash, swelling index and final viscosity were significantly higher ($p < 0.05$) in flour from submerged fermentation. *Lafun* from submerged fermentation had greater sensory quality and higher consumer preference than that of anaerobic fermentation.

Key words: Cassava fermentation, physicochemical, functional, pasting properties.

INTRODUCTION

Cassava, *manihot esculenta crantz*, is a food crop of great importance for the nutrition of over 500 million people in the tropic (Cock, 1982). Its starchy tuberous roots are a valuable source of cheap calories (Cock, 1985; Bokanga, 1999). Additionally in many parts of Africa its leaves and tender shoots are consumed as highly prized vegetables (Bokanga, 1995). Cassava is a highly perishable crop and is usually processed as soon as it is harvested, otherwise spoilage sets in within 48 to 72 h. Cassava has been utilized in several ways and its mode of utilization in Africa shows that nearly three out of four cassava based food are fermented products (Westby, 1994). Natural fermentation of plant materials is widely used in under-developed countries to transform and preserve food crops because of its low technology and energy requirement and the unique organoleptic properties of the final

products (Daeschel et al., 1987; Aro, 2008).

Fermentation has been viewed as a dynamic process during which several catabolic and anabolic reactions proceed simultaneously depending on several factors including substrate, micro flora and environmental factors (Eleazu et al., 2011). During the fermentation of cassava the tissues are softened and disintegration of the tissues by microorganisms results in contact of cyanogenic glucosides, linamarin and lotaustralin with the enzyme linamarase located in the cell wall (Mkpong et al., 1990) leading to the formation of glucose and acetone cyanohydrins which is spontaneously broken down to hydrocyanic acid (HCN) and acetone (Cooke, 1978; Bokanga, 1999; Aworh, 2008). HCN once produced dissipates in the air since its boiling temperature is 25.7°C (Bokanga, 1999). Cassava fermentation consists of two distinct

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methods, aerobic fermentation (heap fermentation) and anaerobic (Hahn, 1992). Heap fermentation is the most widely used processing techniques for reducing cyanogens in Mozambique and Uganda (Essers et al., 1995; Tivana et al., 2007). It involves surface-drying the roots, 1 to 2 h after which they are heaped together, covered with leaves or straws and left to ferment 3 to 4 days until the pieces become moldy, after which they are scrapped, sun-dried and milled to flour. The second method is anaerobic fermentation in which grated cassava tubers is placed in sacks and pressed with stones or a jack between two platforms (Hahn et al., 1987).

Fermentation of cassava has given rise to a wide array of fermented products. In the south-western part of Nigeria, cassava is consumed in the form of hot water flour paste called *Lafun*. The flour is made by allowing peeled tuberous roots of cassava steeped in water to ferment naturally. *Lafun* is produced by submerged fermentation of peeled sliced cassava roots in water for 3 to 5 days (Oyewole and Odunfa, 1988) or by immersing peeled or unpeeled cassava in a stream or stationary water or in an earthenware vessel and fermented until roots become soft (Hahn, 1992) after which the fermented cassava was subjected to sun-drying and milling to powder/flour. The flour is usually turned in freshly boiled water, with no further heating into a stiff porridge consumed with soup (Oyewole and Afolami, 2004). *Corynebacterium manihot*, *Lactobacillus spp.* and *Leuconostoc spp.* are some of the organisms that are involved in fermentation of cassava to *Lafun* (Odunfa, 1985).

Many constraints have been identified in the commercialization of *Lafun* processing. This includes product quality variation from one processor to the other (Oyewole and Sanni, 1995) and differences in processing methods (Akingbala et al., 1991). Idowu and Akindele (1994) enumerated the influence of age and variety of cassava roots as another constraint. Oyewole and Afolami (2004) investigated the influence of use of some new cassava varieties in comparison with local varieties in quality of *Lafun*. Another factor responsible for product variation in *Lafun* is that fermentation process is initiated by chance inoculation by microorganism from the environment (Achi and Akomas, 2006). This study therefore aimed at investigating some of the biochemical changes that occurs when cassava is subjected to submerged fermentation and anaerobic fermentations in the processing of cassava flour, *Lafun* and the influence of these changes on the chemical, functional and pasting characteristics of the products developed from the two fermentation processes.

MATERIALS AND METHODS

Freshly harvested cassava tubers (*Manihot esculenta* crantz) of 12 months old TMS50395 cultivar were obtained from the International Institute of Tropical Agriculture, IITA farm in Ibadan Oyo State, Nigeria.

Processing of cassava tubers

The cassava tubers were sorted, peeled, washed with potable water and drained. 10 kg of the washed tubers were separated into two equal portions. A portion (5 kg) was then cut into slices of 5 to 6 cm length and soaked in a big bowl containing water at ratio of 1:3 w/v. The pieces were completely submerged in water and the bowl uncovered to allow for exposure to air (Kahn, 1992). The second portion were cut into slices, soaked with same quantity of water as in the first process and wrapped in jute sacks placed in a large bowl which was then covered with a polyethylene bag to create anaerobic conditions (Tetchi et al., 2012). Both were left for three (3) days to ferment at ambient conditions (27 to 32°C). Both fermentation conditions are similar but the only difference is the reduced availability and access of surface air to the fermenting tubers caused by the polyethylene covering resulting in anaerobic conditions. The reasoning behind creating this sort of anaerobic condition was to investigate the possible effects this will have on the actions of microorganisms on the fermenting cassava and the resulting product: *Lafun*. During the fermentation, samples of the fermenting tubers and steep water from each fermentation process were aseptically taken out and subjected to different analyses. After fermentation, the pulp was removed, water squeezed out and spread on a tray and sundried for 3 days.

The dried *Lafun* (from submerged and anaerobic fermentations) and a control sample that is traditionally produced sample purchased from a local market were separately milled, sieved and packaged in a polythene bag for analyses.

Chemical analyses

Moisture, ash, crude fibre, crude protein, tritrate acidity were determined by the methods of AOAC, 1990. The pH was measured using pH meter (Unicam 9450 model).

Hydrogen cyanide determination

This was determined by the methods of Oyewole (1990).

Carbohydrate content

Carbohydrate content was calculated by the difference method.

Physical characteristics

Water absorption capacity

Water absorption capacity of the flour samples were determined by the methods of Beuchat (1977).

Swelling index

This was determined according to the method of Ukpabi and Ndimele (1990).

Bulk density (Loose and packed)

These were determined by the methods of Okaka and Potter (1979).

Rheological characteristics

The rheological characteristic of the *Lafun* produced was

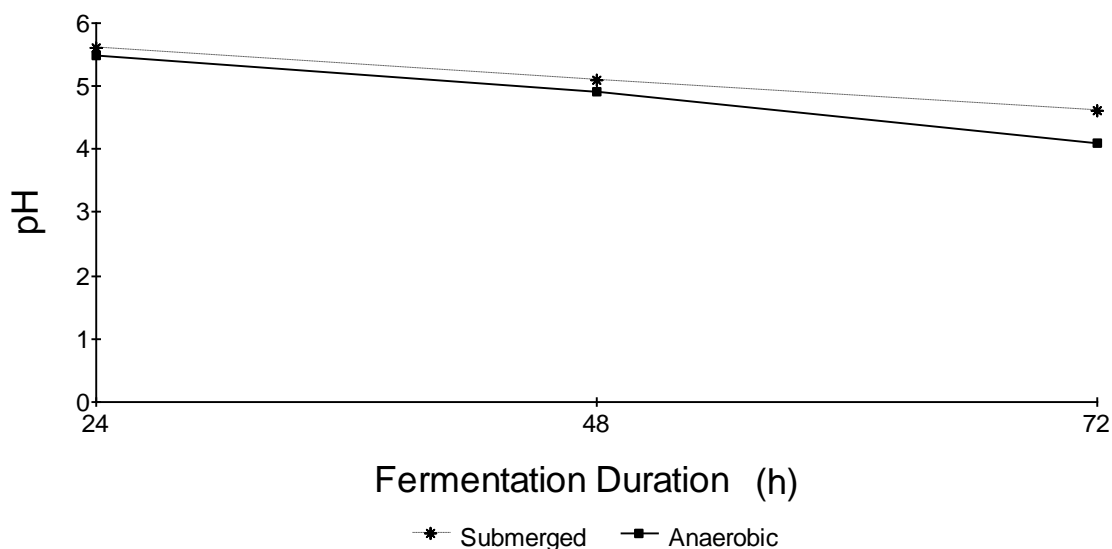


Figure 1. pH of cassava steep water.

determined using Brabender Amylograph. The effects of fermentation on the pasting properties of the flours during heating, cooling and shearing were studied as described by Numfor et al. (1995).

Sensory evaluation

The fermented cassava flour was cooked by briskly turning the flour in freshly boiled water in a pot using wooden turning stick at flour/water ratio 1:4 as described by Oyewole and Afolami (2001) until a consistently smooth paste is achieved. They were served to 10-man sensory panelists who are familiar with the product to evaluate on a 9-point hedonic scale where 9 represents like extremely, 5 represents neither like nor do dislike and 1 represents dislike extremely. The means of sensory scores after being subjected to analysis of variance and separation of means by Duncan's multiple range tests are shown in Table 3.

Statistical analysis

The analysis were performed in replicates and data obtained were subjected to analysis of variance to determine the differences with the aid of the statistical package SPSS (version 15.0) and Duncan's multiple range tests to separate the means at 5% level of significant difference. Microsoft Works spreadsheet was used to plot graphs for presentation of figures.

RESULTS

Changes in pH, HCN and titratable acidity of cassava steep water

The changes in the pH of steep water obtained from fermenting cassava tubers over the period of 72 h are shown in Figure 1. There was no significant difference ($p > 0.05$) in pH within 48 h of fermentation, even though there was a general decrease in pH under both anaerobic and submerged fermentation. A greater decrease in pH

was observed under anaerobic fermentation. The pH decreased significantly ($p < 0.05$) after 48 h of fermentation. pH values were much lower under anaerobic fermentation than in submerged fermentation. Figure 2 shows the changes in titratable acidity of steep water of cassava tubers fermented over a period of 72 h. There were significant differences in titratable acidity ($p < 0.05$) under the different fermentation conditions and over 24 h interval up to the end of fermentation.

Figure 3 shows changes in HCN content of cassava steep water. There were significant differences ($p < 0.05$) in HCN under both submerged and anaerobic fermentation. HCN content differed significantly at 24 h interval till the end of fermentation at 72 h. Under submerged fermentation, HCN reduced from 3.20 ± 0.01 mg/100 g within 24 h of fermentation to 0.98 ± 0.01 mg/100 g at 72 h while a greater reduction to 0.80 ± 0.01 mg/100 g was observed under anaerobic fermentation.

Changes in pH, titratable acidity and HCN of fermenting cassava tubers

Changes observed in pH of cassava tubers fermented between 24 and 72 h are presented in Figure 4. There was no significant differences ($p > 0.05$) between pH under submerged and anaerobic fermentations but between 24 h fermentation interval under both conditions, pH reduced significantly.

Titratable acidity increased significantly as fermentation period increased under both submerged and anaerobic conditions. There were also significant differences ($p < 0.05$) in the values of acidity observed under these conditions. Greater increase was observed for titratable acidity under anaerobic fermentation. HCN content of cassava tubers fermented under submerged condition differed significantly ($p < 0.05$) from values observed under anaerobic

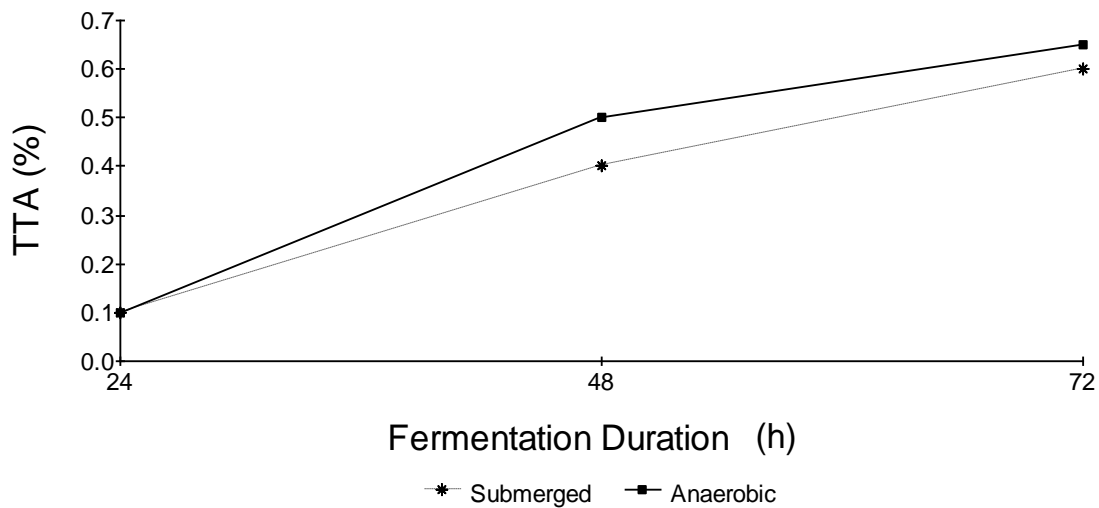


Figure 2. TTA of Cassava steep water.

