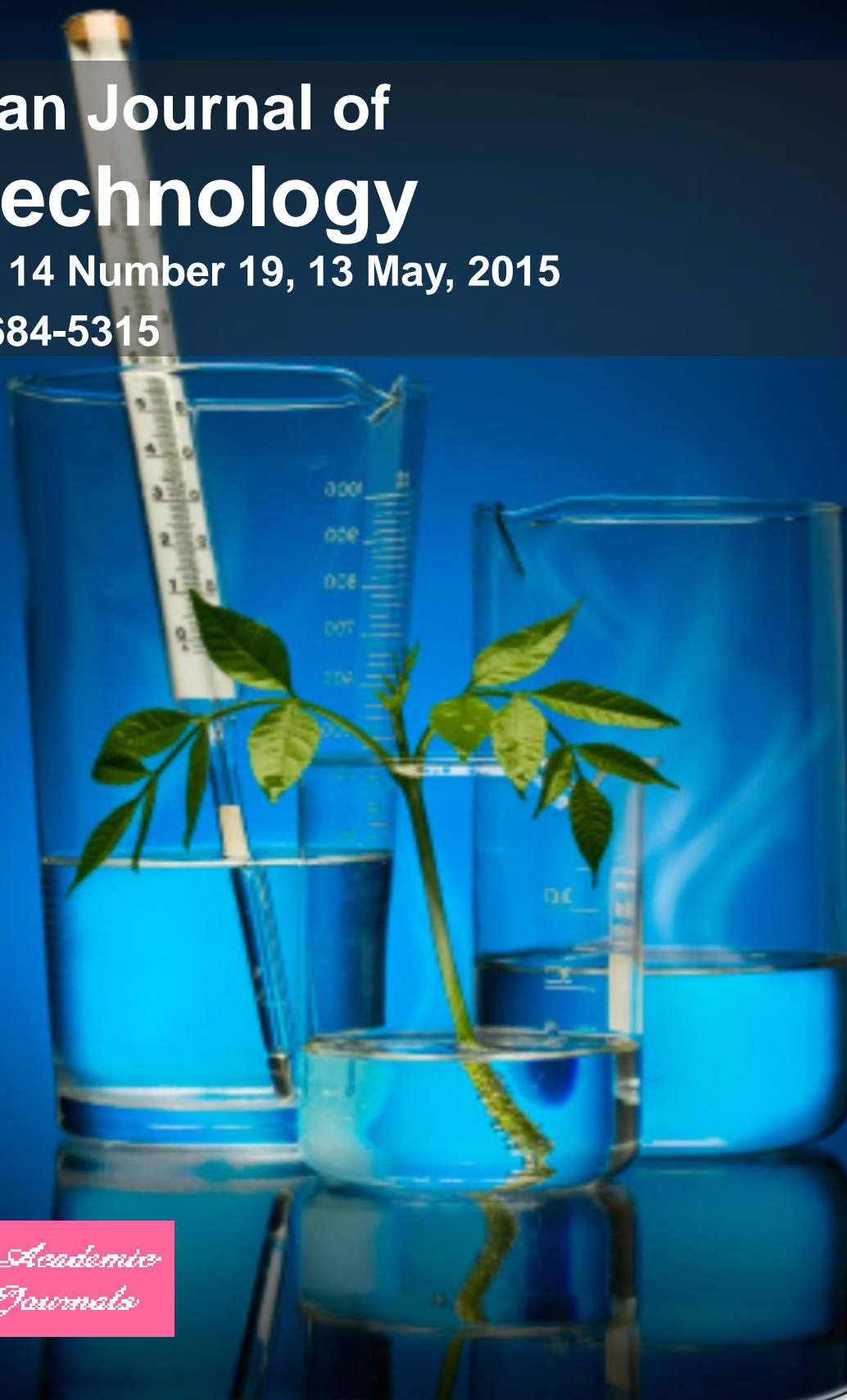


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Full Length Research Paper

Molecular identification of tsetse fly (Diptera: Glossinidae) species based on mitochondrial DNA (COII and CytB) sequences

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Tsetse fly (Diptera: Glossinidae) anti-vector measures are reliant upon accurate identification of species and their subpopulations. Two species were studied, *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* using two mitochondrial DNA: cytochrome oxidase subunit II (COII) and cytochrome b (CytB). Sequencing data were used to perform phylogenetic analysis of the two reared species together with other *Glossina* species' sequences from the DNA data base. For each gene, members of the same species group, *palpalis* or *morsitans* demonstrated a common ancestry and closer relatedness by belonging to one cluster. Within each species group members of the same species clustered together, an indication of common ancestry and relatedness too. In spite of the few mixed clusters, the pattern produced in the phylogenetic trees can provide a good guide to support any other method of *Glossina* identification. It was recommended that evaluations be made to validate other genetic markers that can produce better resolutions to identify tsetse fly species using phylogenetic tree.

Key words: Trypanosomiasis, *Glossina palpalis palpalis*, *Glossina morsitans submorsitans*, cytochrome oxidase II, cytochrome b, neighbour joining tree.

INTRODUCTION

Tsetse flies (Diptera: Glossinidae) are the vectors of trypanosomes, the causative agents of 'sleeping

sickness' or human African trypanosomiasis (HAT) in humans and 'nagana' or African animal trypanosomiasis

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(AAT) in livestock in Sub-saharan Africa. Many consider HAT as one of the major neglected tropical diseases and AAT as the single greatest health constraint to increased livestock production (Vreysen et al., 2013). The tsetse flies belong to the order Diptera, family Glossinidae, and genus *Glossina* (Leak, 1999). *Glossina* ('tongue fly', in reference to the prominent proboscis) species are arranged in three subgenera: *Austenina*, *Nemorhina*, and *Glossina* that correspond roughly with group of species found in different ecological settings. The subgenera often are cited by their group names: the *fusca* group (*Austenina*), the *palpalis* group (*Nemorhina*), and the *morsitans* group (*Glossina*) (McAlpine, 1969; Potts, 1973; Gouteux, 1987). The three subgenera or species groups consist of 33 currently recognized species and subspecies (Gooding and Krafsur, 2005). The major human and animal disease vectors are members of the *palpalis* a riverine, and *morsitans*, a savannah species group (Aksoy et al., 2001). The *fusca* species group are predominantly inhabitants of tropical forests and, as such, rarely come into contact with and feed on domestic animals and humans but they certainly contribute to the maintenance of the reservoir of infection in wild animals (Jordan, 1988; Abila et al., 2008).

Trypanosomiasis is caused by the flagellated protozoa of the genus *Trypanosoma*. Variability in vector competence of AAT or HAT depends on the species of the vector as well as the trypanosomes (Geiger et al., 2005). Economic losses in cattle production are estimated at US \$1 to 1.2 billion and total agricultural losses caused by AAT are estimated at US\$ 4 to 4.75 billion per year (Geiger et al., 2005). It has been predicted that an area of Africa larger than Europe will remain infested and under the threat of trypanosomiasis for the foreseeable future (Geiger et al., 2005). Area-wide integrated pest management (AW-IPM) defined as the integrated use of control tactics against an entire tsetse population within a delineated area (Klassen, 2005) is a highly recommended approach to create tsetse-free zones in Africa. The less controversial interventions under AW-IPM targets parasite and vector controls notably application of the sterile insect technique (SIT) against the vectors (Feldmann and Hendrichs, 2002). Both the parasite and vector control interventions of AW-IPM thus involves genetic control among other strategies. Genetic control aims to alter the reproductive potential of a vector (or parasite) or its vectorial competence such as the sterile insect technique (SIT) (De Deken, www.afrivip.org/sites/default/files/10_tsetse_references.pdf). AW-IPM usually includes genetic analysis to determine the degree of isolation of the populations of any of the vectors species (Vreysen et al., 2013).

Molecular marker applications are being used to differentiate between tsetse fly species, their subpopulations and their evolutionary relationships. A study of genetic relationships of 13 species of the genus *Glossina* inferred from mitochondrial [cytochrome oxidase 1 (CO1),

NADH dehydrogenase 2 (ND 2) and 16S] and nuclear (internal transcribed spacer 1 of rDNA, that is, ITS 1) sequences was conducted and reported by Dyer et al., (2008). Abila et al., (2008) studied levels of genetic differentiation between Ugandan *Glossina fuscipes fuscipes* populations based on COII and CytB, hence the markers are expected to be useful in species identification. An assessment of the possibility of applying sequence analysis of the region coding for CytB as a method of species identification in the field of forensic science was reported by Branicki et al. (2003). DNA originating from individuals in major phyla of vertebrates revealed that the technique is a very sensitive and reliable method for species identification (for vertebrates) and confirms that analysis can be carried out even when there is no reference sample, and the sequences obtained can be assessed through analysis of their similarity to cytochrome b sequences present in the DNA databases. Several genetic markers from mitochondrial DNA [cytochrome oxidase gene (COI, COII, 12S mtDNA)] and nuclear ribosomal DNA (16S rRNA, 28S rRNA) have been used in identification, population genetics and evolutionary studies of different families of myiasis-causing (maggot infestation) flies (Otranto and Stevens, 2002).

Conventionally, tsetse fly species identification is by the use of morphological characteristics in the form of identification keys (Buxton, 1955; Gouteux, 1987; FAO, 1992). This classical species identification relies on minor morphological differences, often challenging for field workers. Other systems of tsetse fly identification have been developed in the last few decades. Wing morphometrics can distinguish between species and subpopulations of tsetse flies and reportedly has the advantage of simplicity of data acquisition and low cost (Rohlf and Marcus, 1993; Solano et al., 1999; Patterson and Schofield, 2005; Camara et al., 2006; Leak et al., 2008; Getahun et al, 2014). For cuticular hydrocarbons identification method, the examination of the potentially stimulating methylalkanes [(via gas chromatography (GC) patterns)] provide reasons for the reproductive isolation of closely related species from each other (Sutton and Carlson, 1997; Getahun et al, 2014) and is used in classification and population studies. While, the phylogenetics method is expected to give greater accuracy when fully developed, a combination of identification methods is expected to give more accurate identification than the single methods. It was proposed that in control programs that involve Sterile Insect Technique (SIT) as suggested for a *G. f. fuscipes* SIT program in Ethiopia, morphological classification alone is not used to classify such populations (Dyer et al., 2011).

In this study mitochondrial DNA (mtDNA) sequence data (COII and CytB) from laboratory reared species were used to determine the sequence relationship between laboratory reared *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* together with sequences

of other tsetse fly species obtained from GenBank databases, to evaluate the possibility of using the phylogenetic relationship to identify and characterize the species and eventually provide information to support Area Wide-Integrated Pest Management (AW-IPM). Previous studies have used different genetic markers to demonstrate that the phylogenetic relationship between the three species groups *palpalis*, *morsitans* and *fusca* are in concordance with their distinct morphological classification and to show genetic differences (and similarities) between different geographical populations of individual species (Patterson and Schofield, 2005; Dyer et al., 2008; Abila et al., 2008; Dyer et al., 2011). The research questions to be answered in this study are: (a) with the COII sequences from the sample *G. palpalis palpalis* and *G. morsitans submorsitans* and their homologous species sequences from the GenBank, can we correctly identify the two species using a phylogenetic tree? and (b) can we do the same based on CytB sequences?

MATERIALS AND METHODS

Laboratory reared tsetse flies used in the study

The two tsetse fly species studied include *G. p. palpalis* and *G. m. submorsitans* which are among the four species of economic importance out of 11 species in Nigeria (FAO, 1992; Leak, 1999). The tsetse flies were collected from the laboratory colony maintained at the Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research (NITR), Kaduna, Nigeria. NITR was established in 1947 as West African Institute for Trypanosomiasis Research (WAITR) (NITR, 2008). However, the increasing need of tsetse flies for research and sterile insect control (SIT) requiring mass dispersal of sterile males necessitated their laboratory rearing. The foundation stock for establishment of a new colony is with the pupae. The *G. palpalis palpalis* colony originated from a waterway in Ija Gwari National Park in Suleija, Niger State, a suburb of Abuja, Nigeria's capital. Evidence of pupae collections from Suleija area was reported by Abubakar et al. (2010). Tsetse flies from NITR colony were used in a SIT project in Nigeria. Pupae from NITR colony of *G. palpalis palpalis* was used for mass-rearing of the species at the International Atomic Energy Agency (IAEA) in Seibersdorf, Austria for the Biological Control (BICOT) project in Nigeria. While, the project office was in Vom, Plateau State, the fly control site was in Lafia Local Government Area, Nassarawa State, Nigeria (Oluwafemi, 2008). IAEA laboratory supplied *G. palpalis palpalis* material to the project through weekly air shipments to Kano, Nigeria in 1988 to 1990. The IAEA colony was continued at a small size until 2009 when it was transferred to Centre International La recherche agronomique pour le développement (CIRAD) insectary in Montpellier, France, while IAEA continued rearing the related species *G. palpalis gambiensis*. For the other laboratory reared species in this study, *G. morsitans submorsitans*, the foundation pupae came from Centre International de Recherche-Développement sur l'Élevage en zone Subhumide (CIRDES), Bobo Dioulasso, Burkina Faso that was set up in 1972. The *G. p. palpalis* of the CIRDES colony originated with pupae that was collected from Samorogouan about 200 km to Bobo Dioulasso in 1980 to 1981. It was brought to NITR in July, 2010.

Tsetse flies depend on warm, vertebrate, antibiotic-free blood as their sole food. Traditionally, tsetse flies are made to feed on live animals (Nash et al., 1968). At NITR the flies in a production cage

were allowed to feed on the blood of a restrained rabbit or goat. An improved system is where the tsetse flies are fed through membranes resembling host skin made of silicone rubber or of agar and parafilm, overlying blood pools poured onto grooved glass plates (Bauer and Wetzel, 1976). The blood is usually collected from abattoirs. This system which is more acceptable to animal welfare groups and more practical (Feldmann and Hendrichs, 2002) is not practiced at NITR. Tsetse fly rearing is simplified in the laboratory because only two developmental stages need to be considered for management - the adult and pupal stages. Tsetse fly reproduction is viviparous as the female gives birth to live offspring. The larva is nourished within the mother and larviposited at an advanced stage of development. The average female lifespan is 100 to 120 days with an average of about 4 pupae per female under laboratory conditions which is less than the optimal yield under natural conditions. A total of 24 teneral tsetse flies were collected from the laboratory, 12 flies for each species with equal number of males and females, although sex was not considered in the identification. Sample collection and laboratory analysis were done between May and June, 2012. Tsetse flies were preserved in 95% ethanol and stored in a freezer following collection before use based on a procedure described for sample preservation for DNA extraction for insects (Schauff, 1986).

Molecular methods

DNA extraction and PCR was done at the Molecular Biology Laboratory of the National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State, Nigeria. Each whole body of the 12 *G. p. palpalis* and 12 *G. m. submorsitans* was used for DNA extraction based on the recommendation that whole body should be used for mitochondrial DNA and only legs for microsatellites analysis (Leak et al., 2008). Homogenate was obtained by grinding a whole fly body in a mortar with pestle, and 1000 µl of phosphate buffer saline (PBS) was added to make a solution. Extraction of genomic DNA was performed using the Zymo Research (ZR) Tissue and Insect DNA MicroPrep™ (Zymo Research Corp. USA) based on manufacturer's instructions. PCR Amplification was initiated for the 400 bp region of the mtDNA cytochrome oxidase II (COII) using the forward and reverse universal invertebrate primer pairs mtD13: COIIF-5'AATATGGCAGATTAGTGCA3' and mtD15: COIIR - 5'TCATAAGTTCARTATCATTG3'. Similarly, amplification was initiated for the 500 bp region of the cytochrome b (CytB) gene using the forward and reverse universal invertebrate primer pairs mtD26: CytB2F - 5'TATGTACTACCATGAGGACAAATATC3' and mtD28: CytB2R - 5'ATTACACCTCCTAATTTATTAGGAAT3' (Simon et al., 1994). Polymerase chain reactions (PCR) were performed in a 25 µl reaction mixtures containing 1 µl of template DNA, 2.5 µl 10X PCR buffer, 0.8 mM dNTP, 2 mM MgCl₂, 0.4 µM of each primer, 1 µl of BSA (Bovine Serum Albumin) and 1 unit of AmpliTaq Gold (Applied Biosystems). Gene Amp PCR^R System 9700 was used for the amplification reactions. Thermal cycler conditions consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min. Reactions were terminated with a final extension at 72°C for 5 min (Abila et al., 2008). PCR products were analyzed by electrophoresis on 2% agarose gel and visualized under ultra violet light.

From the 12 tsetse flies for each species, amplified DNA samples from the PCR products were used for nucleotide sequencing. A total of 44 amplicons were thus used for sequencing. For *G. p. palpalis*, they comprised of 24 amplicons: 12 amplicons for COII and 12 amplicons for CytB, hence all the 12 samples of each gene amplified. For *G. m. submorsitans* they comprised of 20 amplicons: 10 amplicons for COII and 10 amplicons for CytB, hence there was no amplification for 2 DNA samples for COII and CytB, respectively. Sequencing of the 44 amplicons resulted in 88 reactions as

Table 1. BLAST result of *Glossina palpalis palpalis* COII.

Sample	Base pairs	BLAST result			
		Matching species	Gene	% Maximum identity	Accession number
P1a-NITR	341	<i>Glossina f. fuscipes</i> hap 16	COII	93	EU559620
P3a-NITR	284	<i>Glossina f. fuscipes</i> hap 7	COII	94	GU296752
P8a-NITR	345	<i>Glossina f. fuscipes</i> hap 16	COII	93	EU 559620
P9a-NITR	350	<i>Glossina f. fuscipes</i> hap 16	COII	94	EU559620
P10a-NITR	345	<i>Glossina f. fuscipes</i> hap 7	COII	93	GU296752

Table 2. BLAST result of *Glossina palpalis palpalis* COII.

Sample	Base pairs	BLAST Result			
		Matching species	Gene	% Maximum Identity	Accession Number
P1b-NITR	375	<i>Glossina morsitans</i>	CytB	91	KC177594
P2b-NITR	224	<i>Glossina f. fuscipes</i> hap 20	CytB	93	EU562281
P4b-NTR	200	<i>Glossina f. fuscipes</i> hap 20	CytB	93	EU562281
P5b-NITR	277	<i>Glossina morsitans</i>	CytB	95	KC177594
P6b-NITR	268	<i>Glossina f. fuscipes</i> hap 16	CytB	92	EU562277
P8b-NITR	374	<i>Glossina morsitans</i>	CytB	91	KC177594
P9b-NITR	379	<i>Glossina morsitans</i>	CytB	90	KC177594
P10b-NITR	426	<i>Glossina morsitans</i>	CytB	89	KC177594

sequencing was done in the forward and reverse directions to minimize errors. Sequencing was performed by Macrogen USA, Rockville, Maryland using ABI 3730XL (Applied Biosystems) automated sequencer following standard manufacturer's protocols.

BLAST and phylogenetic analysis of mtDNA sequences for relationship between the species

Only sequences that yielded greater than 200 bp were selected for basic local alignment search tool (BLAST) and to generate the neighbour joining trees. All the forward sequences were removed from the analysis as they did not meet this criterion. All the sequences that met this criterion fell within the reverse (minus) sequences but they were converted to forward (plus) sequences using a converter programme, Reverse Complement (The Bioinformatics Organization Inc., 2000). Among them, duplicate sequences with 100% homology were removed before BLAST and phylogenetic analysis. Such duplicate homologous sequences were not submitted to the GenBank. BLAST was done using BLASTN 2.2.27 (Zhang et al., 2000) at the database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>), that is, GenBank. The sequences were aligned with CLUSTAL W (Thompson et al., 1997) with default parameters and scrutinized and edited using MEGA version 4.1 (Tamura et al., 2007). Sequences from the database that best matched that of the samples were collected for phylogenetic analysis. The MEGA 4.1 Kimura-2 parameter model simultaneously calculates the genetic distances between pairs of sequences and constructs a neighbour joining tree (Saitou and Nei, 1987). The bootstrap test of phylogeny (Hillis and Bull, 1993) option of MEGA 4.1 was selected and used.

The sample and GenBank database sequences of the species were combined in MEGA 4.1 to generate neighbour joining tree

separately for the two genes. The following numbers of the sequences were used for the trees: *G. p. palpalis* COII (5), *G. p. palpalis* CytB (8), *G. m. submorsitans* COII (3) *G. m. submorsitans* CytB (3). For the neighbour joining tree, COII sequences of *G. p. palpalis* (5) and *G. m. submorsitans* (3) were used together with COII of *G. f. fuscipes* (2) from the GenBank. This was followed by generating neighbour joining tree for CytB together for *G. p. palpalis* (8) and *G. m. submorsitans* (3), as well as *G. f. fuscipes* (2) and *G. morsitans* (1) from GenBank database. A similar combination of sample DNA sequences and database sequences was used to generate neighbour joining tree for the identification of blow flies (*Cordylobia anthropophaga*), another insect of medical, veterinary and forensic importance (Ogo et al., 2012). DnaSP version 5 was used to generate the haplotypes, an index of DNA polymorphism (Librado and Rozas, 2009).

RESULTS

BLAST of the sequences

Basic local alignment search tool (BLAST) of the DNA sequences of COII and CytB genes of the two species studied revealed only *Glossina* species related to *G. p. palpalis* and *G. m. submorsitans* with percentage identity ranging from 89 to 95% (Tables 1 to 4). No exact sequence of the two species for the two genes were revealed in the GenBank to be used for the phylogenetics identification of the sample species hence only the related species were compared. This also implies that this is the first time that nucleotide database deposits

Table 3. Blast result of *Glossina morsitans submorsitans* CytB.

Sample	Base pairs	BLAST result			
		Organism	Gene	% Maximum Identity	Accession number
M3b-NITR	294	<i>Glossina morsitans</i>	CytB	95	KC177594
M5b-NITR	417	<i>Glossina morsitans</i>	CytB	93	KC177594
M9b-NITR	350	<i>Glossina morsitans</i>	CytB	93	KC177594

Table 4. Blast result of *Glossina morsitans submorsitans* COII.

Sample	Base pairs	BLAST result			
		Matching species	Gene	% Maximum identity	Accession number
M1a-NITR	339	<i>Glossina f. fuscipes</i> hap 7	COII	94	GU296752
M5a-NITR	336	<i>Glossina f. fuscipes</i> hap 7	COII	92	GU296752
M12a-NITR	342	<i>Glossina f. fuscipes</i> hap 16	COII	93	EU 559620

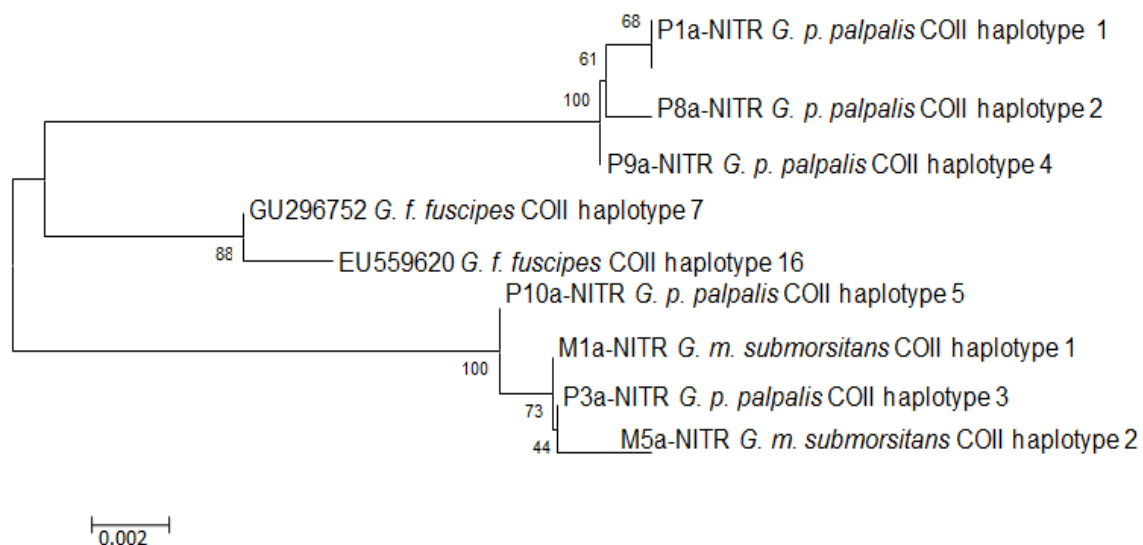


Figure 1. Neighbour joining tree based on sequences of three species' (COII) to identify *Glossina palpalis palpalis* and *Glossina morsitans submorsitans*. *G. p. palpalis* and *Glossina fuscipes fuscipes* (GU2967652 and EU559620) are species of the *palpalis* species group and showed a common ancestry while members of the *G. p. palpalis* and *G. f. fuscipes* species clustered according to their species. *G. m. submorsitans* showed a different ancestry from *palpalis* species group as it belongs to the *morsitans* species group. The scale bar indicates the number of nucleotide substitutions per site.

were made for these species sequences for COII and CytB.

Identification of the species using neighbour joining tree

A neighbour joining tree involving four sequences from three species was generated for COII, the sequences are *G. p. palpalis* and *G. m. submorsitans* from this study

along with *G. f. fuscipes* (GU296752) and *G. f. fuscipes* (EU559620) from GenBank as revealed from BLAST. The three species clustered differently with a few exceptions, that is, P10a-NITR and P3a-NITR which are *G. p. palpalis* species' group members that appeared in the *G. m. submorsitans* cluster (Figure 1). For CytB, the neighbour joining tree involved four species: *G. p. palpalis* and *G. m. submorsitans* from this study along with *G. f. fuscipes* (EU562277, EU562281) and *G. morsitans* (KC177594) from GenBank and the different

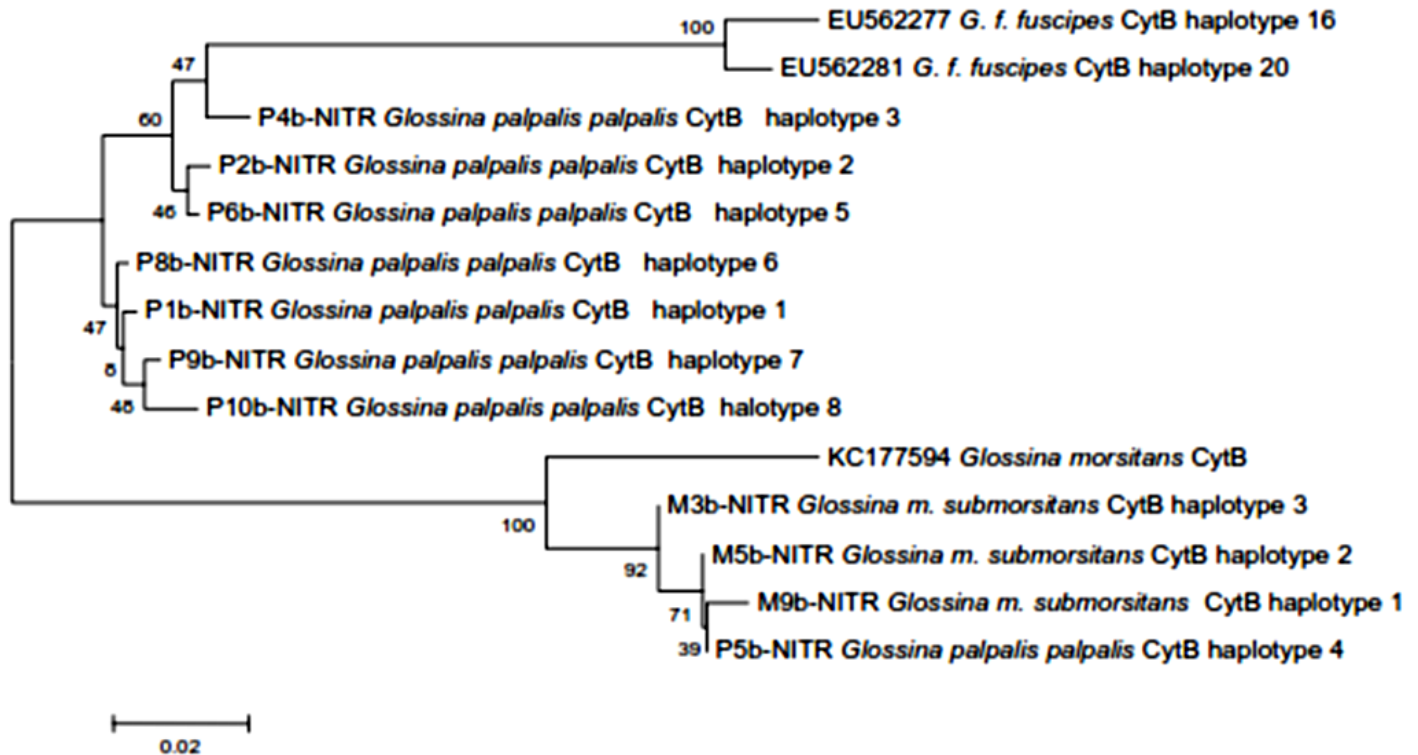


Figure 2. Neighbour joining tree based on sequences of four species' (CytB) to identify *G. p. palpalis* and *G. morsitans submorsitans*. *G. p. palpalis* CytB has a common ancestry with *G. f. fuscipes* CytB (EU562277 and EU562281) from GenBank as both are species of the *palpalis* species group. Members of each species also clustered more closely. *G. morsitans* (KC177594) from GenBank also showed a common ancestry with *G. m. submorsitans* as both are species of the *morsitans* species group. Members of each species also clustered more closely. The only exception was the P5b-NITR, a *palpalis* species member that clustered with the *G. m. submorsitans*. The scale bar indicates the number of nucleotide per site.

species clustered differently with only one exception: P5b-NITR which is a *G. p. palpalis* species that appeared in the *G. m. submorsitans* cluster (Figure 2). It is interesting to note that *G. morsitans* (KC 177594) from GenBank clustered together with *G. m. submorsitans* both of which are species of the *morsitans* species group. The mtDNA sequences of the flies from this study were submitted at the National Centre for Biotechnology Information (NCBI) GenBank database (Accession Numbers *G. p. palpalis* COII KJ013516-20, *G. p. palpalis* CytB KJ013521-28, *G. m. submorsitans* COII KJ207383-5 and *G. m. submorsitans* CytB KJ207386-88) (Table 5). The observed haplotypes are also shown in Table 5.