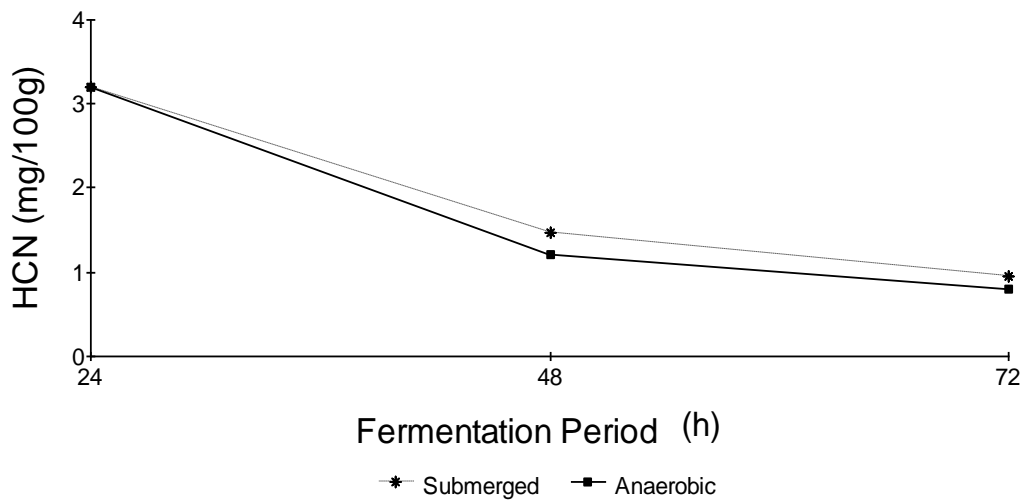


Figure 3. HCN content of cassava steep water (mg/100g).

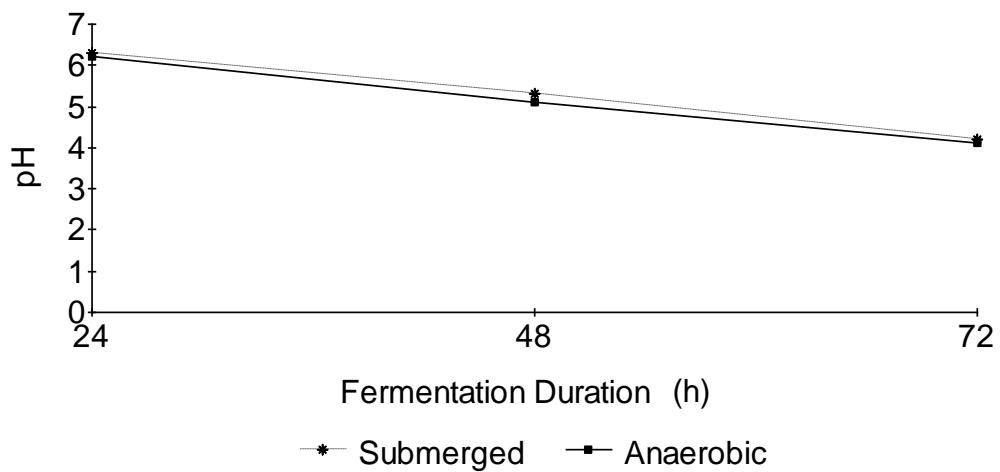


Figure 4. pH of cassava tubers.

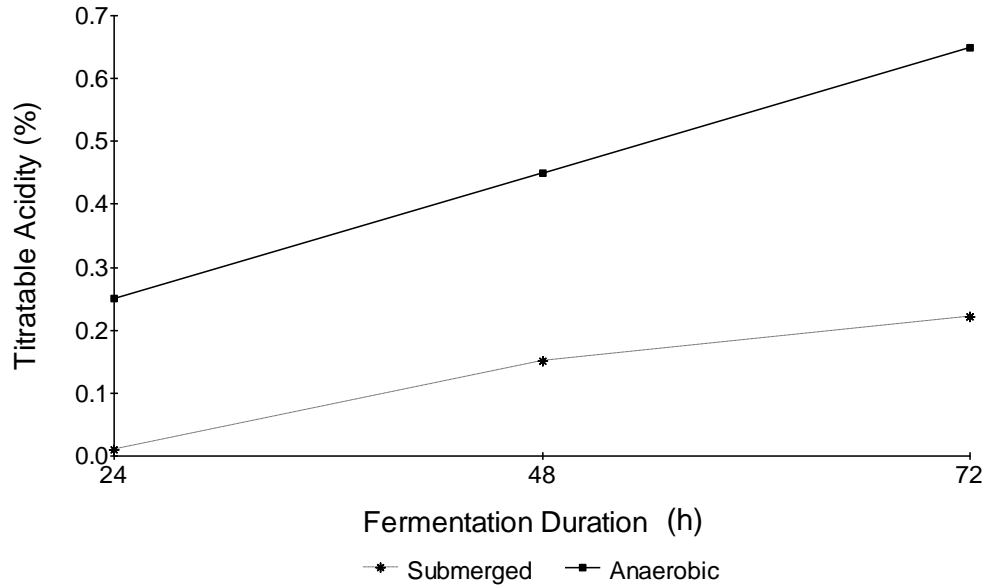


Figure 5. Titratable acidity of cassava tuber.

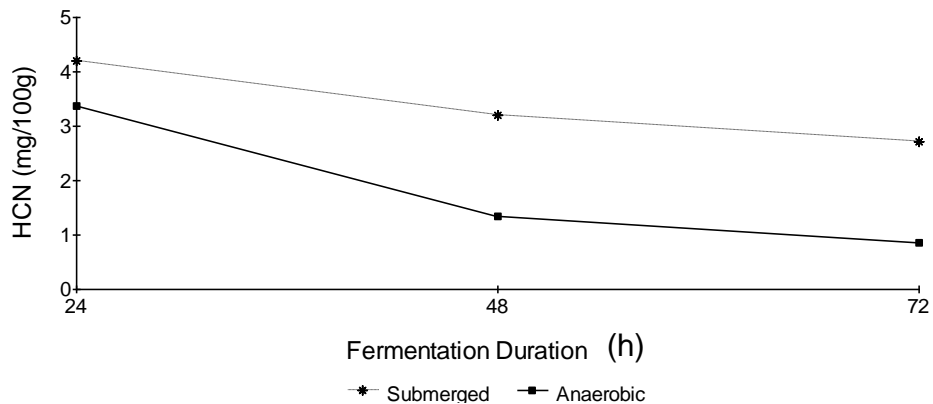


Figure 6. HCN content of cassava (mg/100 g).

fermentation. Within 24 h interval of observations, the HCN content reduced significantly under both fermentation conditions and a higher rate of reduction was observed under anaerobic fermentation as shown in Figure 6.

Changes in physicochemical properties of cassava tuber and flour fermented under different conditions

The chemical and physicochemical characteristics of peeled raw cassava tubers used for this study as well as those of the cassava flour obtained from drying and milling cassava tubers fermented under the two conditions in comparison with those of a market sample (used as control) are shown in Table 1. There was no significant difference ($p > 0.05$) in the pH of the experimental samples and the market sample but the samples

differed significantly in titratable acidity. Highest value was observed for the market sample while the anaerobic fermentation sample had the least titratable acidity ($0.13 \pm 0.00\%$). There was no significant difference ($p < 0.05$) in the HCN acid content of all the flour samples. Moisture content, ash, crude fibre, protein and carbohydrate all differed significantly ($p < 0.05$) among the flour samples. Moisture content was lowest in the anaerobic samples and highest in the submerged samples. Crude fibre was lowest in the anaerobic samples while the market sample had the highest. Ash content was higher in the submerged samples and highest in the market sample. Protein content was least in the anaerobic samples while the market samples had the highest protein content (1.94 ± 0.01 g/100 g). On the other hand, carbohydrate content of the anaerobic sample was higher than that of the submerged fermentation sample and the market sample.

Table 1. Physicochemical characteristics of Peeled cassava tubers and cassava flour under different conditions (% dry weight).

Parameter	Peeled cassava tubers	Market sample flour	Submerged fermented flour	Anaerobic fermented flour
pH	6.71±0.01	4.65±0.07	4.82±0.01	4.70±0.00
TTA (% lactic acid)	0.18±0.00	0.22±0.01	0.18±0.01	0.13±0.00
HCN(mg/100g)	13.83±0.01	1.32±0.01	1.30±0.14	1.28±0.00
Moisture (% wb)	70.10±0.14	12.60±0.14	14.60±0.07	11.60±0.07
Ash (% db)	4.01±0.01	1.00±0.14	0.80±0.00	0.13±0.01
Crude fibre (%db)	1.20±0.01	2.00±0.14	1.49±0.01	0.80±0.01
Protein (%db)	2.64±0.01	1.94±0.01	1.83±0.01	1.78±0.01
Carbohydrate (%)	22.17±0.01	82.46±0.14	81.28±0.01	85.69±0.14

Values are means of duplicate determinations. db means dry basis; wb means wet basis.

Table 2. Functional properties and rheological characteristics of fermented cassava flour under different conditions.

Parameter	Market sample	Submerged fermentation	Anaerobic fermentation
Water absorption capacity (ml/g)	1.40±0.01	1.40±0.01	1.40±0.01
Swelling index	3.00±0.01	4.50±0.01	2.00±0.01
Bulk density (g/ml)			
Packed	0.50±0.00	0.45±0.01	0.50±0.01
Loose	0.38±0.01	0.36±0.00	0.38±0.00
Peak viscosity (RVU)	424.84±0.01	400.45±0.01	400.59±0.01
Trough (RVU)	261.58±0.28	256.08±0.00	252.25±0.00
Breakdown (RVU)	163.25±0.01	144.33±0.01	149.33±0.00
Final viscosity (RVU)	324.08±0.28	320.75±0.01	306.67±0.01
Set back	62.50±0.00	64.66±0.01	54.42±0.01
Peak time (min)	4.94±0.02	4.93±0.00	4.73±0.01
Pasting temperature (°C)	76.85±0.01	76.75±0.01	76.75±0.00

Values are means of duplicate determinations.

The functional properties and pasting characteristics of the fermented cassava flour in comparison with the market samples are shown in Table 2. There was no significant difference ($p>0.05$) in the water absorption of all the samples. Swelling index differed significantly ($p<0.05$) among the samples. Submerged samples had the highest swelling index of 4.5 ± 0.01 , while the anaerobic samples had the lowest (2.00 ± 0.01). There was no significant difference in packed bulk density between the anaerobic sample and the market sample, whereas these two samples differed significantly ($p<0.05$) from the submerged sample. There was no significant difference in loose bulk density between the three samples.

There was no significant difference ($p<0.05$) in the peak viscosity among the samples. Highest peak viscosity was observed in the market sample (424.84 ± 0.01 RVU) and least in the submerged sample (400.45 ± 0.01 RVU). From Table 3, peak viscosity, trough, breakdown, setback, peak time, final viscosity and pasting temperature all differed significantly ($p<0.05$) among the samples.

Peak viscosity was highest in the market sample, followed by the anaerobic sample. There were no significant difference ($p>0.05$) in time to reach peak viscosity between the market sample and the submerged sample but these differed significantly from that of the anaerobic sample. All the samples have significant difference ($p<0.05$) in setback and breakdown viscosities. The submerged fermentation sample had the highest setback of 64.66 ± 0.1 RVU while the highest breakdown was observed in the market sample (163.25 ± 0.01 RVU). No significant difference was observed in the pasting temperatures of submerged fermentation and anaerobic fermentation flours but these differ significantly ($p<0.05$) in comparison with market flour which had a slightly higher pasting temperature.

There was no significant difference ($p<0.05$) in taste of *Lafun* from the cassava flour produced by submerged fermentation and the market sample flour. These two differed significantly ($p<0.05$) in taste and were preferred well than *Lafun* from cassava flour from

Table 3. Sensory evaluation of cooked Lafun fermented under different conditions.

Sample	Taste	Colour	Aroma	Overall acceptability
Market sample	6.27 ^a	7.0 ^a	6.8 ^b	6.5 ^a
Submerged fermentation	7.1 ^a	7.0 ^a	7.6 ^a	6.7 ^a
Anaerobic fermentation	5.6 ^b	4.7 ^b	5.2 ^c	5.4 ^b

Values in the same column with the same superscript are not significantly different ($p>0.05$).

anaerobic fermentation process. Similarly, no significant difference was observed between market sample *Lafun* and the submerged fermentation *Lafun* in colour. *Lafun* from anaerobic fermentation was poorly rated by panelists in colour. Interestingly, aroma of *Lafun* of submerged fermentation process differed significantly from and was preferred over the other flour samples. Finally, in overall acceptability there was no significant difference in overall acceptability between the *Lafun* from submerged fermentation and the market sample though a slightly higher value was observed for the former. *Lafun* from anaerobic fermentation was least rated in overall acceptability.