DISCUSSION

The objective of the study was to evaluate the utility of the two mitochondrial markers (COII and CytB) in identification of the two species by comparison with homologous database sequences in phylogenetic trees. This study is important because anti-vector measures are reliant upon accurate identification of vector species (Aksoy et al., 2001; Dyer et al., 2001). The BLAST

results shown gave good values of percentage identity or homology between the sample species and the species revealed in the GenBank (85 to 95%) while the homology between the sample tsetse fly species for each gene gave 91 to 99%, thus confirming a greater homology within a species than between different species. The availability of homologous species sequences in database is crucial to this identification and characterization method to prove the species' similarity. A bootstrap value of 70% is considered significant evidence for phylogenetic grouping (Hillis and Bull, 1993) and the values obtained in the two neighbour joining trees met this expectation (Figures 1 and 2). In the phylogenetic analysis, the general pattern observed is that the different species groups – *morsitans* and *palpalis* - clustered differently in the phylogenetic tree for each gene showing that each species group has a common ancestry and relatedness with 100% bootstrap values. Further, within each species group, members of each species clustered together thus demonstrating a closer ancestry than the species outside the cluster. For COII (Figure 1), *G. p. palpalis* clustered together with *G. f. fuscipes* both of which belong to the *palpalis* species group while *G. m. submorsitans* which belong to the *morsitans* species

Table 5. Accession Numbers of the mtDNA sequences.

Isolate	Species	Gene	Haplotype	Accession number		
P1a-NITR	<i>G.p. palpalis</i>	COII	Haplotype 1	KJ013516		
P8a-NITR			Haplotype 2	KJ013518		
P3a-NITR			Haplotype 3	KJ013517		
P9a-NITR			Haplotype 4	KJ013519		
P10a-NITR			Haplotype 5	KJ013520		
P1b-NITR	<i>G. p. palpalis</i>	CytB	Haplotype 1	KJ013521		
P2b-NITR			Haplotype 2	KJ013522		
P4b-NITR			Haplotype 3	KJ013523		
P5b-NITR			Haplotype 4	KJ013524		
P6b-NITR			Haplotype 5	KJ013525		
P8b-NITR			Haplotype 6	KJ013526		
P9b-NITR			Haplotype 7	KJ013527		
P10b-NITR			Haplotype 8	KJ013528		
M1a-NITR			<i>G. m. submorsitans</i>	COII	Haplotype 1	KJ207383
M5a-NITR					Haplotype 2	KJ207384
M12a-NITR	Haplotype 3	KJ207385				
M9b-NITR	<i>G. m. submorsitans</i>	CytB	Haplotype 1	KJ207386		
M5b-NITR			Haplotype 2	KJ207387		
M3b-NITR			Haplotype 3	KJ207388		

group clustered separately. Within the *palpalis* species group *G. f. fuscipes* species members formed a separate cluster from the *G. p. palpalis* species. The pattern observed for COII was almost replicated for CytB (Figure 2) where *G. p. palpalis* demonstrated a common ancestry with *G. f. fuscipes* both of which are members of the *palpalis* species group. Within the *palpalis* species group cluster, members of *G. p. palpalis* and *G. f. fuscipes* species formed their own separate clusters. *Morsitans* species group members showed a common ancestry out of which *G. morsitans* (KC 177594) clustered separately from *G. morsitans submorsitans*. This result suggests that the analysis can be used for identification and differentiation of *Glossina* species since any unknown sample species will cluster with known sequences from the database with respect to species and specie group and make identification possible.

In a study based on internal transcribed spacer 2 (ITS2) genetic marker and different species from the *palpalis*, *morsitans* and *fusca* species groups, comparative morphometric analysis of shape variation in the wings of different tsetse species had revealed close accordance with the phylogenetics of the species indicated by DNA sequences where the different *Glossina* species clustered according to their species groups (Patterson and Schofield, 2005). This is supportive of the results from this study. The resulting clusters from this study also agree with Dyer et al. (2008) that reported a phylogeny which confirms the monophyly (having common ancestry) of the morphologically defined

fusca, *morsitans* and *palpalis* subgenera in a study that involved mitochondrial (cytochrome oxidase 1, NADH dehydrogenase 2 and 16S) and nuclear (internal transcribed spacer 1 of rDNA) sequences. Dyer et al. (2011) showed the relative power of different genetic markers to support the monophyly and aid characterization of different species and genetic groups of tsetse flies. The nuclear ribosomal internal transcribed spacer 1 (ITS1) provided support for the monophyly of each of the three tsetse fly species groups hence each group is commonly inherited. However, other nuclear and mitochondrial sequence data did not support the monophyly of the morphological subspecies *G. f. fuscipes* or *Glossina fuscipes quanzensis*.

In spite of the few mixed clusters, the pattern produced in the phylogenetic trees in this study can provide a good guide to support any other method of *Glossina* identification. Further investigation is hereby recommended to include the use of other genetic markers such as mitochondrial cytochrome oxidase 1 (COI), nuclear internal transcribed spacer 1 of rDNA (ITS 1) or ITS 2 and to include controls. The utility of this phylogenetic method in *Glossina* species identification will increase as more deposits of the different economically important species and subpopulation are made in the databases to support identification. Though the phylogenetics method has been used for identification of other organisms such as the use of cytochrome b for vertebrates (Branicki et al., 2003) and some other mitochondrial and nuclear ribosomal DNA sequences for blow flies (Otranto and Stevens, 2002; Ogo et al., 2012),

the application of phylogenetics tree as a method for outright identification of tsetse flies has not been reported. It also remains to be established which markers will work best for tsetse flies identification.

The phylogenetic technique can be employed for identification and characterization of species in tsetse fly control programmes to support the conventional morphological technique that employs some standard identification keys, which may be inaccurate and time consuming even for a trained entomologist.

Conclusion

Accurate characterization and identification of species and their subpopulations is important in the control strategies of tsetse flies. In this study, it was found that it is possible to use the clustering in the neighbour joining tree of the two mtDNA sequences of the species as a means of identification. Members of the same species tended to cluster together implying that they have a common ancestry and relatedness and this can be used for identification if an unknown species is involved. Similarly, species of the same species groups (*palpalis*, *morsitans*) clustered together, demonstrating their common ancestry and supporting the species' identity also. These findings have demonstrated the earlier species groupings that were based on morphological features. In spite of the few mixed clusters, the pattern of the trees can be useful as a method of species characterization and identification while it is hoped that evaluation of other genetic markers will give improved results. Since the other tsetse fly identification methods have also associated challenges of accuracy, time, cost and complexity, they can be complemented with the phylogenetics approach for better results. This study will help to promote genetic study, surveillance and control of tsetse fly populations.

Abbreviations: **AAT**, Animal African trypanosomiasis; **BLAST**, Basic local alignment search tool; **COI**, cytochrome oxidase subunit I; **COII**, cytochrome oxidase subunit II; **CytB**, cytochrome b; **DnaSP**, DNA sequence polymorphism; **HAT**, human African trypanosomiasis; **ITS**, internal transcribed spacer; **MEGA**, molecular evolutionary genetic analysis; **mtDNA**, mitochondrial DNA; **NCBI**, national centre for biotechnology information; **NITR**, Nigeria institute for trypanosomiasis (and onchocerciasis) research; **rDNA**, ribosomal DNA; **SIT**, sterile insect technique; **16S**, the large subunit ribosomal RNA (rRNA); **AW-IPM**, area-wide integrated pest management; **PCR**, polymerase chain reactions; **BSA**, bovine serum albumin; **GC**, gas chromatography.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Determination of long-term effects of consecutive effective fresh chicken manure with solarization and verticillium wilt (*Verticillium dahlia* Klebb) on weed and its control in egg plant

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The aim of the study was to determine the weed density and the most economical way of weed control in eggplant (*Solanum melongena*) fields contaminated with *Verticillium dahliae* (Kleb) after the application of fresh chicken manure and solarization in the second year as the same crop was grown. The effect of solarization on weed and the labor need in weed control continued in a diminishing way in the consecutive observations. With fresh chicken manure (FCM), number of weeds (number m⁻²) decreased but their green and dry biomass (weights g m⁻²) increased. The labor need (d ha⁻¹) to control the weeds decreased. Similar results were also recorded for *V. dahliae* inoculation. As a result of the study, 50% of labor saving was achieved in the plots of solarization and either FCM rate combinations [sol x FCM (12 kg.m⁻²); sol x FCM (6 kg.m⁻²)] compared to the control plots. Achieved savings in labor can afford to cover the costs of solarization and FCM.

Key words: Soil solarization, fresh chicken manure, *Verticillium dahliae*, eggplant, weed, weed control.

INTRODUCTION

Weeds can impact significantly on crop productivity. For long years in Turkey, weed control methods employed in fields against weeds were based on only mechanic methods and the herbicide applications. Thus, producers of some regions do not think of growing crops without

herbicide application (Uygur, 2002). It is impossible to apply these methods in small plots. Although herbicides are accepted to be the most effective and fast solution in weed control, desired results cannot always be harvested. On the contrary, they can cause big environmental

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disorders.

Unconscious use of herbicides results in hardness in weeds. As a result of increased public awareness in environment and the negative effects of the herbicides on human health alternative control methods have been researched (Önen, 2003).

Especially in eggplant (*Solanum melongena*) and intensive vegetable production, one of the most important problems is the yield losses due to weeds, manual control of which requires extra labor. However, this type of weed control cost high (Raffaelli et al., 2011). Instead, through fumigation of the soil just before weed seeds germinate weed problem along with the other soil rooted pathogens can be eliminated (Jarvis, 1993). Nevertheless, fumigants also cause some unwanted side effects. Thus, methyl bromide, a commonly used fumigant, was suggested to be completely abandoned from use in 2005 due to the harmful effect of it to ozonosphere (Katan, 1999).

Instead of these fumigants, solar energy use was considered first (Katan, 1987). By this method which is known as solarization, weed seed intensity was aimed to decrease through covering with plastic in high temperature days in a year for one or two months in order to be heated for pasteurization (Lalitha et al., 2003; Cimen et al., 2010a). The effect of solarization lasts more than one year (Katan et al., 1983; Satour et al., 1989; Candido et al., 2006; Cimen et al., 2010b).

Chicken manure contains significant amounts of nitrogen because of the presence of high levels of protein and amino acids. Owing to its high nutrient content, chicken manure has been considered to be one of the most valuable animal wastes as organic fertilizer (Chen and Jiang, 2014).

This study researched the most economic eggplant production by determining weed seed density in eggplant fields inoculated with *Verticillium dahliae* (Kleb) after the application of FCM and solarization together in the second year.

MATERIALS AND METHODS

Study was conducted in a loamy-clay soil in the research fields of the Department of Plant Protection, Faculty of Agriculture, Dicle University, Diyarbakir, Turkey (latitude 37°53 N, longitude 40°16 E, altitude 680 m above sea level) with dominant semi-arid characteristics during 2010 and 2012 years. The climate in Diyarbakir is dry and hot in summer and cold in winter. For solarization, soil was covered with 0.2 mm transparent polyethylene (PE) for 45 days. Trial was conducted in eggplant fields in 2012 after FCM and solarization application in 2010 and *Verticillium dahliae* (Kleb) inoculation in 2011. *V. dahliae* inoculation was repeated in the same plots in 2012 at the same rate.

Fresh and dry biomass weights of the weeds and the time required for manual weed control were observed. A one square meter quadrat, plant pressing tool and a scale were used in the study. Weed species and eggplant in the trial plots were the study material.

Weed observations were made in the trial plots, arranged in split-split plots trial design with three replications, where solarization, FCM and verticillium inoculation were employed in main, split and

mini plots, respectively. A one-meter quadrat was used for weed count in a total of 36 plots, either of which had a size of 20 m². Weeds within the quadrat were counted by their genus and species and arithmetic means were calculated. Weed density and frequency was calculated according to Odum (1971).

The trial was established in 2010 to measure the effect of three factors, namely solarization, FCM and *V. dahliae* inoculation on the response variable, weed density and frequency. The field was divided into three blocks and each block was further splitted into two whole plots and solarization application cases (applied or not applied) were randomly assigned to the whole plots within each block. Moreover, each whole plot was divided into three split plots and three FCM rates (0, 6 and 12 kg.m⁻¹) were randomly devoted to the split plots within each whole plot. Furthermore, each split plot was bisected into split-split plots and *V. dahliae* inoculation cases (inoculated or not) was randomly assigned to each split-split plot. At each of the split-split plots (*V. dahliae* inoculation cases) 18 observations (3x2x3=18) were performed during the growing season. So a grand total of 36 measurements were available for the analysis.

Determination and counts of the weeds in trial plots

In order for the determination of the weeds, counts were performed in trial plots on 10.05.2012 after 20 months from solarization application (2010) and about 6 months from the first eggplant production season (2011), just before the planting of the eggplant seedlings. In total, 50 weed species of 16 different families were determined. Regarding the density in per square meter the first four weed species in rank were *Sorghum halepense*, *Convolvulus galaticus*, *Convolvulus arvensis* and *Amaranthus blitoides*, respectively. The results were summarized in Table 1 in counts per square meter (number m⁻²).

Fresh and dry weed weights

In a total of four times, weeds were removed from the trial plots, one time before and three times after planting (10.05.2012). The weeds were dried for 12 weeks in greenhouse conditions after their fresh weights were measured. Later, their dry weights were measured using a precise electronic scale. Results were presented in Table 2.

Time required for manual weed removal

Weed clearings from the trial plots were performed manually by two labors on the above mentioned days and the time required for weed removal was determined at every turn. It was later converted into labor inputs per hectare (days ha⁻¹).

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) and LSD through MSTATC computer programme as outlined by Steel and Torrie (1980).

RESULTS AND DISCUSSION

The effect of solarization, FCM and *V. dahliae* inoculation on weeds in eggplant fields before growing season

The list of the weeds determined in the 36 trial plots on 10.05.2012 was given in Table 1. Of the 50 weed species

Table 1. Observation of weed species and their natural distribution in the experimental area (count m⁻²) (2012).

Number	Weed name	Count	Number	Weed name	Count
1	<i>Anagallis arvensis</i>	31	26	<i>Lactuca serriola</i>	44
2	<i>Amaranthus blitoides</i>	95	27	<i>Lallemantia iberica</i>	2
3	<i>Buglossoides arvensis</i>	2	28	<i>Lamium</i> sp.	24
4	<i>Bupleurum rotundifolium</i>	1	29	<i>Lathyrus aphaca</i>	2
5	<i>Cardaria draba</i>	41	30	<i>Lolium</i> sp.	3
6	<i>Carduus pycnocephalus</i>	5	31	<i>Malva neglecta</i>	3
7	<i>Carthamus</i> sp.	1	32	<i>Melilotus</i> sp.	4
8	<i>Centaurea balsamita</i>	8	33	<i>Molucella laevis</i>	6
9	<i>Centaurea iberica</i>	1	34	<i>Myagrum perfoliatum</i>	74
10	<i>Cichorium inthybus</i>	59	35	<i>Neslia apiculata</i>	3
11	<i>Convolvulus arvensis</i>	202	36	<i>Notobasis syriaca</i>	1
12	<i>Convolvulus betonicifolius</i>	9	37	<i>Phalaris</i> sp.	19
13	<i>Convolvulus galaticus</i>	247	38	<i>Poa</i> sp.	4
14	<i>Convolvulus stachydifolius</i>	4	39	<i>Polygonum aviculare</i>	84
15	<i>Conyza canadensis</i>	1	40	<i>Ranunculus arvensis</i>	14
16	<i>Coriandrum</i> sp.	4	41	<i>Sinapis arvensis</i>	39
17	<i>Crepis alpina</i>	44	42	<i>Sisymbrium officinale</i>	3
18	<i>Cynodon dactylon</i>	8	43	<i>Sonchus oleraceus</i>	12
19	<i>Euphorbia aleppica</i>	7	44	<i>Sorghum halepense</i>	260
20	<i>Euphorbia helioscopia</i>	3	45	<i>Tragopogon</i> sp.	4
21	<i>Foeniculum vulgare</i>	1	46	<i>Turgenia latifolia</i>	12
22	<i>Fumaria asepala</i>	17	47	<i>Vaccaria pyramidata</i>	26
23	<i>Galium tricornutum</i>	73	48	<i>Vicia narbonensis</i>	21
24	<i>Hordeum murinum</i>	2	49	<i>Vicia sativa</i>	12
25	<i>Lactuca saligna</i>	12	50	<i>Xanthium strumarium</i>	15

Table 2. Fresh and dry weed weights.

Applications	Total (count/m ²)	Weight (g/m ²)	
		Fresh	Dry
Solarization (Sol)			
Non solarized (-Sol)	27.05	2229	440
Solarized (+Sol)	16.44	1866	347
Fresh chicken manure (FCM)			
Control	24.79	1714	337
6 kg/m ²	19.33	2298	425
12 kg/m ²	21.12	2131	419
Sol x FCM			
*			
- Sol x Control	32.50	1771	366
- Sol x FCM (6 kg/m ²)	22.41	2933	554
- Sol x FCM (12kg/m ²)	26.25	1983	400
+Sol x Control	17.08	1658	308
+Sol x FCM (6 kg/m ²)	16.25	1662	396
+Sol x FCM (12 kg/m ²)	16.00	2279	437
Inoculation (<i>V. dahliae</i>)			
*			
Non inoculated (- Ino)	24.91	1834	348
Inoculated (+ Ino)	17.58	2261	439

Table 2. Contd

Sol × Inoculation			
- Sol × (-) Ino.	29.50	2061	403
- Sol × (+) Ino	24.61	2397	478
+Sol × (-) Ino	20.33	1608	294
+Sol × (+) Ino	12.55	2125	400
FCM × Inoculation			
Cont × (-) Ino	32.25	1504	304
Cont × (+) Ino	17.33	1925	371
FCM (6 kg/m ²) × (-) Ino	22.00	1779	341
FCM (6 kg/m ²) × (+) Ino	16.66	2816	508
FCM (12 kg/m ²) × (-) Ino	20.50	2221	400
FCM (12 kg/m ²) × (+) Ino	21.75	2041	437
Sol × FCM × Inoculation			
			*
- Sol × Cont × (-) Ino	41.50	1725	358
- Sol × Cont × (+) Ino	23.50	1816	375
- Sol × FCM (6 kg/m ²) × (-) Ino	22.33	2091	400
- Sol × FCM (6 kg/m ²) × (+) Ino	22.50	3775	708
- Sol × FCM (12 kg/m ²) × (-) Ino	24.66	2366	450
- Sol × FCM (12 kg/m ²) × (+) Ino	27.83	1600	350
+Sol × Cont × (-) Ino	23.00	1283	250
+Sol × Cont × (+) Ino	11.16	2033	366
+Sol × FCM (6 kg/m ²) × (-) Ino	21.66	1466	283
+Sol × FCM (6 kg/m ²) × (+) Ino	10.83	1858	308
+Sol × FCM (12 kg/m ²) × (-) Ino	16.33	2075	350
+Sol × FCM (12 kg/m ²) × (+) Ino	15.66	2483	525

*, **Significant at 0.05 and 0.01 levels respectively.

given in Table 1 those exceeding the number of plots (36) are of 12 species. They have about 87% of share in grand total. The counts of these 12 species are presented in Table 3.

It is obvious from the table that the effect of solarization on the decrease in weeding before the second eggplant growing season does continue. The results were significant at 5% for *Galium tricorntutum* and *Lactuca* species, and 1% level for the most common 12 weed species in the total. Solarization decreased the weeds about 36% per square meter. This result is in harmony with the findings reported by Katan et al. (1983); Satour et al. (1989); Candido et al. (2006), and Çimen et al. (2010b).

Again it is also evident in the same weed count that FCM application before solarization and *V. dahliae* inoculation during the first eggplant growing season decreased the number of weeds per square meter. However, the interactions among the three factors were found insignificant in dual and triple combinations (Table 4). In a previous study by Çimen and Basaran (2013), it was reported that FCM increased the soil temperature which might be the reason of the decrease of the weeds

determined in this study. Thus, it was reported that broomrape (*Orabanche crenata*), an important problem in cabbage production in Lebanon, was controlled with FCM-Solarization application (Haidar and Sidahmed, 2000). *V. dahliae* is able to infect more than 400 plant species, including annual, herbaceous crops and weeds, as well as fruit, landscape, ornamental trees and shrubs (Pegg and Brady, 2002).

The effect of solarization and FCM on the 51 weed species and their fresh and dry weights (g m⁻²) is presented in Table 5. It was obvious from the table that the effect of solarization, FCM and *V. Dahliae* on weed count is parallel to the common weed count results. Regarding the fresh and dry weed weights obtained from 50 weed species, it was decreased by solarization but increased by FCM and *V. dahliae* inoculation. The most outstanding case here is that *V. dahliae* inoculation decreased the number of weeds as it increased fresh and dry weed weights. This is related to diminishing competition of the weeds between and within the species (Özer et al., 2001) and can be best understood that at the end of the first eggplant production only the weeds tolerant to *V. dahlia* survived in the trial plots and they could

Table 3. Weeds (12 species) have about 87% of share in grand total.

Application	<i>Cardaria draba</i>	<i>Cichorium inthibus</i>	<i>Convolvulus arvensis</i>	<i>Convolvulus galaticus</i>	<i>Crepis alpina</i>	<i>Galium tricornutum</i>	<i>Lactuca serriola</i>	<i>Myagrurn perfoliatum</i>	<i>Polygonum aviculare</i>	<i>Sinapis arvensis</i>	<i>Sorghum halepense</i>	<i>Amaranthus blitoides</i>	Total
Solarization (Sol)						*	*						**
Non solarized (-Sol)	0.02	1.38	3.69	2.52	0.91	1.66	0.66	1.69	1.25	0.91	5.22	1.25	21.22
Solarized (+Sol)	1.11	0.25	1.91	3.91	0.30	0.36	0.55	0.36	1.08	0.16	2.00	1.38	13.41
Fresh chicken manure (FCM)									*				
Control	0.04	0.25	4.62	4.79	1.37	0.91	0.58	1.50	0.50	0.41	4.33	0.29	19.62
6 kg/m ²	0.04	1.70	2.45	2.66	0.29	0.91	0.58	0.70	1.54	0.62	2.91	1.66	16.12
12 kg/m ²	1.62	0.50	1.33	2.20	0.16	1.20	0.66	0.87	1.45	0.58	3.68	2.00	16.20
Sol x FCM													
- Sol x Control	0.00	0.41	4.91	5.33	2.16	1.66	0.83	2.50	0.25	0.66	6.58	0.00	25.33
- Sol x FCM (6 kg/m ²)	0.08	3.08	3.50	0.83	0.33	1.83	0.58	1.41	1.75	1.16	3.75	0.16	18.50
- Sol x FCM (12kg/m ²)	0.00	0.66	2.66	1.41	0.25	1.50	0.58	1.16	1.75	0.91	5.33	3.58	19.83
+Sol x Control	0.08	0.08	4.33	4.25	0.58	0.16	0.33	0.50	0.75	0.16	2.08	0.58	13.91
+Sol x FCM (6 kg/m ²)	0.00	0.33	1.41	4.50	0.25	0.00	0.58	0.00	1.33	0.08	2.08	3.16	13.75
+Sol x FCM (12 kg/m ²)	3.25	0.33	0.00	3.00	0.08	0.91	0.75	0.58	1.16	0.25	1.83	0.41	12.58
Inoculation (<i>V. dahliae</i>)													
Non inoculated (- Ino)	0.86	1.41	2.72	4.75	0.72	0.91	0.69	0.97	1.47	0.36	3.38	2.33	21.61
Inoculated (+ Ino)	0.27	0.22	2.88	1.69	0.50	1.11	0.52	1.08	0.86	0.72	3.83	0.30	14.02
Sol x Inoculation													
- Sol x (-) Ino.	0.05	2.50	3.16	2.77	1.22	1.55	0.66	1.66	1.44	0.66	5.27	2.16	23.16
- Sol x (+) Ino	0.00	0.27	4.22	2.27	0.61	1.77	0.66	1.72	1.05	1.16	5.16	0.33	19.27
+Sol x (-) Ino	1.66	0.33	2.27	6.72	0.22	0.27	0.72	0.27	1.50	0.05	1.50	2.50	18.05
+Sol x (+) Ino	0.55	0.16	1.55	1.11	0.38	0.44	0.38	0.44	0.66	0.27	2.50	0.27	8.77
FCM x Inoculation													
Cont x (-) Ino	0.00	0.25	6.66	7.00	1.91	1.16	0.58	1.16	0.83	0.33	6.58	0.58	27.08
Cont x (+) Ino	0.08	0.25	2.58	2.58	0.83	0.66	0.58	1.83	0.16	0.50	2.08	0.00	12.16
FCM (6 kg/m ² x (-) Ino	0.08	3.25	1.41	4.66	0.25	0.66	0.83	0.58	2.00	0.16	1.91	3.16	19.00
FCM (6 kg/m ²) x (+) Ino	0.00	0.16	3.50	0.66	0.33	1.16	0.33	0.83	1.08	1.08	3.91	0.16	13.25
FCM (12 kg/m ²) x (-) Ino	2.50	0.75	0.08	2.58	0.00	0.91	0.66	1.16	1.58	0.58	1.66	3.25	15.75
FCM (12 kg/m ²) x (+) Ino	0.75	0.25	2.58	1.83	0.33	1.50	0.66	0.58	1.33	0.58	5.50	0.75	16.66

Table 3. Contd.

Sol x FCM x Inoculation													
- Sol x Cont x (-) Ino	0.00	0.50	7.50	6.00	3.50	2.00	0.83	1.83	0.50	0.50	10.66	0.00	33.83
- Sol x Cont x (+) Ino	0.00	0.33	2.33	4.66	0.83	1.33	0.83	3.16	0.00	0.83	2.50	0.00	16.83
- Sol x FCM (6 kg/m ²) x (-) Ino	0.16	6.00	1.83	0.83	0.16	1.33	0.83	1.16	1.66	0.33	3.16	0.00	17.50
- Sol x FCM (6 kg/m ²) x (+) Ino	0.00	0.16	5.16	0.83	0.50	2.33	0.33	1.66	1.83	2.00	4.33	0.33	19.50
- Sol x FCM (12 kg/m ²) x (-) Ino	0.00	1.00	0.16	1.50	0.00	1.33	0.33	2.00	2.16	1.16	2.00	6.50	18.16
- Sol x FCM (12 kg/m ²) x (+) Ino	0.00	0.33	5.16	1.33	0.50	1.66	0.83	0.33	1.33	0.66	8.66	0.66	21.50
+Sol x Cont x (-) Ino	0.00	0.00	5.83	8.00	0.33	0.33	0.33	0.50	1.16	0.16	2.50	1.16	20.33
+Sol x Cont x (+) Ino	0.16	0.16	2.83	0.50	0.83	0.00	0.33	0.50	0.33	0.16	1.66	0.00	7.50
+Sol x FCM (6 kg/m ²) x (-) Ino	0.00	0.50	1.00	8.50	0.33	0.00	0.83	0.00	2.33	0.00	0.66	6.33	20.50
+Sol x FCM (6 kg/m ²) x (+) Ino	0.00	0.16	1.83	0.50	0.16	0.00	0.33	0.00	0.33	0.16	3.50	0.00	7.00
+Sol x FCM (12 kg/m ²) x (-) Ino	5.00	0.50	0.00	3.66	0.00	0.50	1.00	0.33	1.00	0.00	1.33	0.00	13.33
+Sol x FCM (12 kg/m ²) x (+) Ino	1.50	0.16	0.00	2.33	0.16	1.33	0.50	0.83	1.33	0.50	2.33	0.83	11.83

*, **Significant at 0.05 and 0.01 levels respectively.

grow fast and had vitality due to lack of competition of the other weed species, which resulted in an increase in both fresh and dry biomass weights of the weeds.

The effect of solarization, FCM and *V. Dahliae* inoculation on weeds in the fields during the second Eggplant growing season

Weeds were cleaned from the trial plots three times after the solarization during the second eggplant growing season. Fresh and dry weights (g m⁻²) of the collected weeds and the time required for manual elimination (day ha⁻¹) are presented in Figure 1.

It is seen from Figure 1 that solarization decreased the fresh and dry weed weights (g m⁻²) in all of the three weed removals as parallel to its effect on the decrease of weed species (Table 4). As in the case of total weed number total dry weed weight is statistically significant (p<0.01).

Solarization decreased weed dry weight by 27%. Another remarkable result is that the first weed dry weight gradually decreased towards the last dry weight. Also, there was a harmony between the effect of solarization on weed dry weight and the labor input (day ha⁻¹) required for manual weed removal (Figure 1). Similar to the case in dry weed weight, the labor input needed in weed removal gradually decreased from the first to the last. Solarization provided 17% saving in labor input in weed control in the second year.

Even though FCM application before the solarization caused an increase in the fresh and dry weed weights (gm⁻²) (Figure 1), it decreased the labor input required for weed removal (day ha⁻¹). The results of the second observation were significant (p<0.01) in both assessment criteria. However, the relationship between solarization and FCM were not significant in both assessment types.

V. dahliae inoculation, on the other hand, caused a decrease in fresh and dry weed weights

in the first weed removal. But it increased fresh and dry weights in the second and third removals as in grand total (Figure 1). In all of three weed removals the results were significant (p<0.05). The same trend is also seen in labor input for weed removal (Figure 1).

In the plots of triplet combinations among the solarization, FCM and *V. dahliae* inoculation the highest fresh and dry weed weights were seen in applications of “(-)Sol x FCM (6 kg m⁻²) x (-) Ino” and “(-) Sol x FCM (6 kg m⁻²) x (+) Ino” in values close to each other as the lowest dry weight was obtained from the solarization plots of FCM and *V. dahliae* applications “(+) Sol x FCM (6 kg m⁻²) x (-) Ino” and “(+) Sol x FCM (6 kg m⁻²) x (+) Ino” (Table 4 and Figure 1).

As for the manual control of the weeds seen in triplet combination plots, the highest mean labour input in a growing season was 195.13 d.ha⁻¹, which was determined in the control plots where solarization, FCM and *V. dahliae* inoculation were not applied. The lowest labour input, on the other

Table 4. Effect of solarization with fresh chicken manure and verticillium wilt (*Verticillium dahliae* Klebb) inoculation on weed (weight/m²) (2012).