DISCUSSION

The pH and titratable acidity values of steep water and cassava tubers under submerged fermentation and anaerobic fermentation decreased and increased respectively, but a greater decrease in pH and increase in titratable acidity was observed in the steep water from both fermentation conditions signifying that a greater part of the acid produced are leached into the steep water. This has been reported to be attributed to the activities of lactic acid bacteria on carbohydrates in the fermenting cassava (Ogunsua, 1980; Oyewole and Odunfa, 1990; Oyewole and Afolami, 2001). Acid production had been reported to be dependent on microflora and processing conditions (Oyewole, 1990). The microorganisms involved in the *lafun* production include four yeasts: *Pichia onychis*, *Candida tropicalis*, *Geotrichum candida*, and *Rhodotorula* sp.; two moulds: *Aspergillus niger* and *Penicillium* sp.; and two bacteria: *Leuconostoc* sp. and *Corynebacterium* sp. (Nwachukwu and Edwards, 1987). Oyewole and Odunfa (1988) observed that the mould disappeared within 36 h of fermentation while *Bacillus* sp. which was present at the beginning of fermentation decreased drastically as fermentation progressed. The yeasts appeared within 24 to 48 h of the fermentation and increased rapidly. The lactic acid bacteria were implicated throughout the duration of fermentation. *Bacillus* sp., *Corynebacterium* sp., *Candida* sp. and the lactic acid bacteria were considered to play important roles. They have been reported to be responsible for souring of cassava fermented products through lactic acid production (Amoa-Awua et al., 1996; Oyewole and Odunfa, 1988). Some *Bacillus* species were reported to show ability to

breakdown cassava tissues during fermentation process (Padanou et al., 2009) while some yeasts and fungi contribute to cassava tissue breakdown by cellulase production leading to a more intimate interaction between linamarase and cyanogenic compounds of cassava, linamarin and lotaustralin resulting in the formation of glucose and acetone cyanohydrins which is spontaneously broken down to hydrocyanic acid (HCN) and acetone (Cooke, 1978; Bokanga, 1999; Aworh, 2008) and resultant detoxification of cassava. Lactic acid bacteria, yeasts and fungi also contribute to the build-up of the aroma compounds during fermentation (Oyewole, 2001).

Moisture, pH and temperature conditions are critical for the growth of these microorganisms in roots and thus for fermentation. According to Spier et al. (2006) ambient temperature of 30°C combined with high moisture content (90%) lead to highest α -amylase produced by lactic acid bacteria for cassava starch hydrolysis. pH value of below 4.0 has been reported to be optimum for cassava fermentation for *Lafun* production (Oyewole and Odunfa, 1988). An optimum value of 3 mg/kg had been recommended as a safe level for HCH in dried fermented cassava products (Achinewhu et al., 1998).

The greater reduction in TTA observed in cassava tubers under submerged fermentation may be due to the higher water content in the fermenting medium which encourages the activities of the fermenting microflora. Raimbault et al. (1996) explained that interaction of microorganism in the fermenting cassava is likely to be affected by the moisture content of the mass. Reduction in HCN was greater under anaerobic fermentation (3.38 ± 0.001 mg/100 g to 0.86 ± 0.01 mg/100 g) for cassava tubers and 3.2 ± 0.01 mg/100 g to 0.8 ± 0.01 mg/100 g for steep water than 4.2 ± 0.02 mg/100 g to 2.7 ± 0.01 mg/100 g cassava tubers and 3.38 ± 0.01 mg/100 g to 0.86 ± 0.01 mg/100 g observed for submerged fermentation. From this observation, it appears as though anaerobic conditions encourage a greater breakdown of cyanogenic compounds to HCN.

Higher moisture content observed in cassava flour under submerged fermentation after drying for the same length of days with the anaerobic fermentation flour might have been as a result of higher water absorption by the fermenting cassava. Lower crude fibre and protein contents observed in the anaerobic fermented flour could be due to the higher activities of the microflora which break down fibre during fermentation. Reduction in

carbohydrates, ash, and protein during fermentation had been attributed to the leaching of these nutrients into the soaking water and microbial utilization (Oyewole and Odunfa, 1989), though a slight increase in protein content during fermentation was reported by Tivana et al., (2007). Lactic acid fermentation is heterolactic, operating in association with secondary alcoholic and anaerobic fermentation to produce alcohol and organic acid (Raimbault et al., 1996).

Higher bulk densities were observed in the market and anaerobic flours over the submerged fermentation flour. Bulk density is a measure of the heaviness of a flour sample (Oladele and Aina, 2007). Therefore, the latter sample will pack more than other in containers (Azubike et al., 2011). Also higher bulk densities increase the sinkability of powdered material (Ortega -Rivas 2012). There was no significant difference among the samples for water absorption capacities. Water absorption capacity describes the flour- water association ability under limited water supply (Oladele and Aina, 2007) and is expressed as gram of water absorbed (retained) per gram of sample.

The water absorption capacity obtained from this study was much lower than those reported for unfermented cassava flour (Azubike et al., 2011). Higher water absorption capacities of 6.0 g water/g sample and 9.5 g water/g sample were observed respectively for red and white sweet potato flour (Onuh et al., 2004). Swelling index describes the volume a particular quantity of *Lafun* will occupy when mixed with a specific quantity of water. Swelling index was highest in the submerged fermentation flour. Swelling index illustrates the relationship between the length of fermentation and the different fermentation conditions.

From this study it was observed that even though all the *Lafun* samples had the same water absorption capacities they differed significantly in swelling index. The submerged fermentation flour showed potentials for higher swelling power above the other two flour samples. This signifies that the possible changes caused by fermentation in the morphology of the starch moieties in the resultant flour is the inducement of higher swelling features and fermentation beyond four days causes a reduction in swelling index of yam flour (Iwuoha, 2004). The higher swelling index in *Lafun* from submerged fermentation will be of economic benefit for and consumers because only a small quantity will produce very large mass of product when the product is cooked by end user/consumer. Similar peak viscosity and water absorption values were observed for the submerged fermentation and anaerobic fermentation flours, while a higher peak viscosity was observed for the market sample (424.84 ± 0.01). It has been observed that peak viscosity usually occurs at the equilibrium between granule swelling which increases viscosity and granule rupture and alignment due to mechanical shear which causes its decrease (Ayernor, 1985, Bolade 2009). Numfor et al. (1995) studied the effect of pH on the pasting properties

of starch by acidification with citric acid and observed that peak viscosity was not affected by pH between 6.9 and 4.5, however on lowering to 3.5, a reduction in peak viscosity suggested that enzyme action reduces peak viscosity of native (unfermented) starch. The rheological change also reflects greater internal stability of the fermented starch granules resulting in reduced swelling and amylose leaching.

Breakdown viscosity is regarded as a measure of the degree of disintegration of starch particles or paste stability during heating (Dengate, 1984; Bolade, 2009). The submerged fermentation flour with the lowest breakdown value was more resistant to heat and shear force followed by the anaerobic flour. This would guarantee a more stable cooked paste. Highest final viscosity was in the market sample (324.08 ± 0.28), closely followed by the submerged fermentation flour. Higher final viscosity is attributed to aggregation of amylose molecules in the paste (Miles et al., 1985). Highest set back was observed in submerged fermentation flour (64.66 ± 0.01) and lowest in anaerobic fermentation flour (54.42 ± 0.01) respectively. The lower value for anaerobic flour may be due to the higher rate of reduction in titratable acidity and HCN which implies faster breakdown of starch and other carbohydrate during fermentation, thereby leading to reduction in the amount of starch to be gelatinized. It has been observed that stimulated enzymes in soaked cereals grains are capable of causing partial hydrolysis of starch molecules (Akingbala et al., 1987). Similar but significantly different pasting temperatures were observed in all the flours samples. Higher temperature of gelatinization reflects greater internal granule stability (Numfor et al., 1995). The pasting temperatures observed were in the range of 76.76 ± 0.01 to $76.85 \pm 0.01^\circ\text{C}$ for all the samples. These values were higher than those observed for fermented cassava starch by Numfor et al., 1995, but they are comparable to (73.4 to 74.8°C) observed for maize flour (Bolade, 2009). Lower pasting temperature implies that the flour will gelatinize faster that is, at reduced temperatures. The observed low pasting temperatures may probably be due to enzyme activity stimulated by the fermentation process which has broken down the matrix embedding the starch granules, thus allowing the granules to swell freely and gelatinize faster. Similar pasting temperatures (74.95 ± 0.87 to $76.85 \pm 0.44^\circ\text{C}$) were observed by Oyewole and Afolami (2001) when different cultivars of cassava were subjected to fermentation.

The higher value of acidity of *Lafun* sample from submerged fermentation and the market sample must have positively impacted on the taste and aroma of the products as they were significantly highly rated than the anaerobic sample. It has been observed that consumers of *Lafun* described good quality *Lafun* as one with little or no odour, having characteristic white colour and texture which is non-sticky to the hand (Oyewole and Afolami, 2001). In all the attributes, *Lafun* from submerged

had the better consumer acceptability than *Lafun* from anaerobic fermentation which is comparable to the market sample.

Conclusion

Consequently to our investigation, it could be observed that even though both types of fermentation led to a considerable decrease in pH and increase in titratable acidity in *Lafun*, submerged fermentation had a higher positive impact on nutritional and functional qualities of the cassava flour. The biochemical changes that occurred led to a higher ash content (which is indicative of the mineral content), a relatively higher protein and fibre content than those observed in anaerobic fermentation. Also submerged fermentation produced *Lafun* of higher swelling index and higher final viscosity. It would be apparent that submerged fermentation produced a better quality *Lafun* than anaerobic fermentation as confirmed by consumers' preference for *Lafun* from submerged fermentation. Therefore we advise that for optimum quality *Lafun*, cassava should be subjected to aerobic fermentation.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

***Mycobacterium tuberculosis* complex identification by polymerase chain reaction from positive culture in patients from Jamot and Mbalmayo district hospitals**

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Tuberculosis (TB) is a contagious and infectious disease of which the causative agents are mycobacteria. Up to one third of the world's population is infected by these bacteria. Its control and follow up treatment are achieved by the use of antituberculosis drugs after tests and diagnosis. The last two decades have been marked by the development of new techniques for detection and characterization of *Mycobacterium tuberculosis* complex (MTBC). Conventional tests to differentiate MTBC from non tuberculosis mycobacteria (NTM) such as Para nitro benzoic acid (PNB) inhibition tests are often time consuming. This study aim at using PCR amplification of specific markers (*hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*) for more rapid detection of MTBC in positive cultures. The study was conducted in Jamot Hospital, the largest urban treatment center for tuberculosis in Cameroon and in Mbalmayo District Hospital, a small rural district hospital. Mycobacterial culture was performed on all smear positive sputa. All positive cultures were subjected to drug susceptibility testing (DST) using the indirect proportion method. On the same way, MTBC were differentiated from other mycobacteria using the PNP inhibition test. DNA extracted from positive cultures was subjected to PCR amplification using specific primers (*hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*). Analysis of PCR products was done by agarose gel electrophoresis. A total of 79 smear positive pulmonary tuberculosis patients were enrolled at the two sites. Drug susceptibility carried out showed that among the samples analyzed, 68 (86.08%) were susceptible to all TB drugs tested, while 11 (13.92%) were resistant to at least one of them. Resistance to streptomycin was the most frequent (8.86%), followed by resistance to isoniazid (5.06%). Identification by PCR using specific markers as *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* revealed that the mycobacterium strains belonged to the MTBC. In short, identification by PCR using these specific markers revealed that mycobacterium species responsible for pulmonary tuberculosis in patients from Jamot and Mbalmayo District Hospital belonged to the MTBC. Also PCR technique is more rapid compared to the PNP inhibition test.

Key words: *Mycobacterium tuberculosis* complex, polymerase chain reaction (PCR), Cameroon.

INTRODUCTION

Tuberculosis (TB) remains a major global health problem caused mainly by *Mycobacterium tuberculosis*. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people). The probability of developing TB is much higher among people infected with HIV. The number of TB deaths is unacceptably large given that most are preventable. The rate of new TB cases has been falling worldwide for about a decade, achieving the millennium development goal global target. TB incidence rates are also falling in all six WHO regions. The rate of decline (2% per year) remains slow. An estimated 1.1 million (13%) of the 8.6 million people who developed TB in 2012 were HIV-positive. About 75% of these cases were in the African Region (WHO, 2013). The majority of cases worldwide in 2012 were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26 and 12% of total cases, respectively (WHO, 2013).

Cameroon is an independent nation situated in the Central Africa. Tuberculosis (TB) has emerged as a major public health problem in Cameroon and other border countries due to economic decline and the general failure of TB control, and other health services following the economic crisis in the year 1980s. The current incidence of TB in Cameroon has been reported to be about 238 cases per 100 000 population (WHO, 2013). The emergence of multidrug-resistant TB (MDR-TB) has also been documented in Cameroon. According to the National TB Control Programme (NTBCP) in 2012; 25, 576 new sputum smear-positive were reported among all TB cases despite the implementation of the directly observed treatment short course strategy (DOTs) by the NTBCP in 2003 (WHO, 2013).