Applications	1. (1.07.12)		2. (07.08.12)		3. (01.11.12)		Total	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Solarization (Sol)		*			**	**		**
Non solarized (-Sol)	495	156	327	89	358	87	1181	333
Solarized (+Sol)	474	123	193	59	223	60	892	243
Fresh chicken manure (FCM)			**					
Control	397	138	177	61	233	67	808	268
6 kg/m ²	514	130	313	82	318	76	1146	289
12 kg/m ²	543	151	290	79	321	77	1155	308
Sol x FCM								
- Sol x Control	388	148	195	63	230	63	813	276
- Sol x FCM (6 kg/m ²)	655	172	417	105	434	105	1506	383
- Sol x FCM (12kg/m ²)	441	147	370	100	411	94	1223	342
+Sol x Control	405	128	160	59	237	72	803	260
+Sol x FCM (6 kg/m ²)	373	87	209	59	202	48	785	195
+Sol x FCM (12 kg/m ²)	645	154	210	58	231	61	1087	275
Inoculation (<i>V. dahliae</i>)	*		*	*	*	*		
Non inoculated (- Ino)	597	151	227	65	233	58	1058	276
Inoculated (+ Ino)	372	128	293	83	348	89	1014	300
Sol x Inoculation								
- Sol x (-) Ino.	584	176	268	76	284	72	1136	324
- Sol x (+) Ino	406	136	387	103	432	103	1226	342
+Sol x (-) Ino	610	127	187	54	182	45	980	227
+Sol x (+) Ino	338	119	199	63	265	76	803	259
FCM x Inoculation								
Cont x (-) Ino	396	156	133	46	221	59	751	262
Cont x (+) Ino	398	120	222	76	246	76	866	273
FCM (6 kg/m ²) x (-) Ino	704	145	306	80	207	51	1218	278
FCM (6 kg/m ²) x (+) Ino	325	114	306	84	428	101	1073	300
FCM (12 kg/m ²) x (-) Ino	692	153	242	69	271	65	1206	283
FCM (12 kg/m ²) x (+) Ino	394	148	338	89	371	90	1104	328
Sol x FCM x Inoculation								
- Sol x Cont x (-) Ino	422	205	161	52	205	54	790	311
- Sol x Cont x (+) Ino	355	92	228	75	254	73	837	240
- Sol x FCM (6 kg/m ²) x (-) Ino	880	201	383	99	270	71	1534	372
- Sol x FCM (6 kg/m ²) x (+) Ino	430	143	450	111	598	139	1479	394
- Sol x FCM (12 kg/m ²) x (-) Ino	449	121	259	77	376	91	1085	290
- Sol x FCM (12 kg/m ²) x (+) Ino	433	174	482	122	445	96	1361	393
+Sol x Cont x (-) Ino	370	108	105	40	237	64	712	213
+Sol x Cont x (+) Ino	441	149	215	77	238	80	896	306
+Sol x FCM (6 kg/m ²) x (-) Ino	527	89	230	62	145	32	902	184
+Sol x FCM (6 kg/m ²) x (+) Ino	220	85	189	56	259	64	668	206
+Sol x FCM (12 kg/m ²) x (-) Ino	935	185	226	60	165	39	1327	285
+Sol x FCM (12 kg/m ²) x (+) Ino	355	123	194	56	297	84	846	264

*, ** Significant at 0.05 and 0.01 levels respectively.

Table 5. Effect of solarization with fresh chicken manure and verticillium wilt (*Verticillium dahliae* Klebb) inoculation on weed control manually (work day/ha) (2012).

Applications	1. (1.07.12)	2. (07.08.12)	3. (01.11.12)	Total
Solarization (Sol)		**		
Non solarized (-Sol)	83.56	41.31	27.77	152.66
Solarized (+Sol)	74.88	29.51	21.41	125.81
Fresh chicken manure (FCM)				
Control	97.74	41.49	26.56	165.79
6 kg/m ²	66.66	33.68	23.78	124.13
12 kg/m ²	73.26	31.07	23.43	127.77
Sol x FCM				
- Sol x Control	100.34	46.87	29.51	176.73
- Sol x FCM (6 kg/m ²)	67.01	35.76	28.12	130.90
- Sol x FCM (12kg/m ²)	83.33	41.31	25.69	150.34
+Sol x Control	95.13	36.11	23.61	154.86
+Sol x FCM (6 kg/m ²)	66.31	31.59	19.44	117.36
+Sol x FCM (12 kg/m ²)	63.19	20.83	21.18	105.20
Inoculation (<i>V. dahliae</i>)	*			
Non inoculated (- Ino)	78.23	33.79	20.48	131.48
Inoculated (+ Ino)	80.20	37.03	28.70	145.95
Sol x Inoculation	*			
- Sol x (-) Ino.	83.56	43.98	24.30	151.85
- Sol x (+) Ino	83.56	38.65	31.25	153.47
+Sol x (-) Ino	72.91	23.61	16.66	113.19
+Sol x (+) Ino	76.85	35.41	26.15	138.42
FCM x Inoculation				
Cont x (-) Ino	103.12	41.31	26.73	171.18
Cont x (+) Ino	92.36	41.66	26.38	160.41
FCM (6 kg/m ²) x (-) Ino	63.54	28.81	14.93	107.29
FCM (6 kg/m ²) x (+) Ino	69.79	38.54	32.63	140.97
FCM (12 kg/m ²) x (-) Ino	68.05	31.25	19.79	119.09
FCM (12 kg/m ²) x (+) Ino	78.47	30.90	27.08	136.45
Sol x FCM x Inoculation	**			
- Sol x Cont x (-) Ino	105.55	57.63	32.63	195.13
- Sol x Cont x (+) Ino	95.13	36.11	27.08	158.33
- Sol x FCM (6 kg/m ²) x (-) Ino	64.58	33.33	19.44	117.36
- Sol x FCM (6 kg/m ²) x (+) Ino	69.44	38.19	36.80	144.44
- Sol x FCM (12 kg/m ²) x (-) Ino	80.55	40.97	21.52	143.05
- Sol x FCM (12 kg/m ²) x (+) Ino	86.11	41.66	29.86	157.63
+Sol x Cont x (-) Ino	100.69	25.00	21.52	147.22
+Sol x Cont x (+) Ino	89.58	47.22	25.69	162.50
+Sol x FCM (6 kg/m ²) x (-) Ino	62.50	24.30	10.41	97.22
+Sol x FCM (6 kg/m ²) x (+) Ino	70.13	38.88	28.47	137.50
+Sol x FCM (12 kg/m ²) x (-) Ino	55.55	21.52	18.05	95.13
+Sol x FCM (12 kg/m ²) x (+) Ino	70.83	20.13	24.30	115.27

*, ** Significant at 0.05 and 0.01 levels respectively.

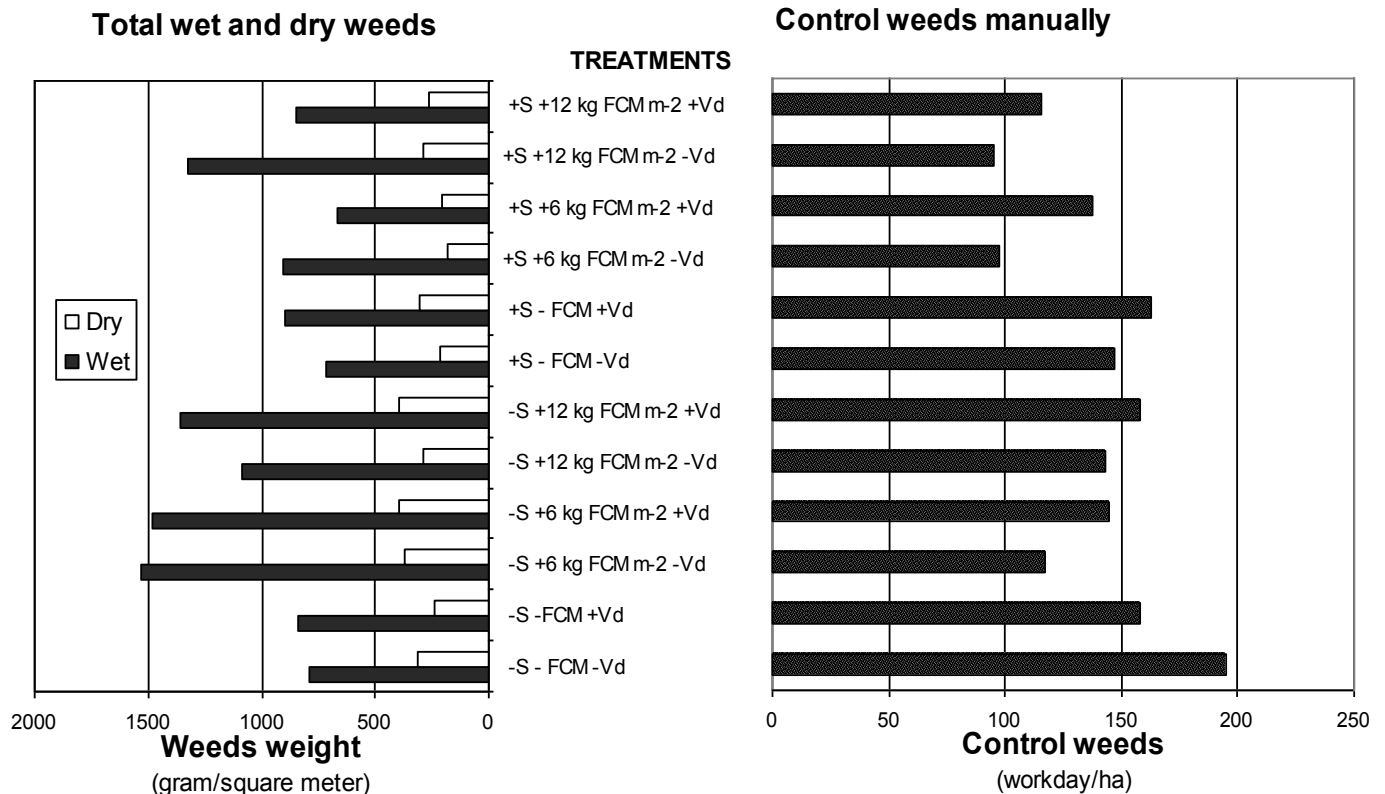


Figure 1. Effect of solarization with fresh chicken manure and verticillium wilt (*Verticillium dahliae* Klebb) inoculation on weed and its control manually (2012).

hand, was determined to be 95.13 and 97.22 d.ha⁻¹ for the plots where two FCM rates (12 kgm⁻² and 6 kgm⁻², respectively) and solarization applications were performed but seedlings were not infected with *V. dahliae*, the combinations were “+Sol x FCM (12 kgm⁻²) x (-) Ino” and “+Sol x FCM (6 kgm⁻²) x (-) Ino” (Figure 1). Both applications saved labour input about 50% compared to control plots.

Conclusion

In this study, the effect of together application of FCM and solarization on weed control gradually decreased in successively eggplant grown fields for two production season. In a previous study, in the high eggplant yielding applications of “+Sol x FCM (12 kgm⁻²) x (-) Ino” and “+Sol x FCM (6 kgm⁻²) x (-) Ino” 50% of labor savings was achieved in weed control, which may compensate the solarization and both FCM application costs. However, it was concluded that the achieved labor saving is not sufficient for adoption of these practices by the producers especially in places where hidden unemployment rate is quite high.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

***Pleurotus pulmonarius* cultivation on amended palm press fibre waste**

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In the last few decades, rapid global demand for edible oils has resulted in a significant increase in the land area of oil crop cultivation. In the process of extraction of palm oil from oil palm fruit, biomass materials such as palm pressed fibre (PPF) are generated as waste products. This research was undertaken to evaluate the use of palm pressed fibres as the substrates for the cultivation of *Pleurotus pulmonarius* which currently use sawdust. Seven different substrates (A to G) were prepared from saw dust, palm press fibre (PPF), palm press fibre ash (PPFA), distilled water and Hoagland solution either alone or in combinations. These substrates were combined to investigate a probable effect of substrate combination on yield of *P. pulmonarius*. The highest yields were observed from substrates D (comprising 50% PPF, 50% PPFA and water with a mean fresh weight of 95.0 g) and substrate F (comprising of 50% PPF, 50% PPFA and Hoagland solution with a mean fresh weight of 89.20 g). The performance of substrate combination of PPF, PPFA and water however compare favourably with that of PPF, PPFA and Hoagland solution combination under all growth and yield parameters investigated. Therefore, this study has revealed that with optimum use of the biomass generated from the palm waste, prevention of environmental pollution problems and conversion of low quality waste biomasses into a valuable high protein food can be achieved.

Key words: *Pleurotus pulmonarius*, substrates, palm pressed fibre, palm press fibre ash, Hoagland solution.

INTRODUCTION

Oil palm is a fast-growing crop, which grows in the tropical lowlands where rainfall is distributed evenly. It can grow in a wide range of soil types with relatively low

pH but is susceptible to high pH (Hartley, 1988). In the last few decades, rapid global demand for edible oils has resulted in a significant increase in the land area of oil

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Abbreviations: PPF, Palm pressed fibre; PPFA, palm press fibre ash.

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crop cultivation, including oil palm (Yacob, 2008). Due to increasing demand for palm oil, enormous quantities of wastes/bye products are generated which may include palm kernel shell, palm kernel cake, decanter cake, empty fruit bunch, palm press fibre, palm oil fuel ash, palm oil mill sludge and palm oil mill effluent. Thus, a need for the sustainable management of products, which if left un-attended to, may perhaps lead to environmental problems. While research is on-going on the use of by-products as biofuels, the products are sometimes incinerated or released directly on field (Tabi et al., 2008). These practices create environmental pollution problems as incineration emits gases with particulates such as tar and soot droplets of 20 to 100 microns and a dust load of about 3000 to 4000 mg/nm (Igwe and Onyegbado, 2007). Indiscriminate dumping of empty fruit bunch also causes methane emission into the atmosphere. In recent time composting and vermicomposting have been gaining grounds as good options for the management of these wastes because they are organic in composition (Yusri et al., 1995; Thambirajah et al., 1995; Danmanhuri, 1998). To also minimize pollution effect of such waste, newer usage ought to be investigated.

Pleurotus pulmonarius commonly known as Indian Oyster, like other spp in the genus, have been reported to directly breakdown lingo-cellulosic materials (Zadrazil, 1978) which makes them economically viable in biotechnological conversion of wastes to high quality protein food (Onuoha et al., 2009). The ability to bio convert lignocelluloses materials as substrates results from the presence of lignocellulolytic or fibrolytic enzymes such as xylanases, cellulases and lacasses (Sun et al., 2004) which convert cellulose and lignin into useful carbohydrates for energy generation by the fungi (Baysal et al., 2003). Mushrooms are seasonal organisms and are always available in short supply (Onuoha et al., 2009). While, mushrooms such as *Pleurotus* spp are commercially produced and sold in markets in Asia, America and Europe, they are still being hunted for in forests and farmland for sale in Africa (Onuoha et al., 2009) hence the need for their commercial production. *P. pulmonarius* is selected for this study because it is one of the species commonly eaten in Nigeria (Zoberi, 1972).