Tuberculosis is an infectious disease caused by a mycobacterium belonging to *M. tuberculosis* complex (MTBC) which includes: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti* (Singh et al., 2006). Identification of *M. tuberculosis* complex is performed routinely by conventional techniques taking into account cultural (growth rate, colonies morphology and pigmentation) drug susceptibility testing (DST) and biochemical characters. Conventional methods for differentiation of MTBC are time consuming thus, it is imperative to develop and implement a rapid technique that is able to

identify the MTBC.

Advances in molecular biology and knowledge on the genome of *M. tuberculosis* complex have enabled the development of faster identification techniques. Molecular methods like PCR are increasingly used for rapid laboratory diagnosis of TB; different target sequences have been evaluated for detection of MTBC/differentiation between MTBC and NTM (van Embden et al., 2000). In the present study, a combination of 5 targets sequences (hupB, IS6110, IS1081, oxyR, rpoB) was used to detect MTBC in positive cultures by PCR and agarose gel electrophoresis. The five targets were used to see the results are identical to those of the conventional methods and conclude that each of the markers can be used to differentiate the MTBC to other mycobacteria.

MATERIALS AND METHODS

Study setting and design

This was a cross-sectional study involving all pulmonary smear positive patients, age ≥ 15 years attending Jamot and Mbalmayo District hospitals from April to June 2010. All patients underwent physical examination and their histories were recorded. The chosen study sites was Jamot and Mbalmayo District hospitals. All the sputa collected from the enrolled patients were evaluated for acid fast bacilli (AFB). Sample processing, confirmatory microscopy, culture, drug susceptibility testing and quality control were performed at the Mycobacteriology Laboratory of the Centre Pasteur of Yaoundé (Cameroon).

A study questionnaire was designed for patient data collection at each study site including sex, age, marital status, educational level, residence (urban vs. rural) and clinical data (previous TB treatment). The study was funded by Central Africa Network for Tuberculosis, AIDS/HIV and Malaria (CANTAM) a network sponsored by the European Developing Countries Clinical Trials Partnership (EDCTP).

Ethical clearance and administrative authorization

Ethical clearance N° 126/CNE/SE/09 and an administrative authorization N° 631. 7-10 were respectively obtained from the Cameroon National Ethic Committee and the Cameroonian Ministry of Public Health. Signed informed written consent was obtained from each enrolled patient.

Sample processing

Ziehl-Neelsen and/or auramine smear examinations were performed at the recruitment sites (Soini and Musser, 2001). Only samples with the highest smear grade were transported in a cold box to the Centre Pasteur du Cameroun (CPC, Yaoundé) for confirmatory microscopy, culture and drug susceptibility testing (DST). Each specimen was submitted to a decontamination step

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Table 1. Characteristics of different primers.

Target gene	Primers	Primer sequence	Size (bp)	Reference
<i>hupB</i>	<i>hupBF</i>	5'GGAGGGTTGGGATGAACAAAGCAG-3'	645	Prabhakar et al. (2004)
	<i>hupBR</i>	5'GTATCCGTGTGTCTTGACCTATTTG-3'		
<i>IS6110</i>	<i>IS6110F</i>	. <i>IS6110F</i> 5'-CCTGCGAGCGTAGGCGTCGG-3	123	Eisenach et al. (1990)
	<i>IS6110R</i>	<i>IS6110R</i> 5'-CTCGTCCAGCGCCGCTTCGG-3 '		
<i>IS61081</i>	<i>IS61081F</i>	5'CGACACCGAGCAGCTTCTGGCTG-3'	306	Niyaz et al. (1998)
	<i>IS61081R</i>	5'GTCGGCACCACGCTGGCTAGTG-3'		
<i>oxyR</i>	<i>OxyRF</i>	5'-GCGTCTGGTCGCGTAGGCAGTG-3'	473	Mokaddas et al. (2007)
	<i>OxyRR</i>	5'GTGAAGTAGTCGCCGGGCTGCT-3'		
<i>rpoB</i>	<i>rpoBF</i>	5'TACGGTCGGCGAGCTGATCCAAA-3'	235	Mokaddas et al. (2007)
	<i>rpoBR</i>	5'ACAGTCGGCGCTTGTGGGTCAAC-3'		

using lauryl sulfate sodium. Quality control procedures (internal and external) were conducted at the mycobacteriology laboratory of the CPC.

Sputum culture and identification

After centrifugation, the sediment of the decontaminated product was used to inoculate two Löwenstein Jensen culture media, with an additional one supplemented with a 0.65% solution of sodium pyruvate. Cultures were incubated at 37°C, read weekly for growth and considered negative when no colony was obtained after 8 weeks of incubation. The PNB inhibition test was used to distinguish non tuberculous mycobacteria from *M. tuberculosis* complex.

Drug resistance assays

For all positive cultures, drug susceptibility testing was performed using the indirect proportion method on Löwenstein Jensen media at the following drug concentrations: isoniazid (H1: 1 mg/ml and H2: 2 mg/ml), streptomycin (S: 4 mg/ml), Rifampicin (R: 40 mg/ml), ethambutol (E: 2 mg/ml), ofloxacin (2 mg/ml), kanamycin (K1: 30 µg/ml and K2: 20µg/ml) (Canetti et al., 1963). Drug resistance was defined as growth on a drug containing medium greater than or equal to 1% for INH and RIF, and 10% for SM and EMB (Canetti et al., 1963).

DNA extraction

Colonies were scraped from *Lowestein-Jensen* slopes, collected into Eppendorf tubes containing Tris-EDTA (10 mM, 1 mM, pH 8) and heated for 30 min at 90°C. After centrifugation at 13,000x g, the supernatant was collected into a new tube and kept at -20°C until further use.

PCR amplification of target sequences and detection of PCR products by gel electrophoresis

The specific primers of markers *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* were used (Table 1). A total of 13 µL PCR mix per sample

was prepared with 6.25 µL of ready mix RedTaq, 5.625 µL of PCR water, forward and reverse primers of 0.125 µL in equal quantities and 1 µL of lysate. Distilled water was used as negative control and *M. tuberculosis* H37 Rv strain was used as positive control. The amplification according to each target sequence was done as follow:

For *hupB* and *rpoB* targets, initial denaturation was done at 95°C for 10 min, followed by 35 cycles at 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), and a final extension at 72°C for 7 min.

For *IS6110* target, initial denaturation was done at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

For *IS1081* target, initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min were done.

For *oxyR* target, initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min were done.

After amplification, 5 µL of the amplicon were mixed with 15 µL of loading buffer for electrophoresis on 2% agarose gel prepared with ethidium bromide. Visualization was done under the ultraviolet transilluminator (BIO-RAD).

RESULTS

From April to June 2010, 79 smear positive pulmonary tuberculosis cases were enrolled at both study sites. Males represented 53.16% (42/79) of the case versus, 46.84% (37/79) for females. Patient's age ranged from 15 to 60 years.

Culture and phenotypic characteristics

All 79 smear positive samples yielded positive cultures as shown in Figure 6 after two weeks for some samples and one month for others. Then, PNB identification and Valid DST results were available for all the 79 strains after one month. All the above samples were sensitive to PNB. However, 68 strains (86.08%) were sensitive to the six



Figure 1. PCR amplicon profiles with *hupB* primers (100 bp, DNA ladder; Column 1, positive Control; Column, 2-15: Amplicons).

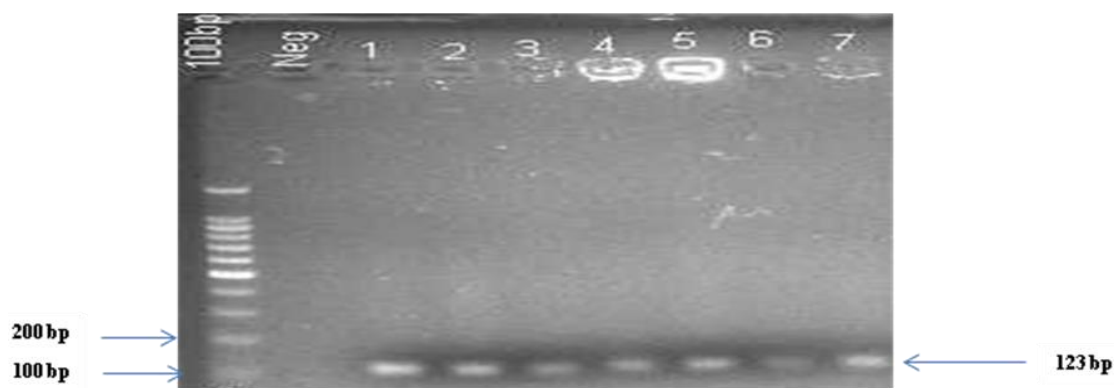


Figure 2. PCR amplicon profiles using *IS6110* primers (100bp: DNA ladder; Neg, négative Control; Column 1, positive Control; Column 2-7, Amplicons).

drugs tested against 11 strains (13.92 %) resistant to at least one drug. Resistance rates were highly observed respectively to streptomycin (8.86%) and isoniazid (5.06%). No resistance to rifampicin, ethambutol, ofloxacin and kanamycin was noted.

PCR amplification of target sequences *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*

The results of the detection of PCR products by agarose gel electrophoresis have allowed visualization, for each primer, a band of different size and intensity. Thus, application of amplification's technique on *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* markers by PCR, followed by electrophoresis on 2% agarose gel allowed the identification of all samples tested. The target bands, specific for each primer of the *M. tuberculosis* complex were expressed in base pairs (bp) (Figures 1 to 5).

The expected sizes of PCR products were 645 bp for *hupB* after 122 min of amplification, 123 bp for *IS6110* after 102 min, 306 bp for *IS1081* after 102 min, 473pb for *oxyR* after 102 min and 235 bp for *rpoB*. PCR products from positive control and all target sequences obtained with all 79 samples had the sizes expected after an hour of electrophoresis. Nothing was obtained from negative controls. Thus, the analysis of 79 amplified samples by gel electrophoresis showed that all bands had the same base pair (bp) numbers according to each primer as shown in the Figures 1 to 5.

DISCUSSION

In countries with a high prevalence of tuberculosis, the majority of cases of pulmonary tuberculosis in adults are sputum smear-positive (Ait-Khaled and Enarson, 2003). From this study with 79 clinical evidences of pulmonary

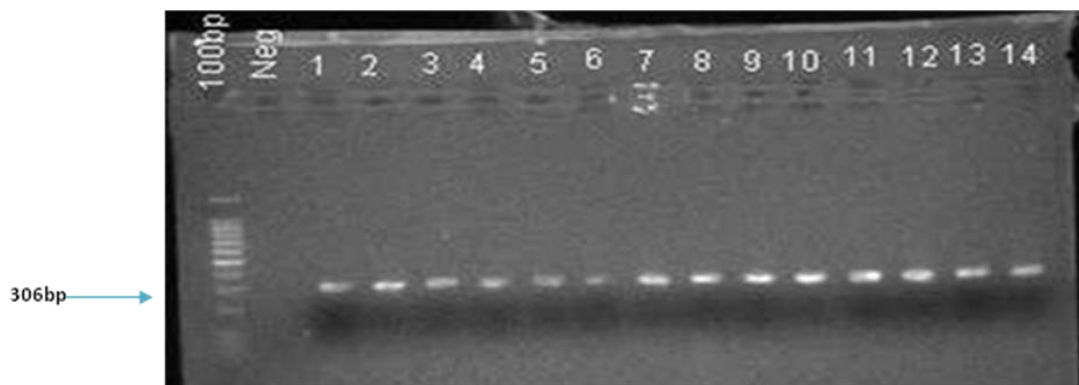


Figure 3. PCR amplicon profiles using *IS1081* primers (Neg, negative control; 1, positive control).