A wide range of plant waste such as saw dust, paddy straw, bagasse, cornstalks, waste cotton, banana stalks and leaves can be used for *Pleurotus* mushroom production without a requirement for costly processing methods and enrichment materials (Chang and Miles, 2003). In Nigeria, the traditional substrate for cultivation of *P. pulmonarius* is sawdust (Onuoha et al., 2009). The low availability of sawdust coupled with the pollution effects of oil palm waste are reasons while the usability of oil palm fibre for the production of *P. pulmonarius* ought to be looked into. *Pleurotus* spp may be a good candidate in this study because; they are efficient degraders of lignocellulosic materials, easy to grow with simple technology, short life span, available and native to Nigeria

Table 1. Substrate combination for *P. pulmonarius* cultivation.

Substrate	Composition of substrate
A	100% Sawdust + water
B	100% PPF + water
C	100% PPFA + water
D	50% PPF + 50% PPFA + water
E	100% PPF + Hoagland
F	50% PPF + 50% PPFA + Hoagland
G	100% PPFA + Hoagland

PPF; Palm press fibre; PPFA; Palm press fibre ash.

(Stanley et al., 2011).

MATERIALS AND METHODS

The spawn (fungal) spores of *P. pulmonarius* used for the cultivation of the fungus were collected from Federal Institute of Industrial Research Oshodi, Lagos State in Nigeria. The sawdust was obtained from Sabo market sawmill Ogbomoso, Oyo State, palm pressed fibre was obtained from a palm oil processing industry in Masifa, Ogbomoso, Oyo State in Nigeria while all salts and reagents used were of analytical grade and supplied by Sigma Aldrich through Labtrade Chemicals Limited, Nigeria. Seven different substrate combinations were prepared as shown in Table 1 with each substrate combination prepared in triplicate.

Substrate preparation

One kilogram (1000 g) of each substrate was used for the mixed substrates, they were in equal proportion of 500 g (Oei, 2005) for each component and were done using a weighing balance (APX 200, Denver Instrument, Arvada, Colorado). Dried palm pressed fibre (PPF) were chopped into smaller pieces of 2 to 4 cm while palm pressed fibre ash (PPFA) were prepared by burning PPF to ash and allowed to cool. 1000 g individual substrate or a combination of 500 g each of PPF and PPFA for mixed substrate was added to 295 ml of sterile distilled water or Sterile Hoagland solution. Substrates were mixed until all the water was absorbed and packaged in a separate polythene bags. The bags of substrates were then compressed and closed with PVC necks which were covered with cotton and wrapped with papers to prevent entry of insects. The bags were pasteurized at 100°C for 8 h to avoid microbial contamination and were allowed to cool and inoculated with about 8 g of spawn. The substrate were subsequently placed vertically in a spawn running room maintained at 25°C and watered daily to maintain a relative humidity between 70 to 80% for spawn colonization while mycelia density was measured according to the method of Kadiri (1998). After the colonization, the upper parts of the bags were opened for fructification. The method employed was as describe by Sarkar et al. (2007).

Data collection

The yield of *P. pulmonarius* on the different substrate combination was determined by recording the number, weight, diameter of pileus and size of the fruit bodies after sprouting. The measurements from the various replicates were added and their mean value

Table 2. Reagent composition of modified Hoagland solution used in experimental design.

Reagent	Amount of reagent in water (Stock) (g/L)	Amount of stock in 1 L of water (ml/L)
KNO ₃	202	2.5
Ca(NO ₃) ₂ ·4H ₂ O	236	2.5
Fe-EDTA	15	1.5
MgSO ₄ ·7H ₂ O	493	1
NH ₄ NO ₃	80	1
H ₃ BO ₃	2.86	1
MnCl ₂ ·4H ₂ O	1.81	1
ZnSO ₄ ·7H ₂ O	0.22	1
CuSO ₄	0.05	1
Na ₂ MoO ₄ ·2H ₂ O	0.12	1
KH ₂ PO ₄	136	0.5

Hoagland and Arnon, 1950.

calculated. Other data collected include time of mycelia growth after inoculation and mycelia growth rate after 14 days, initial day of bottom formation, fruiting body formation day and average number of fruiting body.

Number and height fruit bodies

Number of fruit bodies was obtained by directly counting the number of fruit bodies on each substrate. The height was measured in centimeters using a steel ruler of dimension 50 cm by 2.5 cm (Dongguan Hust Tony Instrument Co. Ltd, Guandong, China) from the base of the stipe to the pileus.

Diameter of the pileus

This was also measured in centimeters with ruler from one edge of the pileus across the stipe to the other edge.

Fresh and dry weight of fruit bodies

This was done using an electrical weighting balance (APX 200, Denver Instrument, Arvada, Colorado).

Biological efficiency

This was calculated as:

$$\frac{\text{fresh weight of harvested mushroom}}{\text{substrate weight}} \times 100$$

Preparation of Hoagland solution

Hoagland solution was prepared as indicated in Table 2 according to the method of Hoagland and Arnon (1950) using the following salts KNO₃, MgSO₄·7H₂O, KH₂PO₄, Fe-EDTA, H₃BO₃, CuSO₄, ZnSO₄·7H₂O, MnCl₂·4H₂O, Na₂MoO₄·2H₂O, Ca(NO₃)₂·4H₂O.

Location of experiment

The experimental set up was carried out at the laboratory complex

of the department of Pure and Applied Biology, Ladoké Akintola University of Technology Ogbomoso Oyo State, Nigeria. The University is located at the lower limit of southern guinea savannah zone, between Latitude 8°10'N and Longitude 4°10'E.

Proximate and mineral content analyses

These were carried out at Nigeria Stored Product Research Institute (INSPRI), Ilorin, Nigeria according to standard methods.

Statistical analysis

Analysis of variance (ANOVA) was further employed to assess similarities and differences between the growth parameters.

RESULTS AND DISCUSSION

In this research *P. pulmonarius* was cultivated on seven combinations of substrates consisting of Palm press fibre (PPF), Palm press fibre ash (PPFA), sawdust, Hoagland solution and water. The effect of substrate combination on mycelia and fruit body initiation as well as bottom formation and number of mushroom fruit body is presented in Table 3 while Table 4 shows the effect of various substrates combination on other growth parameters. Figure 1 shows the growth of *P. pulmonarius* on substrate A, D and F.

Mycelia initiation and growth assessment

Mycelia initiation after inoculation on various substrates vary between 48 to 72 h with substrates E, G and F having the least period of mycelia initiation of 48 h while A, B, C and D have mycelia growth initiation period of 72 h. Mycelia were abundant on substrate F, moderate on substrate A and B, and scanty on substrates C, D, E and G after 14 days.

Bottom initiation and fruit body formation

Earliest bottom initiation was recorded on day 25 on substrates D and F followed by Substrate C on day 26, substrate A and B on Day 27, and substrate E and G on day 28 and 29, respectively. Earliest mushroom fruiting body were observed on day 26 on substrates D and F followed by substrate C on day 27, substrate A and B on Day 28, and substrate E and G on day 30.

Number of fruit body, height of stipe and diameter of pileus

Average number of fruiting body was observed to be higher on substrate D (27) and F (31), followed by A (16) and E (18), while the least were recorded on substrate B (15), G (13) and C (12). Highest height of stipe were

Table 3. Effect of substrate combination on mycelia and fruit body initiation as well as bottom formation and number of mushroom fruit body.

Substrate	Mycelia initiation (days)	Mycelia growth after 14 days	Bottom initiation (day)	Fruit body formation (day)	Average no of fruit body formed
A	3	++	27	28	16
B	3	++	27	28	15
C	3	+	26	27	12
D	3	+	25	26	27
E	2	+	28	30	18
F	2	+++	25	26	31
G	2	+	29	30	13

+, Scanty growth of mycelia; ++, moderate growth of mycelia; +++, abundant growth of mycelia.

Table 4. Effect of various substrate combinations on growth parameters measured.

Substrate	Average height of stipe (cm)	Average diameter of pileus (cm)	Average fresh weight of fruit bodies (g)	Average dry weight of fruit bodies (g)	Biological efficiency (%)
A	1.40±0.23 ^a	2.80±0.21 ^a	13.60±3.91 ^a	5.47±3.91 ^a	1.37±0.39 ^a
B	4.70±0.81 ^{bc}	5.37±0.93 ^a	14.13±1.02 ^a	5.47±1.02 ^a	1.43±0.09 ^a
C	1.60±0.10 ^a	1.60±0.20 ^a	4.30±1.80 ^a	1.50±1.80 ^a	0.45±0.15 ^a
D	8.00±0.24 ^c	13.47±2.98 ^b	95.37±8.69 ^b	24.20±8.69 ^b	9.53±0.87 ^b
E	1.30±0.11 ^a	2.80±0.21 ^a	13.60±3.91 ^a	5.87±3.91 ^a	1.37±0.39 ^a
F	7.00±1.48 ^c	12.50±2.95 ^b	89.20±8.63 ^b	21.87±8.63 ^b	8.93±0.84 ^b
G	1.80±0.40 ^a	2.90±0.40 ^a	4.90±1.80 ^a	1.60±1.80 ^a	0.55±0.15 ^a

Mean values followed by the same alphabets in the column are not significantly different by Duncan's Multiple range Test (DMRT) ($P \leq 0.05$).

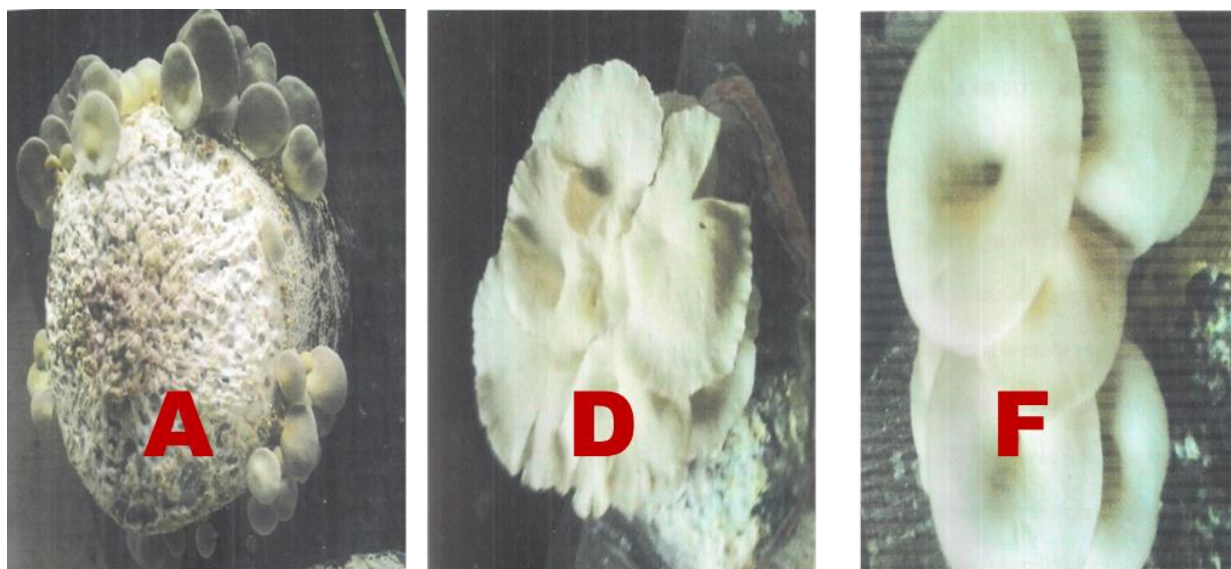


Figure 1. *P. pulmonarius* growing on substrate A (100% Sawdust + water), D (50% PPF + 50% PPFA + water) and F (50% PPF + 50% PPFA + Hoagland).

recorded on substrate D and F as 8 and 7 cm, respectively, while the least height were recorded on

substrate A and E as 1.4 and 1.3 cm. Substrate B, C and G have heights of 4.7, 1.6 and 1.8 cm, respectively. Best

fruit bodies of average diameter 13.5 and 12.5 cm were equally recorded on D and F while the least were recorded on A and E as 1.2 and 2.8 cm. Substrate B, C and G have average diameter of pileus recorded as 5.3, 3.2 and 2.9 cm, respectively.

Fresh and dry weight of fruit bodies

The mean fresh weight recorded on substrate D and F were 95.0 and 89.2 g followed by that of substrates B (14.1 g), A (13.6 g) and E (13.6 g). The least amount was recorded on substrates C (4.3 g) and G (4.9 g). The mean dry weight recorded on substrate D and F were 24.6 and 21.9 g followed by that of substrates B (5.4 g), A (5.4 g) and E (5.9 g). The least amount was recorded on substrates C (1.5 g) and G (1.6 g). The highest yield of *P. pulmonarius* was recorded on substrates D and F with average fresh weight of fruit bodies recorded as 95.37 and 89.20 g, respectively. Substrates D and F also recorded the highest average number of fruit body, height of stipe and diameter of pileus (27, 8 and 13.5 cm for D) and (31, 7 and 12.5 cm for F). The yields of *P. pulmonarius* recorded for D and F in this work is greater than the highest recorded for the growth of *P. pulmonarius* cultivated on sawdust, cassava peels and oil palm fibre (15 g) in the work of Onuoha et al. (2009), that of *P. pulmonarius* cultivated on corncorb and rice bran substrates (53.2 g) in the work of Stanley et al. (2011) and that of *Pleurotus ostreatus* cultivated on cotton waste, rice straw and sawdust (4.3 g) substrates in the work of Jonathan et al. (2012). Onuoha et al. (2009) recorded highest number of fruit body, height of stipe and diameter of pileus as 6, 2.6 and 5 cm on sawdust while Stanley et al. (2011) recorded highest number of fruit body, height of stipe and diameter of pileus as 12, 3.6 and 5.5 cm and no growth was recorded on palm press fibre substrate only. This result therefore compare favourably with such previously published work. Oil palm fibre have been reported to improve the mineral content of soil such as N, K, and organic C, and to improve C:N ratio (Akinyele et al., 2013) which have been reported to favour the growth of *P. pulmonarius*. Oil palm fibre has also competed favourably in enhancement of mineral content of substrates than sewage and animal dung (Mbah and Mbagwu, 2006). Fibre has lesser phosphorus content which inhibit the uptake of micronutrients and contain a high C:N ratio which favours the growth of mushroom (Zadrazil, 1980; Onuoha et al., 2009).

The outstanding performance of *P. pulmonarius* on substrate D and F amended with oil palm ash may be due to the potential of ash to influence substrate pH and enhance nutrient bioavailability as yield and nutrient availability have been associated with pH (Altomare et al., 1999). Wood ash has previously been used to amend soil pH (Lerner and Utzinger, 1986; Naylor and Schmidt, 1986; Hakkila, 1989) and supply plant nutrient (Ohno and Erich, 1990; Mbah and Nkpaji, 2010). Ashing has also

been reported to release most nutrient otherwise locked up within the body of substrate (Mehdi et al., 2013). Ash has been reported for potential use in organic agriculture and as good source of K, P, Mg, Ca and micronutrients (Kakier and Summer, 1996; Demeyer et al., 2001). Wood ash has shown significant impact on growth and yield of maize in the absence of synthetic fertilizers (Nottidge et al., 2005; Mbah and Nkpaji, 2010). Patterson (2001) reported that ash application up to 25 Mgha⁻¹ significantly increased barley and canola yield, and canola oilseed yield. Effective and timely utilization of ash for maintaining soil quality and reducing the harmful effects of acidification of surface waters have also been reported (Fransman and Nihlgard, 1995; LeBlanc et al., 2006). The ability of ash to increase bioavailability of N, P, K⁺, Ca²⁺ and Mg²⁺ have been documented (Mehdi et al., 2013).