Figure 4. PCR amplicon profiles using *oxyR* primers (Neg, negative control; A, positive control; B-N, amplicons)

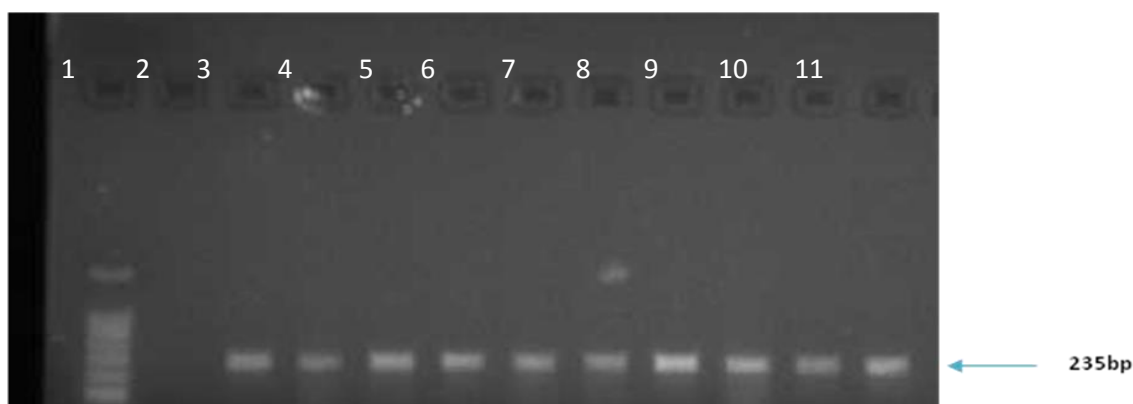


Figure 5. PCR amplicons profile using *rpoB* primers (100 bp, DNA ladder, Column 1, negative control; Column 2, positive control; 3-11, Amplicons)

tuberculosis confirmed, men were more infected 42/79 (53.16%) than women 37/79 (46.84%). The experience of the World Health Organization (WHO) since 1997 in

assisting high TB burden countries to set up standardised TB surveillance systems shows that globally, men account for a higher proportion of notified TB cases (The

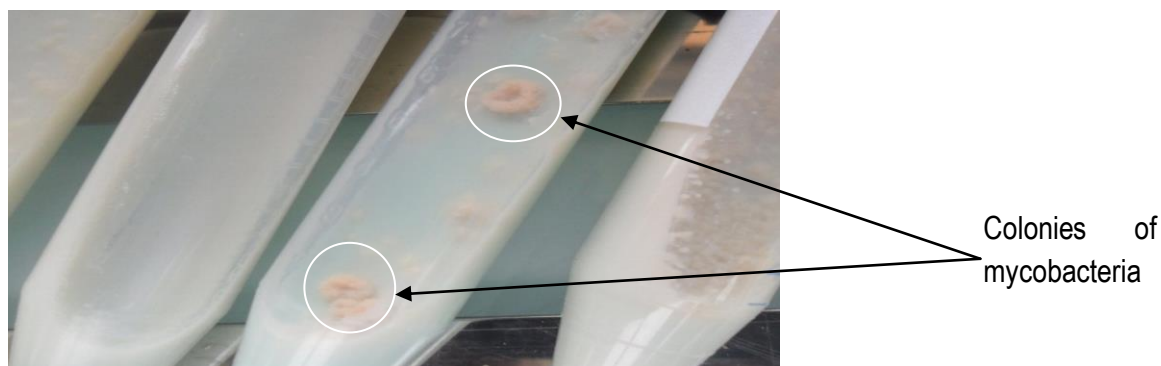


Figure 6. Aspects of some mycobacterial cultures growing.

Union, 2008). These results are similar to another recorded in South Africa which showed a high predominance of pulmonary TB in male (Dheda et al., 2009). This is due to specific factors related to socio-cultural phenomena (Godfrey-Faussett et al., 2000) that contribute to the acceleration of the TB epidemic and in addition, the general factors such as poor health infrastructure, a poor organization and management, poverty, weak health systems and poor management of human resources.

For culture, growth of *M. tuberculosis* complex species was inhibited by p-nitrobenzoic acid (PNB), thus confirming their presence. However, non-tuberculous mycobacteria are resistant (Giampaglia et al., 2007).

The use of PCR amplification technique for the routine diagnosis of TB has been improved in recent years. The results and interpretation of PCR may be shortly summarized. The results obtained here are similar with respect to amplicon sizes to those of Prabhakar et al. (2004) for *hupB* (645 bp); of Eisenach et al. (1990) and Sekar et al. (2008) for *IS6110* sequence (123 bp); of Van Soolingen et al. (1992) and Bahador et al. (2005) for *IS1081* (306 bp); finally of Mokaddas et al. (2007) for *oxyR* (473 bp) and *rpoB* (235 bp).

Since *rpoB* has been published to have correlation between its mutations and rifampicin resistance (Halse et al., 2010) and used in differentiation of MTBC from non-tuberculosis mycobacteria, it is an essential gene that is uniformly present in all mycobacterial species. The chance occurrence of nearly identical sequences in the *rpoB* gene corresponding to MTBC-specific primers may result to misidentification of some non tuberculous mycobacteria as MTBC (Mokaddas and Ahmad, 2007).

For primer *oxyR*, the expected target characterizing the presence of *M. tuberculosis* complex species in all sample tested confirms the results reported by Mokaddas et al. (2007) who used the same primer to differentiate *M. tuberculosis* complex species of non-tuberculous mycobacteria and got the same band at 473 bp.

The insertion sequences for instance *IS1081* and *IS6110* were reported to be specific for *M.*

tuberculosis complex and hence are extensively exploited for laboratory detection of the agent of tuberculosis and for epidemiological investigations based on polymerase chain reaction.

Amplification of the fragment of the *IS6110*, which is (Das et al., 1995) specific for the *M. tuberculosis* complex, belongs to IS_3 family and is found in almost all members of the *M. tuberculosis* complex. Most strains of *M. tuberculosis* carry 10 to 15 copies of *IS6110*, this characteristic helps to increase sensitivity of PCR over that obtained in amplification of a single DNA sequence (Gill et al., 2012).

Despite the results with others primers in this study, PCR targeting *IS6110* has shown higher positivity than PCR for other targets. Also the methodology of PCR for *IS6110* has been widely carried out in different technical set up and has been proved to be simple and reproducible, compared to methodologies for PCR (Negi et al., 2007) targeting other gene sequences.

The similarity at points of migration on the gel with identical bands, 645 bp for *hupB* leads us to conclude that the species belong to *M. tuberculosis* complex. Knowing that the *IS6110* sequence identifies the *M. tuberculosis* complex and the *HupB* establishes the differences between *M. tuberculosis* and *M. bovis* (Prabhakar et al., 2004; Mishra et al., 2005), it is likely that *M. tuberculosis* is responsible for tuberculosis in patients involved in this study since MTB is the most common species and most frequently encountered in the Centre region of Cameroon (Assam et al., 2011). However to be sure, a study of genomic characterization using techniques such as line-probe assays should be required.

This study demonstrates the wide distribution of the *M. tuberculosis* complex species in the areas of Jamot and Mbalmayo District Hospitals. These results confirm the specificity of the PCR and the identity of all strains tested as species belonging to the *M. tuberculosis* complex. For this study, PCR technique has proven useful for the detection of *M. tuberculosis* complex with a sensitivity of

100% both for all strains. This may indicate that a single agent would be responsible for TB in all patients, therefore homogeneity of the mycobacterial population. The high activity of the PCR would be obtained due to multiple copies of the target sequence frequently present in the genome of mycobacteria. The routine hospital's technique requires use of conventional species-specific identification, for instance PNB in the growing media of mycobacteria to identify the species present since most mycobacterial infections are still caused by MTBC. Then, conventional species-specific identification and proper patient management are delayed due the slow growing nature of mycobacteria (Mokaddas and Ahmad, 2007). The nucleic acid amplification using specific primers provides a good opportunity to identify the *M. tuberculosis* complex from cultures of MTB. The amplification of the target sequence is a quick and relatively simple and the results are available within a day instead of several weeks as the identification tests with PNB. As PCR can detect both living and dead mycobacteria (Bauman et al., 2003), some patients may remain PCR-positive for mycobacterial DNA for several months after treatment. This study shows the importance of PCR in the diagnosis and identification of mycobacterial species, and thus can allow establishment of an appropriate anti-tuberculosis chemotherapy early. The introduction of the PCR technique in health services may increase the number of tuberculosis cases treated and can also reduce the time required for diagnosis of tuberculosis.

Conclusion

In short, identification by PCR using specific makers as *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* revealed that the mycobacterium species responsible for pulmonary tuberculosis in patients from Jamot and Mbalmayo District Hospital belonged to the MTBC. This technique is more rapid compared to the identification test with Para nitro benzoic acid (PNB).

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Novel lipid-based dermal microgels of Neobacin[®]

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This study investigates the potential of novel microgels based on solid lipid microparticles (SLMs) as a sustained delivery system for neobacin[®], a topical antibiotic drug powder. Matrices generated from sunseed oil and goat fat (1:9, 2:8 and 3:7) was surface-modified with Phospholipon[®] 90G and employed to formulate SLM-based microgels. The microgels were characterized in terms of *in vivo* wound healing in rats, *in vitro* permeation, membrane drug retention studies and antimicrobial activity against various microorganisms using standard cup-plate agar diffusion method. The 3:7 microgels exhibited sustained release property, achieving 34% drug permeation over 12 h, 64% membrane drug retention and largest growth inhibition zone diameters (IZD) on all organisms, whereas commercial neobacin[®] gel achieved 35% drug permeation at 4 h and 72% membrane drug retention. *In vivo* wound healing followed this order 3:7>1:9>2:8 better than neobacin[®] powder. Neobacin[®] microgel formulation despite rapid degradation possessed greater wound healing and antimicrobial property than the conventional powder form of neobacin[®].

Key words: Microgels, surface-modified solid lipid microparticles, sustained release, neobacin[®].

INTRODUCTION

Skin is the largest, easily accessible organ of the body for local and systemic drug administration with numerous advantages compared to other routes (Souto et al., 2011; Reszko et al., 2009). However, it is an excellent barrier that naturally prevents transport of molecules into and out

of the body despite several strategies proposed to overcome the barrier functions of the *stratum corneum* (Higaki et al., 2005; Trommer and Neubert, 2006).

Nano/micro carriers have shown skin penetration enhancement properties and include nano/micro-

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Abbreviations: SMSLMs, Surface-modified solid lipid microparticles; SLMs, solid lipid microparticles; P90G, Phospholipon[®] 90G; IZD, inhibition zone diameters; IU, International units; MWCO, molecular weight cut-off; SRMS, solidified reverse micellar solutions.

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emulsions, solid lipid nano/micro-particles produced from lipid materials of various fatty acids, triglycerides, oils, phospholipids and waxes of GRAS status (Acosta et al., 2011; Lv et al., 2009). However, these liquid or colloidal dispersions have low viscosity, thus providing only a short-term adherence onto the skin; hence inconvenient for applications. Incorporation into semi-solids (for example, creams) or hydrogels can increase adhesivity and convenience for use. Recently, many products that take advantage of nanotechnology have been manufactured (Lv et al., 2009; Senyigit et al., 2010; Eskandar et al., 2010; Pardeike et al., 2009; Korting and Schafer-Korting, 2010).

In practice, a wide range of antibacterial action is provided by the overlapping spectra of bacitracin and neomycin (neobacin[®]) in the treatment of infections. Neobacin[®] which is available as a powder, 5 g for wound dressing, contains 5 mg of neomycin sulphate and 250 IU of bacitracin zinc in each gram of powder. But as a dressing powder, it is without moisture, thus impedes fast migration of epithelial cells to the wound surface thereby delaying healing as a result of scab formation. In addition, it causes allergic dermatitis especially in disrupted skin (Fox et al., 2006; Dainiak et al., 2010; Atiyeh et al., 2009). Development of an alternative to powder-type neobacin[®] dosage form that would provide sustained delivery is a major challenge. We report here, surface-modified solid lipid microparticles (SMSLMs) of neobacin[®] generated from a blend of liquid (sunseed oil) and solid (goat fat) lipids at 1:9, 2:8 and 3:7 respectively, surface modified with phospholipid and Tween 80 as a mobile surfactant. The objective of the study was to confer some hydrophobic character to the freely water soluble neomycin sulphate to enhance its permeation through the skin for better drug release. To improve adhesivity, convenience and moist-driven wound healing devoid of scabbing, we therefore, encapsulated the SMSLMs into Carbopol 940[®] hydrogels (microgels).

MATERIALS AND METHODS

The materials include Neobacin[®] powder (Drugfield Pharmaceuticals Ltd, Nigeria), Carbopol 940[®] (B. F. Goodrich, U.S.A.), propylene glycol, triethanolamine (Spectrum Chem. Mfg. Corp., California), ethanol (BDH, England), polysorbate 80 (Uniqema, Belgium), Phospholipon[®] 90G (Phospholipid GmbH Köln, Germany), sunseed oil (double refined) vitamin A fortified (Kelwaram Chanrai Group Lagos, Nigeria), and goat fat (Pharmaceutics Laboratory, University of Nigeria). Distilled water (Lion water, Nigeria) was used for all preparation.

Lipid matrix preparation and formulation of SLMs

Goat fat was obtained as earlier described (Attama et al., 2007; Nnamani et al., 2010; Attama and Müller-Goymann, 2006). Mixtures of sunseed oil and goat fat in the ratio of 1:9; 2:8 and 3:7 were prepared by fusion at 60°C on a thermo-regulated water bath

shaker (Heto, Denmark) and stirred until solidification. Surface-modification was done with 20% phospholipid (SRMS). The lipid matrices were characterized in a differential scanning calorimetry (DSC) machine (NETZSCH DSC 204 F1, Germany) at 35-190°C under a 20 ml/min nitrogen flux at a heating rate of 10°C/min. All determinations were baseline-corrected.

SLMs were formulated to contain 5% w/w of each SRMS, 1.5% w/w polysorbate 80 and enough distilled water to make 100% (w/w). By adding neobacin[®] (10% w/w) to each of the SRMS prior to SLM production by melt emulsification, neobacin- loaded SLMs were obtained. The thermal properties of all SLM formulations were determined by DSC.