From previously reported works, various agricultural wastes reported for cultivation of mushrooms have been documented to include; cotton waste, sugar cane bagasse, wheat straw, rice straw, paper waste, saw dust and cassava peels (Onuoha et al., 2009; Jonathan et al., 2012). Previous attempts in cultivation of *P. pulmonarius* on oil palm fibre have met various degrees of difficulties. This report therefore adds to the history of successful cultivation of *P. pulmonarius* on rarely utilized substrate such as oil palm fibre. The work of Onuoha et al. (2009) which reported the previous trial of cultivation of *P. pulmonarius* on oil palm fibre recorded no growth while the limited growth recorded on the mixture of oil palm fibre, cassava peel and sawdust substrate was attributed to the presence of cassava peel and sawdust component of the substrate. This result negates the observation of Okwujiako (1992) and Onuoha et al. (2009) that asserts that sawdust is the best artificial substrate for the growth of *P. pulmonarius* and which remains the current practice in Nigeria. The addition of fibres in substrates used for agricultural cultivation has been reported to increase the mineral contents of such substrates such as C, N, P and K. The comparative mineral contribution of fibres to substrate correlates with the mineral composition of the oil palm fibre applied to such substrates (Akinyele et al., 2013).

Analysis of variance (ANOVA), proximate, mineral content analysis and biological efficiency

The analysis of variance (ANOVA) of the growth parameters measured and biological efficiency obtained by separating and subjecting the mean results to Duncan Multiple Range Test (DMRT) indicates that substrate combination in treatment D and F resulted in optimal yield of *P. pulmonarius*. The mean of D and F were significantly different from those recorded on all other substrates combination. The mineral content and proximate analysis of *P. pulmonarius* cultivated on substrates D and F were then investigated for further comparison. The percentage (%) proximate analysis of *P. pulmonarius*

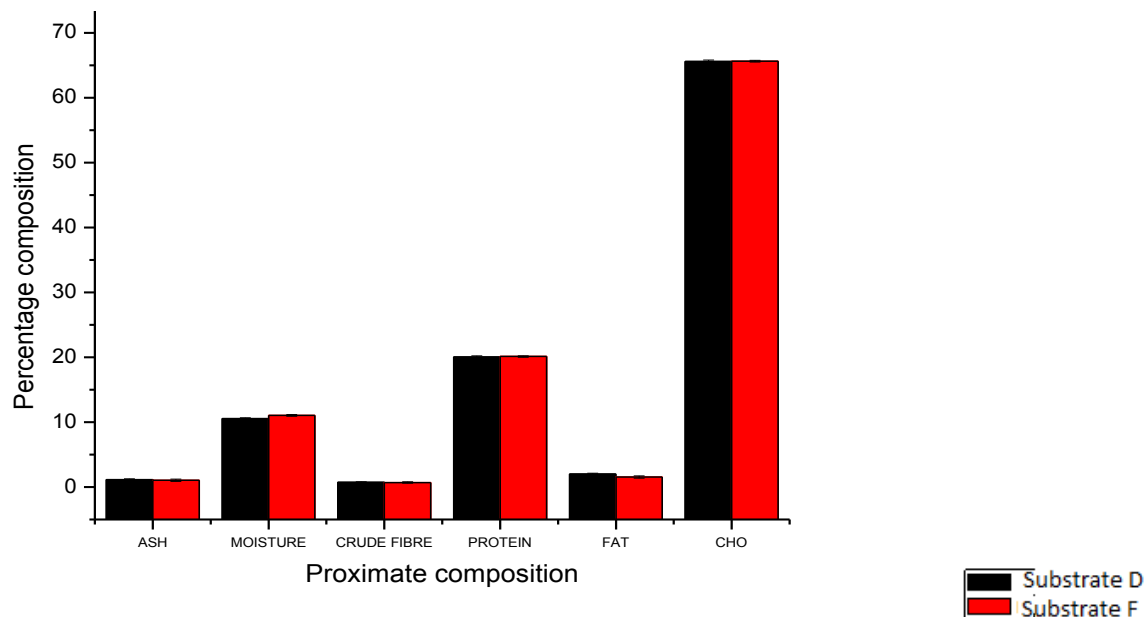


Figure 2. Proximate composition of mushroom obtained from substrate D (50% PPF + 50% PPFA + water) and F (50% PPF + 50% PPFA + Hoagland). Values are the means \pm standard deviation of three replicate determinations.

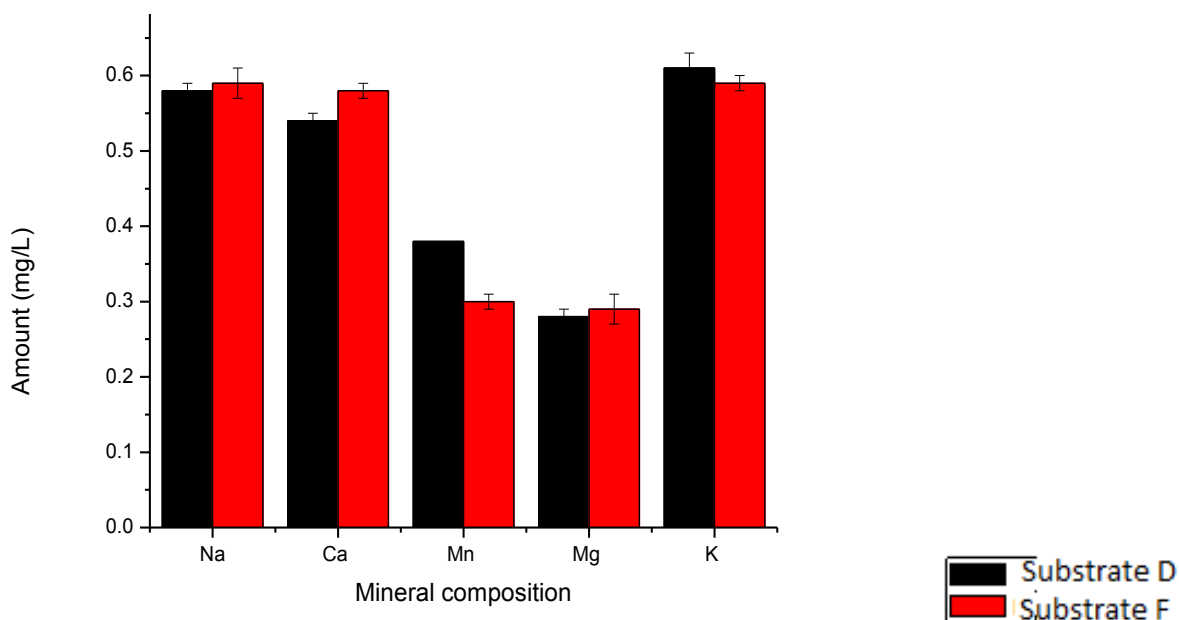


Figure 3. Mineral composition of mushroom obtained from substrate D (50% PPF + 50% PPFA + water) and F (50% PPF + 50% PPFA + Hoagland). Values are the means \pm standard deviation of three replicate determinations.

cultivated on substrate D and F shows similar results (Figure 2) with mushroom on substrate D containing Ash (1.14), moisture (10.54), crude fibre (0.71), protein (20.03), fat (2.00) and carbohydrate (55.59), while that cultivated on substrate F contains Ash (1.03), moisture (11.03), crude fibre (0.70), protein (20.13), fat (1.53) and

carbohydrate (65.59). The mineral content analysis of *P. pulmonarius* cultivated on substrates D and F also shows similar result (Figure 3) with D containing in mg/L of Na (0.58), Ca (0.54), Mn (0.38), Mg (0.28), and K (0.61) and F containing in mg/L of Na (0.59), Ca (0.58), Mn (0.3), Mg (0.29) and K (0.59). Results indicate a similar proximate

and mineral composition for mushroom cultivated on substrate D and F.

The similarity in the proximate and metal mineral contents of *P. pulmonarius* despite its cultivation on different substrate and Hoagland solution may be attributed to the ability of the mushroom to extract sufficient amount of nutrient from those locked within the organically rich lignocellulolytic fibre substrate for its yield and quality. Ability of various fungi to extract mineral nutrients from their solid phase compounds to satisfy nutritional requirements have been reported (Glowa et al., 2003). Fungi have been reported to degrade and solubilize minerals from rocks, metallic zinc, basalt, alumino-silicates, biotite, microcline and chlorite (Sterflinger, 2000; Glowa et al., 2003) by the production of organic and inorganic acids, alkalis, CO₂ and other complexing agents (Sterflinger, 2000; Cunningham and Kuiack, 1992; Goldstein, 1995). Organic acids, peptides, proteins, phenolics, ligninolytic enzymes, chitin (Altomare et al., 1999) and other complexing agents provides both sources of solubilization and metal chelating anion to complex the metal cation (Devevre et al., 1996). The solubilization, extraction and absorption by fungi have been explain to include biomechanical weakening and biochemical solubilization (Kumar and Kumar, 1999; Sterflinger, 2000; Hoffland et al., 2004; Gadd, 2010) while other mechanisms involved include acidolysis, complexolysis, redoxolysis and mycelia metal accumulation.

Highest biological efficiency was recorded for substrates D (9.5%) and F (8.9%) followed by A (1.4%), B (1.4%) and E (1.4%) while the least biological efficiency was recorded against substrates C (0.4%) and G (0.5%). The result shows that substrate D is better converted and supports a higher yield of mushroom that is significantly similar to that of substrate F but differs from that of all other substrate investigated and reveals the suitability of the combination of ash, water and palm press fibre (Substrate D) over Ash, Hoagland solution and palm press fibre (Substrate F), and other substrate combination investigated. The above result suggests that substrate D may contain higher nutrient suitable for *Pleurotus* cultivation than substrate F and other substrates being investigated. Previous report have shown that improved growth is directly associated with amount of nutrient concentration until a threshold after which the effect of such addition may not be further noticed (Kang and Iersel, 2004). Kang and Iersel (2004) reported increase in shoot and total dry weight with increasing nutrient solution concentrations until a threshold after which little or no additional increase in dry weight was observed. The improved yield observed in substrate D and F therefore suggests that the two substrates provided sufficient amount of nutrient needed for improved yield of *Pleurotus* mushroom. This result may explain why mushrooms grow naturally and are picked on fields by locals on burnt palm trees in Nigeria. The performance of substrate combination of palm press fibre ash, water and palm press fibre

however compare favourably with that of palm press fibre ash, Hoagland and palm press fibre combination under all growth and yield parameters investigated.

This result corroborates the findings of Ajibade et al. (2013) which noted that addition of chemical fertilizers during cultivation may not necessarily give corresponding high yield. This result also confirms the usability of palm waste in the improvement of soil fertility and cultivation of mushroom as reported by Tabi et al. (2008); Sudirman et al. (2011) and Ajibade et al. (2013). The differences in the nutritional composition of substrate used have been suggested for the variation in the results obtained as reported by Tabi et al. (2008). Factor such as amount of nitrogen, carbon content and substrate particle size have also been suggested to contribute to yield of cultivated mushrooms. From this research, all substrates applied in this study show potentials for *Pleurotus* mushroom cultivation either alone or in combination as all substrates produce *Pleurotus* fruit bodies. However, Substrate D with the composition of 50% of PPF and 50% of PPFA and water yielded the highest amount (fresh/dry weight, average diameter of pileus and height of stipe) of *Pleurotus* fruit bodies as well as improved mycelia, bottom initiation, and fruit body formation day per replicate compared to the other substrates. With optimum use of the biomass generated from the palm waste, it will not only solve the environmental pollution problem but it can also offer a promising way to convert low quality bio-masses into a valuable high protein food.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Evaluation of soil microbial communities as influenced by crude oil pollution

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Impact of petroleum pollution in a vulnerable Niger Delta ecosystem was investigated to assess interactions in a first-generation phytoremediation site of a crude oil freshly-spilled agricultural soil. Community-level approach for assessing patterns of sole carbon-source utilization by mixed microbial samples was employed to differentiate spatial and temporal changes in the soil microbial communities. Genetic diversity and phenotypic expressions were measured for a more holistic perspective. The 5'-terminal restriction fragments generated after Csp digestion of 16S rRNA gene correlated with observed DNA concentrations in the community profile and revealed loss of diversity with pollution. Crude oil pollution significantly reduced phosphomonoesterases and respiratory activities and values were pH dependent. There were no expressed dehydrogenases activity in initial spill site but were enhanced with phytoremediation. Factor analysis of predictors and independent variables indicates that respiratory, alkaline phosphatase and β -glucosidase activities could be used to explain underlying factors. Positive soil – microbes – plant interactions were observed.

Key words: Species diversity, impact of crude oil pollution, soil – biota interactions, ecosystem monitoring, genetic diversity.

INTRODUCTION

Ecosystem functioning as described by Green et al. (2004), is incomplete without soil micorganisms, as they affect the chemical, biological and physical characteristics of soil. The anthropogenic influences, stemming from increased industrialization, oil exploration activities, sabotage and illegal refining of petroleum (Nwaichi et al., 2010) in oil – rich regions, on

biogeochemical cycles could impede on soil quality evaluation (Li et al., 2005). Phytoremediation, the use of plants to remove, degrade or separate hazardous substances, has been described by Nwaichi et al. (2011) to rely simply on the plant capability to accumulate large quantities of a certain contaminants or to take up and transpire large amount of water from soil and

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Abbreviations: TTC, 2,3,5-Triphenyltetrazolium chloride; TPF, triphenyl formazan; PNP, para-nitro phenyl phosphate; PCR, polymer chain reaction; ENT, effective number of types.

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groundwater. They also reported the possible contributions from production and secretion of plant enzymes, or stimulation of microbial biotransformation within the rhizosphere to this emerging and environmentally – friendly technique. The study of complex diversity pattern of the microbial community in parched and inundated soil environment has proven to be a herculean task. This may have arisen from inadequate biological classification of such environment. Ovreas (2000) described species diversity to consist in species richness, total of species present, evenness, and the distribution and these complement genetic diversity and ecosystem biodiversity in understanding soil microbial diversity. A wholistic understanding of soil microbial communities have been broadly defined by biochemical-based techniques and molecular-based techniques (Kirk et al., 2004; Abbasi et al., 2010). Biochemical methods accounted for their phenotypic expressions (e.g., respiration, enzymes and catabolic potential) while the use of signature lipid biomarkers (SLB), like phospholipid fatty acids (PLFA), and molecular biology (nucleic acid technologies) gives information on the microbial community composition based on groupings of fatty acids (Broughton and Gross, 2000) and genetic diversity, respectively. Molecular methods have the advantage of obtaining information about uncultivable organisms and can be applied to study complex trophic interactions in the field and to address underlying ecological questions.

In order to evaluate various strategies employed by microorganisms to adapt to changed environmental conditions under wide perturbations, this study seeks to evaluate the impact of crude oil pollution on soil microbial community, relevant biochemical indices, possible interactions thereof and the effects of phytoremediation on such ecosystem.