Characterization of SLMs

Particle size, injectability, time-dependent pH stability and storage stability studies of samples stored at room and refrigeration temperatures were carried out. The drug encapsulation efficiency was determined indirectly after centrifugation in a membrane concentrator (MWCO 5000, Vivascience AG, Hannover, Germany). Drug concentration of the aqueous continuous phase was determined by a UV/Vis spectrophotometer (Unico 2102, England) at 252 nm considering the initial amount of drug loaded using the equation:

$$EE (\%) = \frac{\text{Real drug loading}}{\text{Theoretical drug loading}} \times 100 \quad (1)$$

Preparation of microgels

Carbopol 940[®] (1% w/w) was dispersed in sufficient distilled water and stirred on a magnetic stirring device (SR 1UM 52188, Remi Equip., India) at 600 rpm for 30 min. Propylene glycol (10% w/w) was added and the mixture further neutralized with triethanolamine to pH 5.5. Neobacin[®] drug powder was incorporated into one batch of hydrogel while plain hydrogel batch (not containing drug or SLMs) was also produced. To prepare microgels, Carbopol 940[®] was dispersed in 85 ml of the SLMs (with/without drug) and stirred thoroughly to ensure uniform dispersion. All products were dispensed in lacquered aluminium tube, securely closed and stored at room temperature until used. All formulations were 100% dispersions according to Table 1.

Characterization of microgel formulations

Physical examination

The semi-solid formulations were physically examined for colour, homogeneity, spreadability and consistency. The pH was also evaluated.

In vitro permeation studies

Franz diffusion cells with a receiver compartment volume of 20 ml and effective diffusion area of 2.84 cm² were used to evaluate drug delivery characteristics from the selected compositions. A Millipore membrane (0.22 µm), (Millipore Corporation, Billerica, MA) was used. The receptor phase (phosphate buffer solution, PBS, pH 7.4) was continuously stirred and kept at a temperature of 37 ± 0.1°C during the experiments. 0.5 g of the semi-solid products equivalent to 1 g of drug powder was spread uniformly on the membrane

Table 1. Composition of various optimized SLMs and semi-solid formulations.

Ingredients (g)	SMSLM compositions (g)						Neobacin hydrogel	Plain hydrogel	Neobacin [®] powder
	SLM-0			SLM-neobacin [®]					
Sunseed oil	1	2	3	1	2	3	-	-	-
Goat fat	9	8	7	9	8	7	-	-	-
Lipid blend	4	4	4	4	4	4	-	-	-
Phospholipon [®] 90G	1	1	1	1	1	1	-	-	-
Neobacin [®]	-	-	-	10	10	10	10	-	-
Polysorbate 80	1.5	1.5	1.5	1.5	1.5	1.5	-	-	-
Distilled water	88.5	88.5	88.5	73.5	73.5	73.5	-	-	-
Microgel compositions (g)									
SMSLMs	85	85	85	85	85	85	-	-	-
Carbopol 940 [®]	1	1	1	1	1	1	1	1	-
Propylene glycol	10	10	10	10	10	10	10	10	-
Triethanolamine	4	4	4	4	4	4	4	4	-
Distilled water	-	-	-	-	-	-	75	85	-

placed in the donor compartment. At appropriate time intervals, 1 ml of the sample was withdrawn from the receiver compartment and the same amount of fresh solution was added to maintain sink condition. Each experiment was run in three independent cells. The samples were analyzed spectrophotometrically at a wavelength of 252 nm and the concentration of neobacin[®] in each sample was determined from a standard curve. Each data point represents the average of three determinations. The release study was carried out for 12 h period.

Skin retention studies

The Millipore membrane (0.22 µm) from each product batch of *in vitro* permeation study was removed, gently scraped with spatula to remove all remaining residues and further cleaned with cotton wool soaked in PBS before crushing. To the crushed Millipore membranes contained in different volumetric flasks, 50 ml of ethanol was separately added and mechanically shaken in a water bath shaker (Heto Denmark) at 37°C for 1 h. After which they were filtered using a Whatman No. 1 filter paper and the filtrate analyzed using UV spectrophotometer. The concentration that was retained and further eluted from the membrane was calculated using standard Beer's plot and compared with the drug concentration that permeated through the skin *ab initio*.

In vivo wound healing assay

The wound-healing properties of the microgel formulations were investigated on wound-inflicted rats and the efficacies of the formulations were compared. Thirty six adult (age: 6-10 weeks old) Wistar albino rats of both sexes (180 -250 g) were divided into nine groups of four rats each. Four groups served as controls, one group as standard and the other four as test groups. The hair around the left flank was shaved with shaving sticks and swabbed with cotton wool. A template disc (diameter 30 mm) was placed and outlined on the skin. Full thickness wounds were produced by excising the skin with sterile surgical blades. The wounds were immediately covered with a superficial smear of the semi-solid preparations (0.25 g)

equivalent to 0.5 g of drug powder. A comparative assessment was done using the commercial drug powder. The treatment was in accordance with Table 1. The animals were on normal feed, clean water, and the formulations were smeared on the afflicted wounds each alternate day. Wound diameter measurements were taken before each application in triplicates and the average taken. Housing was by battery cage system. This animal experiment complied with the University of Nigeria recommendation of the committee for safe handling of experimental animals and the protocol approval was obtained.

In vitro antimicrobial activity of the formulations

The antibacterial activity of various semisolid formulations of neobacin[®] against various clinical isolates of microorganisms (*Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Escherichia coli*) was evaluated by the standard cup-plate method and the inhibition zone diameters measured. The organisms were seeded in sterile nutrient agar, swirled for uniform distribution and allowed to set at room temperature. Cork borer of diameter 6 mm was used to bore holes in the set agar. A stock solution of neobacin[®] (0.25 µg/ml) was prepared by dissolving 50 mg of neobacin[®] powder in a litre of distilled water and two-fold serial dilution was done to obtain different dilutions of the drug. The various neobacin[®] concentrations were dropped into the wells of the seeded plates, and incubated after 30 min at a temperature of 37°C ± 0.5 for 24 h.

Statistical analysis

All experiments were performed in replicates for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and Student's t-test were performed on the data sets generated using SPSS. Differences were considered significant for p < 0.05.

RESULTS

The thermal property of goat fat has been described

(Attama et al., 2007; Nnamani et al., 2010; Attama and Müller-Goymann, 2006) whereas sunseed oil had an endothermic peak at 39.3°C. The physical mixtures of sunseed oil and goat fat at 1:9, 2:8, and 3:7 respectively showed melting peaks at 52.13, 51.4 and 50.9°C (Figure 1). The SRMS of 1:9 matrices melted at 51.3°C whereas that of 2:8 had peak at 51.2°C while SRMS 3:7 melted at 48.7°C. The DSC thermogram of pure neobacin[®] powder showed two sharp endothermic peaks at 149°C with an enthalpy of -4.711 mW/mg and 214.5°C with an enthalpy of -4.29 mW/mg while an exothermic peak appeared in between the endothermic ones at 176.8°C with an enthalpy of -0.1252 mW/mg.

For SLM-O formulated from 1:9 matrix, two broad endothermic peaks were observed at 78.0 and 123.7°C with enthalpies of -25.94 and -8.425 mW/mg, respectively. The drug-loaded SLMs produced from this matrix showed endothermic peaks at 53.8 and 80.6°C. The melting endotherms of the SLM-O from SRMS 2:8 showed broad peaks at 52.6, 89.9 and 116.7°C while the corresponding drug-loaded SLMs showed sharper endothermic peaks at 57.4, 86.3 and 122°C. However, the SLM-O produced from SRMS of 3:7 showed broad endothermic peaks at 88.8 and 118.9°C while the corresponding SLM-drug-loaded sample showed an endothermic peak at 126.0°C but with an earlier shoulder of broad peak which occurred at 108.2°C due to an unstable modification.

Table 2 shows that the SMSLMs containing no neobacin[®] (SLM-O) were larger than those containing neobacin[®] (SLM-Drug). This trend was again observed when the samples were re-analyzed after one month of storage. Photomicrographs (Figures 2 and 3) showed that the SLMs were smooth, well-formed and monodispersed. It was observed that after 3 months, all the batches formulated with SRMS 1:9 had pH in the range of 4.90±0.05 to 5.30±0.01; those formulated with SRMS 2:8 had pH in the range of 4.80±0.05–5.25±0.10, whereas the pH of SLMs formulated with SRMS 3:7 ranged from 4.70±0.10 to 5.50±0.1. The syringeability study showed that the drug-loaded SLMs that appeared smaller in size required bigger gauge needle (23 G) to be syringeable within 20 s while the non-drug-loaded SLM samples (SLM-O) were syringeable with smaller needle of 25 G.

Table 2 shows the encapsulation efficiency of the formulations. It was evident from the table that the drug encapsulation efficiencies in the formulations were very poor and varied inversely with the proportion of sunseed oil to goat fat (1:9>2:8>3:7) respectively.

The microgels had uniform colour, is somewhat white for the microgels, cream to off-white colour for the plain hydrogel containing neobacin[®] powder (neobacin[®] gel) and transparent for gel without neobacin[®]. They had good consistency and were quite spreadable with pH constantly in an acceptable range of 5.5 ± 0.25.

Figure 4 comparatively depicts the release of neobacin[®] from all formulations. The result shows that neobacin[®] gel achieved a maximum release of 35% at 4 h attaining faster peak than those encapsulating SMSLMs; but immediately degraded just at the point the microgels were starting to release the embedded drug at 10 h. The microgels generally showed slow release of the entrapped drug and those formulated from the 3:7 SRMS matrices achieved maximum release of 34% at 12 h, despite the low percentage encapsulation efficiency.

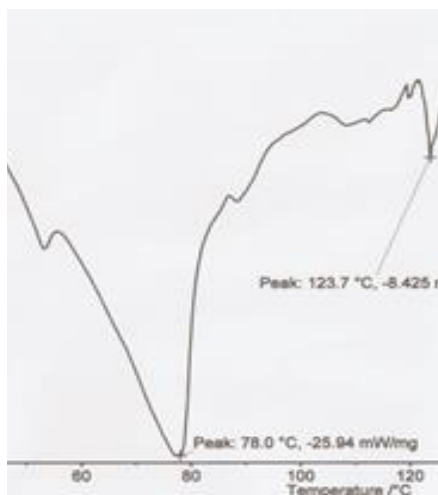
Table 3 shows the result of the Millipore membrane retention studies. Neobacin gel had the highest concentration of neobacin[®] (72%) retained on the membrane followed by neobacin microgel formulated from SLMs of SRMS 3:7 matrix (64%) while SLMs of 1:9 SRMS matrix had the least (27%).

The result of the *in vivo* wound healing study is depicted in Figure 5. It is discernible from the figure that the wounds of the rats treated with neobacin microgels (3:7>1:9>2:8) healed faster than neobacin[®] powder. At day 12, maximum healing of 70% was recorded for the formulation produced from 3:7 SRMS whereas the drug powder could only achieve 60 % wound healing. Neobacin[®] gel also achieved 60 % release though at day 8 instead of day 12.

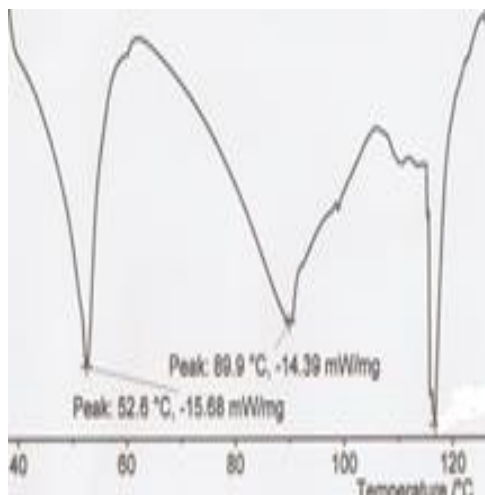
The formulations had various degrees of microbial growth inhibition (Table 4). Among the neobacin microgels, the batch formulated from SRMS 3:7 had the highest growth inhibition diameters (IZD) on all microorganisms followed by the neobacin[®] gel formulation while that of 2:8 microgel was the least. The drug powder showed the highest IZD of 26.11 mm on *Staphylococcus aureus* only, followed by the neobacin microgels of 3:7 which recorded 25.32 mm on the same organism (Figure 5).

DISCUSSION

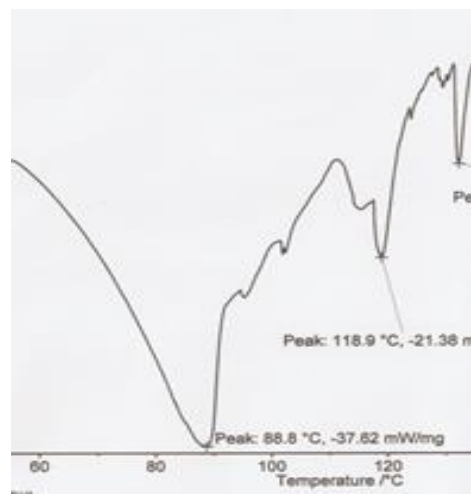
Surface-modification of the physical mixtures by phospholipid (SRMS) generated matrices that melted at lower temperatures (Attama et al., 2007; Nnamani et al., 2010; Attama and Müller-Goymann, 2006). The characteristic thermogram of neobacin[®] powder could be due to the fact that it is a combination drug. This shows that neobacin[®] loadings resulted in lower temperature of melting suggesting that the matrix generally produced less ordered crystals that required lower energy to overcome the lattice forces than the more rigid (crystalline) substance entirely contained in the SLM-O. The high enthalpy values generally observed with the drug-loaded SLMs suggest low entrapment of neobacin[®] in the matrices (especially the 3:7) due to inherent solubility problem of the drug. Although the SLMs were stable, monodispersed, smooth and well-formed, some appeared clustered showing tendency of an intending



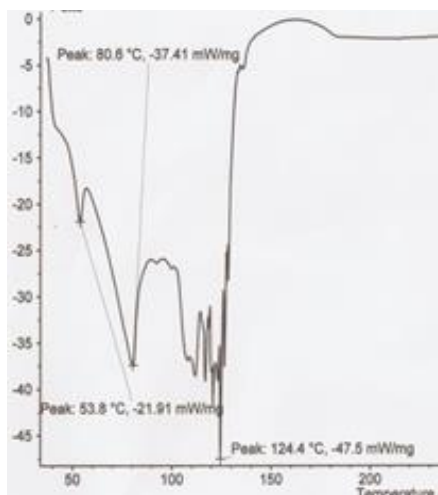
SLM-O from SRMS 1:9



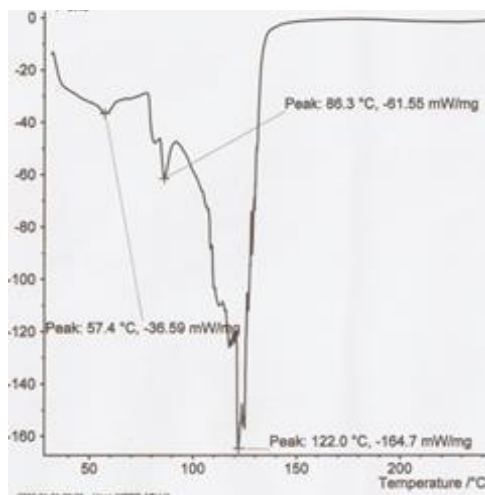
SLM-O from SRMS 2:8



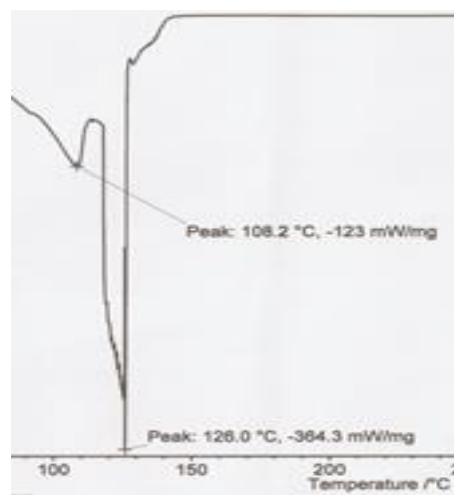
SLM-O from SRMS 3:7



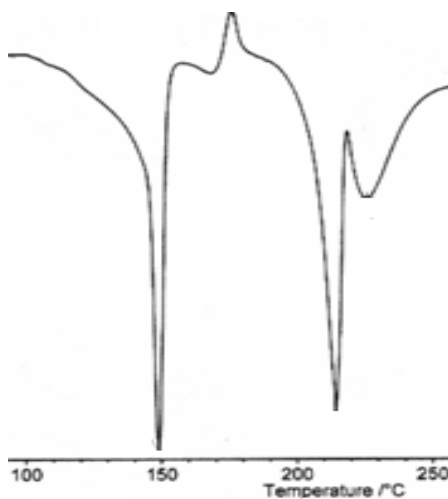
SLM-Drug from SRMS 1:9



SLM-Drug from SRMS 2:8



SLM-Drug from SRMS 3:7



Pure neobacin® powder

Figure 1. DSC thermograms

Table 2. Properties of the formulated SLMs.

Batch	LM	Average particle size (µm)		EE (%)	S (Guage)	Thermal properties	
		4°C	25°C			M.pt (°C)	Enthalpy (-mW/mg)
SLM – O	1:9	21.67±0.01	32.35±0.02	-	25	78; 123.7; 131.8	25.94; 8.42; 5.38
SLM – O	2:8	20:68±0.01	28.16±0.02	-	25	52.6; 89.9; 116.7	15.68; 14.39; 17
SLM – O	3:7	19.69±0.01	19.85±0.01	-	25	85.8; 118.9; 132.3	37.62; 21.38; 13
SLM – D	1:9	12.50±0.02	19:17±0.03	5.9	23	58.8; 80.6; 124.4	21.91; 37.4; 47.5
SLM – D	2:8	15.00±0.01	20.02±0.03	5.0	23	57.4; 86.3; 122	36.59; 61.55; 164
SLM – D	3:7	18.08±0.01	19.83±0.01	4.3	23	126.0; 108.2	123; 364.3

LM = lipid matrix; EE = encapsulation efficiency; S = syringeability; SLM-D = neobacin[®]-loaded solid lipid microparticles; SLM-O = Zero-drug SLMs; M.pt = melting point, values represented with corresponding enthalpies in order of appearances.

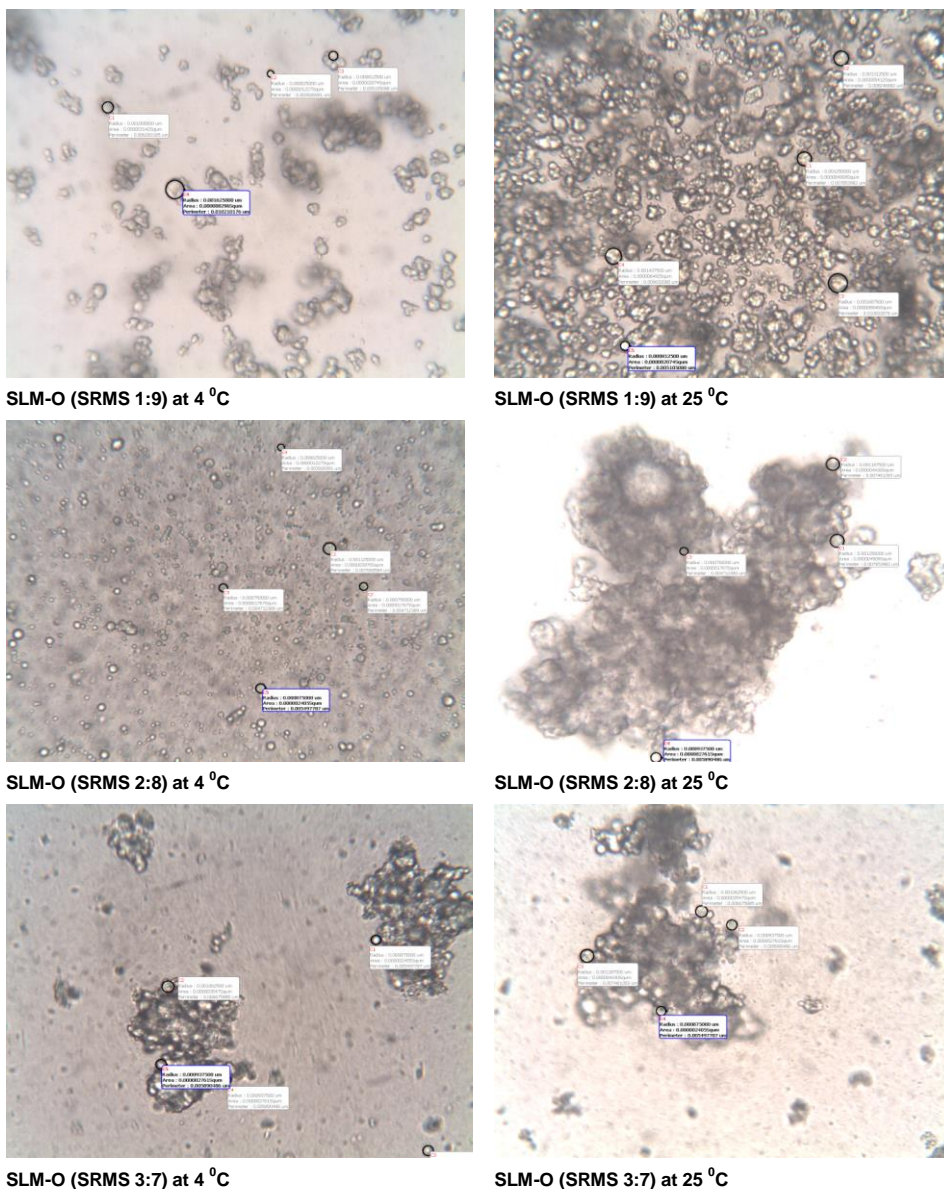
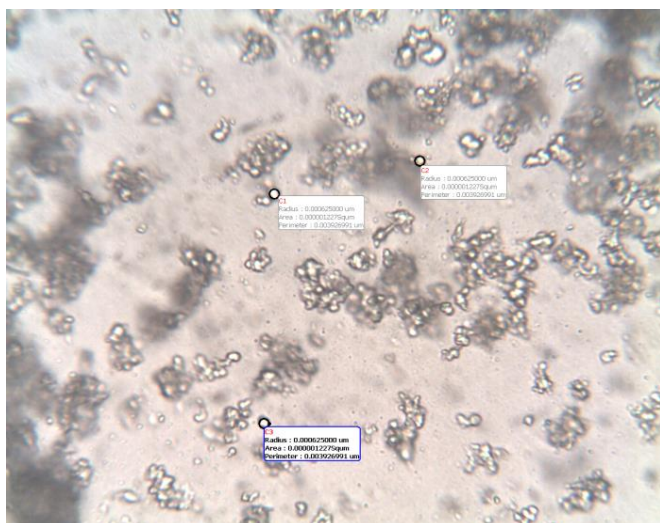
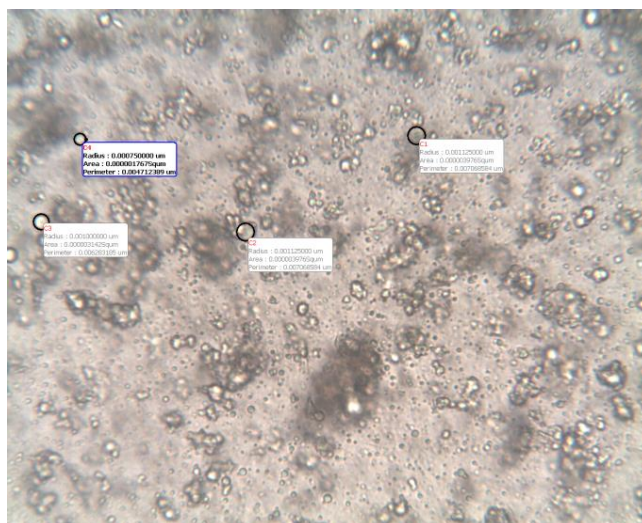


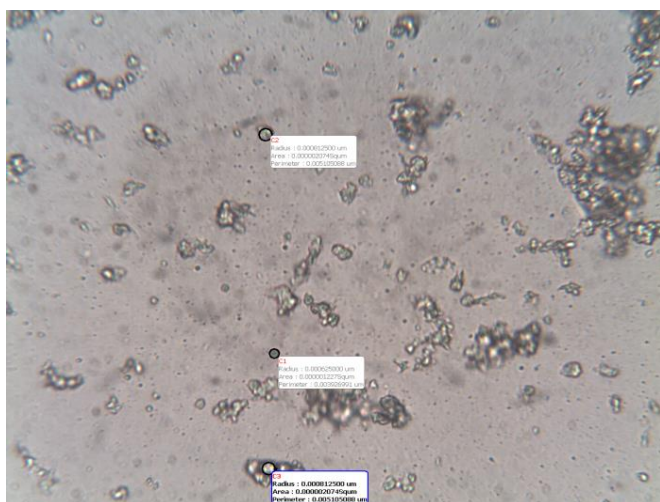
Figure 2. Photomicrographs of zero-drug SLMs at different storage temperatures (X100).



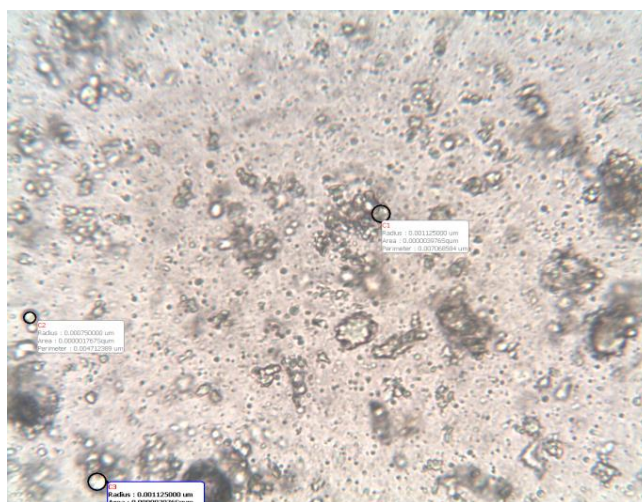
SLM-Drug (SRMS 1:9) at 4°C



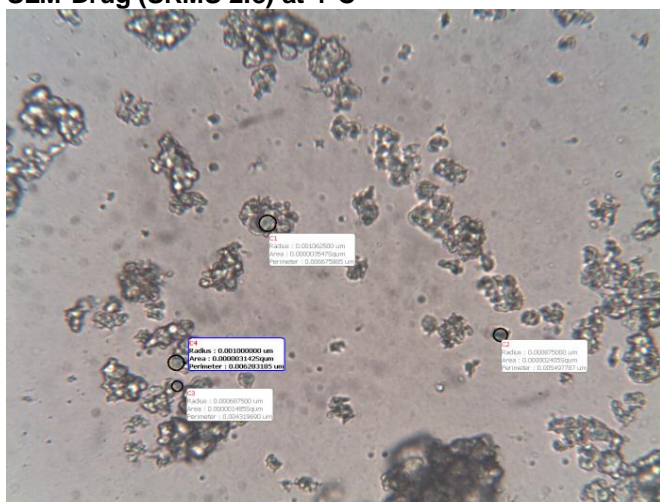
SLM-Drug (SRMS 1:9) at 25°C



SLM-Drug (SRMS 2:8) at 4°C



SLM-Drug (SRMS 2:8) at 25°C



SLM-Drug (SRMS 3:7) at 4°C



SLM-Drug (SRMS 3:7) at 25°C

Figure 3. Photomicrographs of drug-loaded SLMs at different storage temperatures (X100).

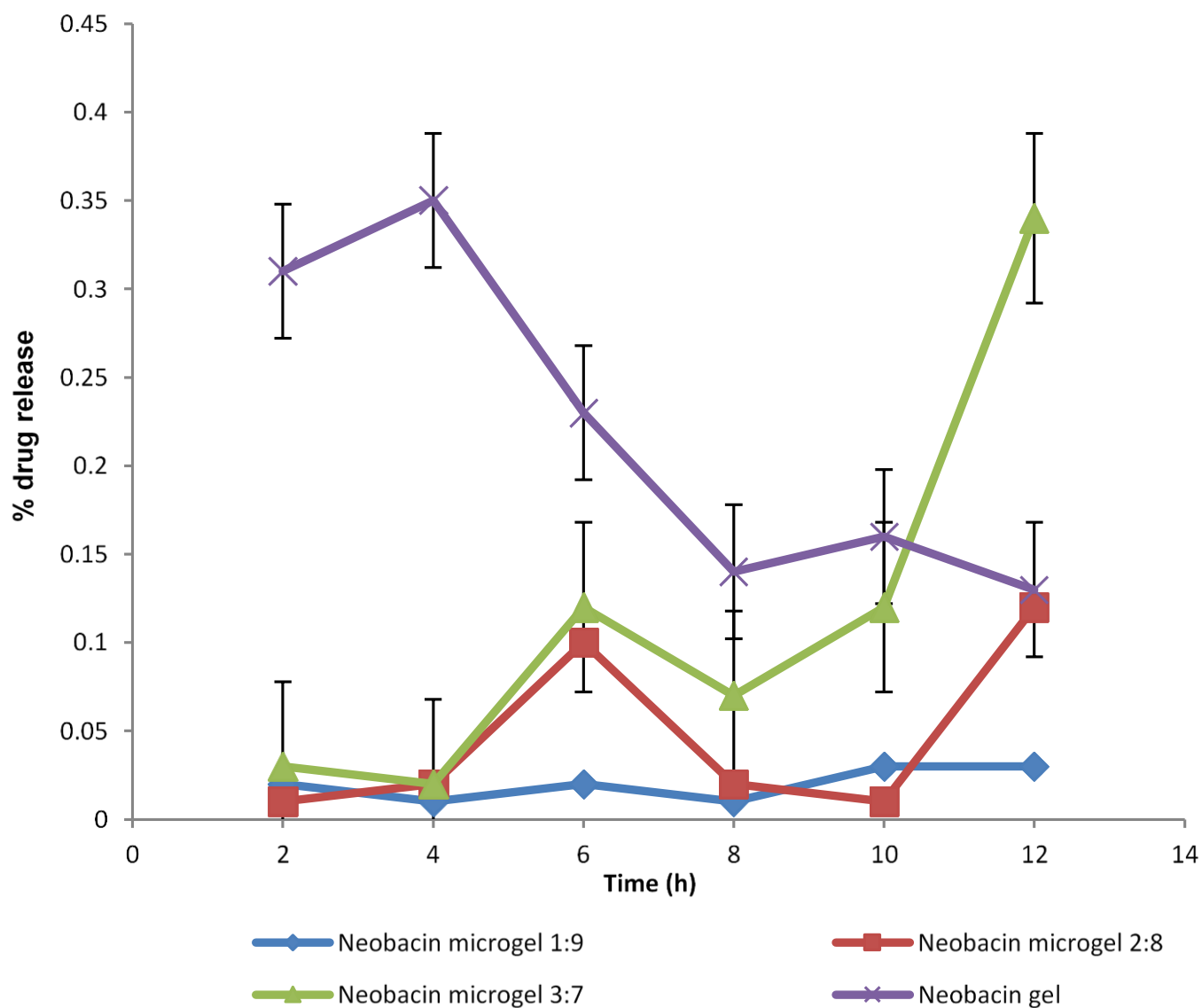


Figure 4. *In vitro* drug release study.

Table 3. Skin retention study.

Formulations	Concentration (%)
Neobacin microgel 1:9	27
Neobacin microgel 2:8	36
Neobacin microgel 3:7	64
Neobacin [®] gel	72

particle agglomeration which may not likely be stable upon extensive storage in the form of SLMs. Photomicrographs indicated that SLM-O formulations without neobacin[®] (Figure 2) were bigger than SLM-drug loaded

formulations (Figure 3). However, their shapes were best preserved at lower temperature rather than at room temperature. The result of the syringeability study suggests that even though particles of SLM-O batches

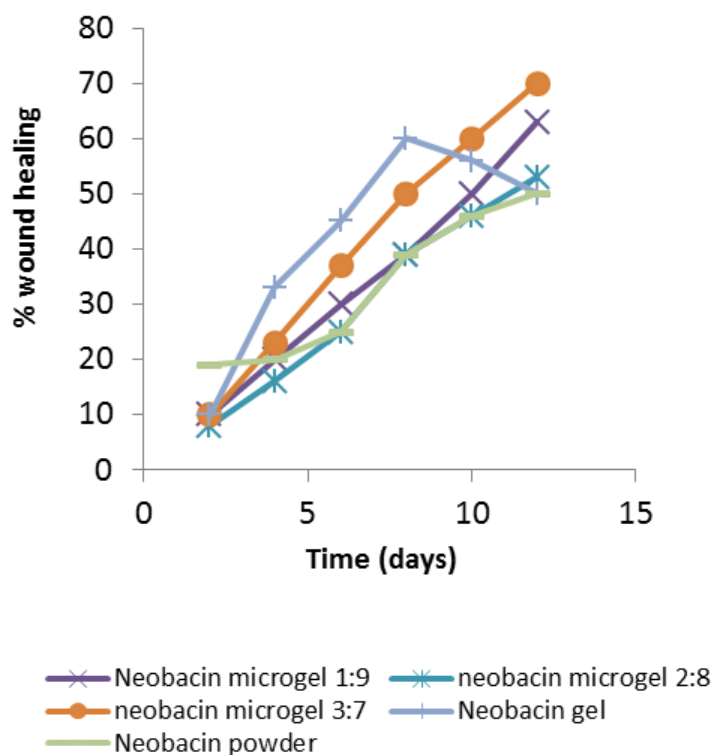


Figure 5. Percentage wound healing.

Table 4. Antimicrobial activity of neobacin[®] from topical drug delivery systems.

Formulations	Inhibition zone diameter, IZD (mm)*				
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. typhi</i>	<i>K. pneumonia</i>	<i>E. coli</i>
Neobacin microgel 1:9	21.13±2.58	15.11±0.98	11.22±0.76	20.12±3.04	19.±0.7
Neobacin microgel 2:8	15.23±0.76	16.33±0.78	10.12±0.93	17.11±0.85	17±0.9
Neobacin microgel 3:7	25.32±1.64	23.33±1.97	23.23±1.56	24.33±1.88	24±1.3
Neobacin [®] gel	23.21±1.75	21.23±1.99	23.33±1.60	23.33±1.55	23±1.5
Gel alone	15.36±0.92	15.21±0.83	15.11±0.37	15.12±0.74	15±0.8
Neobacin [®] powder	26.11±2.07	21.32±1.44	22.14±1.56	23.23±1.75	22±0.6

*Values are average of three determinations.

appeared bigger, they were more collapsible upon syringing to conveniently pass through smaller needles of 25 G while the SLM-Drug samples could not, perhaps due to the little drug entrapped in the lipid domain of the microparticles. The encapsulation efficiency was very poor and varied inversely with the proportion of sunseed oil to goat fat (1:9>2:8>3:7) respectively. This could be due to low solubility of the drug in the lipid melt, which agrees with literature that hydrophilic drugs could be only 5% entrapped in a lipid particle and good loading capacity is a result of high solubility of the drug in the lipid melt (Sanna et al., 2003; Bondi and Craparo, 2010; Muchow et al., 2008; Souto et al., 2004). Moreover, the

encapsulation efficiency values confirm the observations from the thermal analysis.

The microgels generally showed higher and a more prolonged release of the entrapped drug than the neobacin[®] gels. In addition, since microgels formulated from the 3:7 SRMS matrices achieved maximum release of 34% at 12 h, despite the low percentage EE, it means that it was available in the aqueous medium conveying the microparticles, closely attached to the carrier surface, in the vicinity of the surfactant layer, so that upon encountering the gel matrix of the polyacrylic acid, it got properly embedded in its 3-D matrix (Zhu et al., 2009; Feng et al., 2009; Kapoor and Chauhan, 2008; Bachhav

and Patravale, 2009; Padula et al., 2009). Therefore to improve the skin uptake and targeting, a semi-solid vehicle as we have demonstrated remains a standard approach which according to some report suggests that hydrogels exhibit high stability and adhesivity in addition to low toxicity (Bachhav and Patravale, 2009; Padula et al., 2009; Bhalekar et al., 2009).

The result of skin retention study showed that some neobacin[®] permeated through the membrane while reasonable amount was retained on the Millipore membrane (0.22 μm). This confirms the earlier observation on the size of the micro-particles and further agrees with literature that particles whose diameter were greater than 10 μm remained on the skin surface whereas those within 3 and 10 μm selectively penetrated the follicular ducts and those smaller than 3 μm randomly distributed into the hair follicles and stratum corneum (Schaefer and Lademann, 2001). Our observation is in consonance with literature reports that greater drug retention in the skin was observed while a lower concentration of drug could permeate through (Bhalekar et al., 2009; Puglia et al., 2008; Zhu et al., 2008). However, the drug release pattern is governed by the type of solid lipid matrix, not the hydrogel type (Paolicelli et al., 2009). Chen and co-workers as well as other researchers also observed that some processing parameters can affect the properties of the hydrogel such as the order of mixing the gelators (Chen et al., 2007; Sanna et al., 2010; Feng et al., 2009).

The result obtained from the *in vivo* wound healing study agrees with those of Fox et al. (2006) and Atiyeh et al. (2009) that wounds heal best when kept clean and moist rather than from dry powdery environment which forms hard scab and acts as a barrier to the development of new tissues. Semi-solid formulation provides moist environment which maintains epithelial cell viability to 97% (Atiyeh et al., 2009), and enables healing epithelial cells to freely migrate across the wound bed, promoting wound healing from the sides as well as the bottom of the wound. Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contraction is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. A wound without moisture will form scab (Fox et al., 2006; Atiyeh et al., 2009), as was observed from the drug powder-treated wounds. This is because the epithelial cells had to migrate from under below the scab to reach moisture that enabled them to heal the wounds but in so-doing, the epithelial cells migrated longer distances and, as a result, did not survive well, thus delayed wound healing. Better wound healing property of

the microgel formulations could additionally be attributed to their occlusive nature which enhanced adherence of the drug formulation to the wound surface (Wolf et al., 2009). Moreover, the results of the antimicrobial study indicated that neobacin microgel formulated with sunseed oil:goat fat (3:7 matrix) was the overall best, having consistently inhibited the growth of all tested microorganisms, followed by the neobacin[®] gel and lastly conventional neobacin[®] powder.

Interestingly, the neobacin[®] gel had maximum drug permeation of 35% at 4 h prior to degradation achieving 72% drug retention on the membrane, whereas neobacin microgel that was prepared from 3:7 SMSLM had slow initial release that intercepted the degrading neobacin[®] gel at 10 h prior to its achieving a maximum *in vitro* drug permeation of 34% and skin retention of 64%. In an outlook study exploiting both fast and delayed release of neobacin[®], a complementary system of neobacin[®] gel and microgel prepared from 3:7 SMSLM would be recommended and if packaged in separate tubes could carry instruction as "After wound cleaning, apply neobacin[®] gel first and after 4 h, repeat application with microgel".

Conclusions

This study has shown that microgels are better for wound healing than dry drug powder. Neobacin[®] gel formulation despite rapid degradation was also superior to the conventional powder form of neobacin[®].

Conflict of interests

The authors have not declared any conflict of interests.

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