MATERIALS AND METHODS

The study area consists of a two – week old crude oil spilled (and burnt afterwards) soil in Oshie community in the Niger Delta region of Nigeria. This community is a host to a major oil company in Nigeria. Eye witnesses suspects sabotage as the cause of spill. 20 cm depth soils from this site were randomly collected (using soil auger) and bulked following a field survey and set up in a phytoremediation pot experiments (in replicates of three) using *Vigna subterranea*, *Hevea brasiliensis*, *Cymbopogon citratus* and *Fimbristylis littoralis*. *F. Littoralis* and *H. Brasilensis* were chosen for their prevalence in study area while the use of *V. subterranea* has been reported by Nwaichi et al. (2010; 2014). Black polyethylene bags were used for potting and free drains were made possible for the growth period by narrow perforations at the base, to avoid water logging in pots. pH was determined on site using Hanna micro pH meter by probe insertion in soil solution (1:5). Temperature was not regulated to mimic natural environments. Seedlings for *V. subterranea* and *H. brasiliensis* were raised on moist cotton spread to exclude contamination while young *C. citratus* and *F. littoralis* were sourced from University of Port Harcourt botanical garden. An agricultural soil in the same region with history of no pollution constituted control regimes. Chemical and physical characterization of control and polluted soils were performed before the start of the

experiment. After 90 days with watering, based on need, plants were harvested and rhizospheric soils were collected for laboratory analyses. These were transported in ice chests to Institute of Agrophysics Poland for analysis. Different soil regimes (2 mm screen and air dried) from planted and unplanted, polluted and unpolluted variants were subjected to preparations for various analyses discussed: Assay for protease activity was adapted from reported protocol by Alef and Nannipieri (1995). In order to determine the amino acids released after incubation of soil with sodium caseinate for 2 h at 50°C using Folin-Ciocalteu reagent, 5 ml of 1% substrate (sodium caseinate in TRIS HCl buffer pH 8.1 (prepared one night before and kept in fridge), was added into test only. Only 5 ml of TRIS HCl buffer pH 8.1 was added to control. After centrifugation for 2 min at 200 rpm, absorbance readings (578 nm) of 96 Corning plates containing 200 µl samples in a Spectrophotometer (Infinite M200 PRO TECAN) were taken with buffer as blank.

Measurement of dehydrogenase activity was adapted from reported protocol by Alef and Nannipieri (1995). This method is based on the estimation of the 2, 3, 5-Triphenyltetrazolium chloride (TTC) reduction rate to Triphenyl formazan (TPF) in soils after incubation at 30°C for 24 h. TTC and TPF are light-sensitive so beaker was shielded and all procedures were performed under diffused light. Results were corrected for control and calculation was done for p-nitrophenol per ml of the filtrate by reference to the calibration curve.

Phosphomonoesterases activity assay was adapted from reported protocol by Alef and Nannipieri (1995). The method is based on the determination of p-nitrophenol released after the incubation of soil with p-nitrophenyl phosphate for 1 h at 37°C. To 1 g dry soil, 0.25 ml Toluene was added, then 4 ml buffer after 10 min in a fume chamber for tests and control. To test, 1 ml substrate (para-nitro phenyl phosphate, PNP) was introduced and all samples incubated for 1 h at 37°C. Thereafter, 1 ml substrate was added to control. An aliquot of 1 ml 0.5 M CaCl₂ and 4 ml 0.5 M NaOH were added to test and control and shaken with Multi RS – 60 BIOSAN Programmable rotator-mixer/ shaker for 3 min and were centrifuged (Ependorf Centrifuge 5810R) at 4000 rpm for 10 min. Microplates containing 200 µl samples were read off in a spectrophotometer at 485 nm using buffer as blank. For buffer preparation, correction of 0. M HCl and 1 M NaOH mixture were corrected to pH 6.5 and 11 for acid and alkaline phosphatases, respectively.

Respiratory activity determination was adapted from reported protocol by Alef and Nannipieri (1995). Substrate (Glucose) induced method was employed. Evenly, 1 ml glucose solution was added onto the soil in respiratory flasks and autoclaved (Fedegari Autoclave AG and Classic Prestige Medical Autoclave) at 121°C for 20 min. Titration was done using 0.1 M HCl and Phenolphthalein indicator, while shaking and titre values were recorded for calculation.

Assay for B- glucosidase activity was adapted from reported protocol by Alef and Nannipieri (1995). This was based on the principle of released p-nitrophenol estimation, after soil incubation for 1 h at 37°C. Supernatant (200 µl) in well plates were read off spectrophotometrically at 400 nm. For buffer, 200 µl Modified universal buffer was used.

Community level profiling, CLLP was done using BIOLOG Microstation™ Biotek Instruments USA. One gram soil was added to 99 ml sterile peptone water and calibrated using a peristaltic pump, and then autoclaved (SterilClave 18 BHD Caminox 2009) at 121°C for 20 min alongside tips and tubes. On 24 h intervals, 9 daily readings (27°C incubation) were taken. Carbon sources are as provided by manufacturer.

DNA extraction was done immediately following FastDNA® SPIN kit for Feces and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA following manufacturer's protocol). DNA concentration thereafter was determined using Nano Drop 2000 Spectrophotometer (Thermo Scientific). These were stored at -80°.

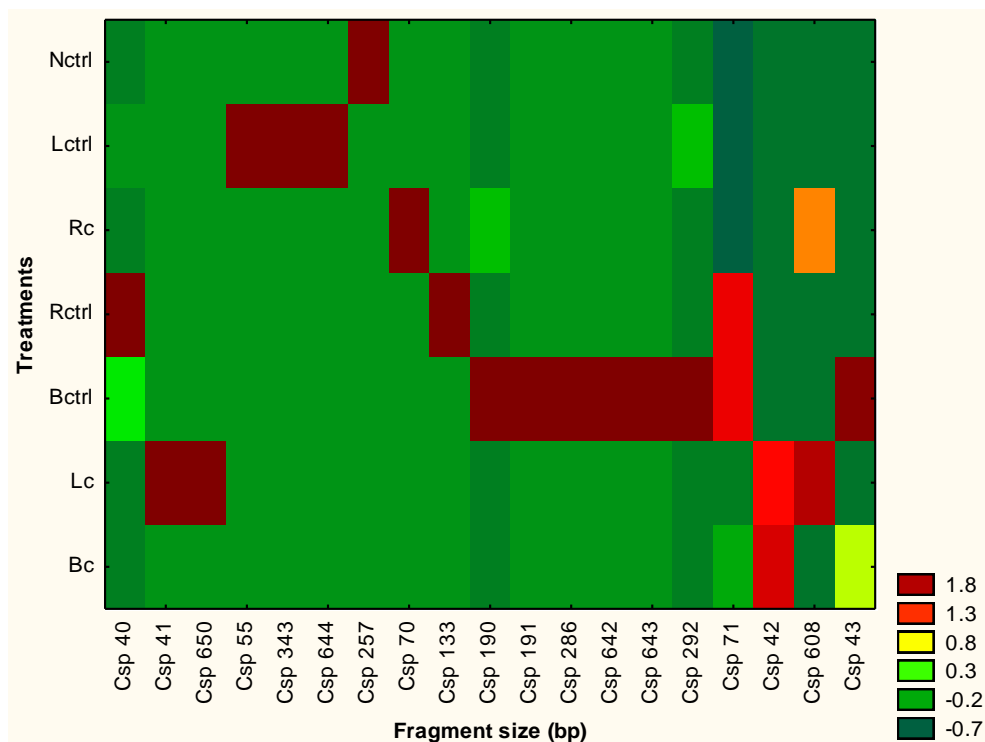


Figure 1. Cluster representation of tRFLP profiles from the study. Ctrl, c, and N denote control, crude oil polluted soils and 'no plant', while, B, L, and R represents Bambara, Lemon grass and Rubber plants respectively.

For PCR AOA (Polymerase Chain Reaction – Ammonia Oxidizing Archea), dilution of DNA to concentration of 2 ng/μl in nuclease free water was made and samples centrifuged in 'short' mode at 12000 rcf. Manual specific instruction for AB Applied Biosystems Veriti 96 Well Fast Thermocycler was followed. ExoSAP-IT® for ABI Affymetrix® USB® products (Source: Exonuclease 1-Recombinant) was used for purification of products and kit procedure was followed. Restriction endonuclease kit protocol was followed for digestion. Optimization of samples were done as described by He et al. (1994), Roux (2003) and Traugott et al. (2006). Terminal restriction fragment length polymorphism (t-RFLP) AOA was done using a DNA sequencer (AB Applied Biosystems HITACHI 3130) and manufacturer's protocol was followed. T-RFs with a size < 40 bp and 1% area were excluded from analyses.

Analysis of variance for data for triplicate determinations, principal component and factor analyses were elaborately done using STATISTICA v 10.

RESULTS AND DISCUSSION

From the results of the molecular technique based on polymer chain reaction (PCR), considerable differences between variants both with respect to the number of detected taxonomic units and to the structure of their distribution (Figure 1) were seen. In the polluted soils, there were fewer communities in comparison to unpolluted counterparts and could be employed in unravelling the numerical structure. Similar community distribution were seen for *H. brasiliensis* cultivated soils,

possibly because it is a native species to the study site where polluted soils were sourced. In a similar study but with PCB contamination, Patyka and Kruglov (2008) made similar observations. For comparison, the T-RFLP profile analysis of DNA extracted from control soil (Figure 1), in which levels of Hydrocarbons contaminants are negligible, differed considerably in both number and sizes of T-RFs and in their peak heights. Generally, DNA concentrations were observed to correlate with fragments present in the community profile, per treatment (data not shown).

Therefore, these peak heights, are reflective of the relative abundance of those ribotypes that are preferentially amplified during PCR and to an extent, may give an insight on the relative abundance of those ribotypes in the community. This is subject to any latent bias from PCR amplification. A more detailed analysis of the ecological parameters of the assemblage of prokaryotes showed the highest genetic diversity, determined by Shannon's index, in soil where Lemon grass was grown in an unpolluted soil and the lowest in the initial agricultural soil adopted as control, which was left unplanted. In this study, the equitability (evenness) index was relatively low and decreased with the area (Figure 1) occupied by the community. This means that the diversity and evenness in this experiment from the contaminants undisturbed habitat are much higher than in those from contaminants highly disturbed habitat. The

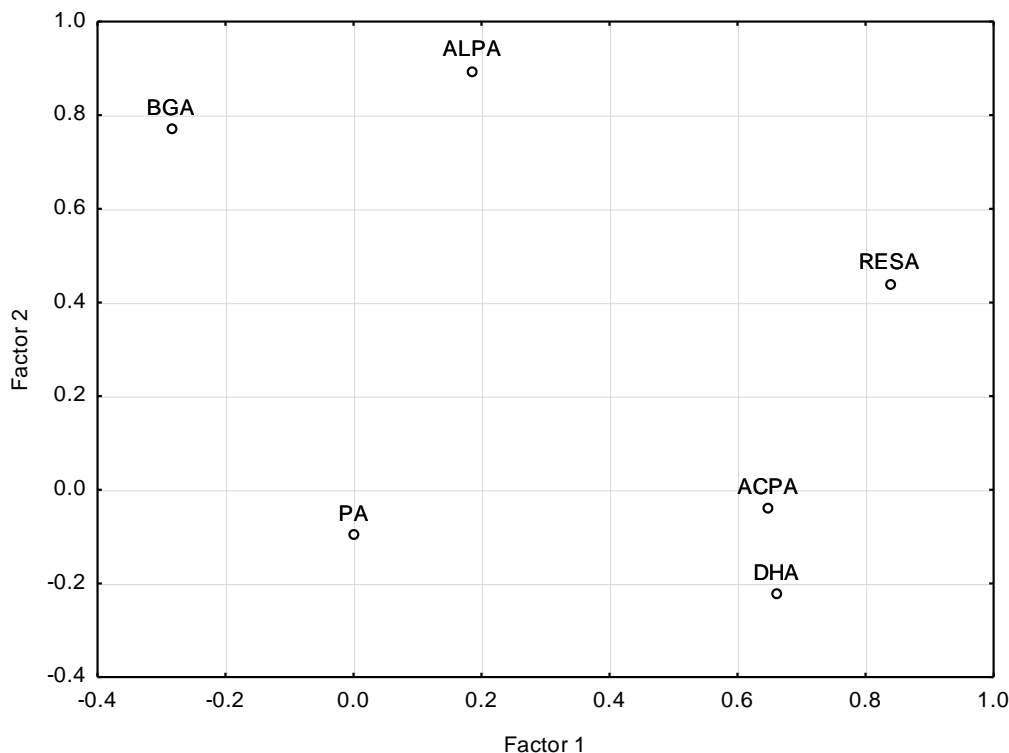


Figure 2. Factor analysis of predictors and independent variables for biochemical parameters. PA, DHA, ACPA, BGA, ALPA and RESA denote protease, dehydrogenase, acid phosphatase, β -glucosidase, alkaline phosphatase and respiratory activities respectively.

advantages with plant – soil - microbial interactions cannot be overemphasized. The unpolluted planted soils not only have greater number of species present, but the individuals in the community are distributed more equitably among these species. True diversity (Tuomisto, 2010) and effective number of types (ENT), revealed an equivalent diversity as a community with 4, 3 and 5 equally - common species for unpolluted soils cultivated with *V. subterranea*, *H. brasillensis* and *C. citratus*, respectively. There was a shift however to 3, 2 and 3 with pollution effects for *V. subterranea*, *H. brasillensis* and *C. citratus* – cultivated regimes, respectively.

Biochemical indices using factor analysis (Figure 2), which identifies "invisible" factors that represent the hidden organization or "organizing principle" of whatever is being measured with a number of observable measures (here dehydrogenases, proteases, phosphomonoesterases, β -glucosidases and respiratory activities) underscores the semblance of respiratory, alkaline phosphatase and β -glucosidase activities to hidden factors. Factor scores or "factor loadings" indicate how each "hidden" factor is associated with the "observable" variables used in the analysis (Tucker and MacCallum, 1997). Factor loading of 0.84 indicates that respiratory activity can be used to describe hidden Factor 1; in other words, Factor 1 has characteristics, very similar to respiratory activity. Other observable measures

were not useful in describing Factor 1. Similarly, factor loadings of 0.89 and 0.77 indicate that Factor 2 has characteristics, very similar to alkaline phosphatase and β -glucosidase activities. At 95% confidence level, analysis of variance among plants and between treatments indicated significant differences between unplanted polluted and unpolluted soils, *H. brasillensis* and *V. subterranea* - planted (Figure 3) for respiratory activity. Observed acidic soil pH influenced recorded phosphomonoesterase activity. Uniform activity were recorded for dehydrogenases and were more marked between planted and unplanted regimes (Figure 4) in *H. brasillensis* - planted unpolluted soils. This again, is attributed to low pH values with least value recorded in unplanted polluted soil.

In the BIOLOG system, 95 different carbon sources were used to produce a metabolic profile of microorganisms. The profiles obtained using community samples were differentiated properly by statistical analyses. Diversity index measurements, as well as PCA analysis were done for a higher degrees of resolution (Balser, 2000) between soils in order not to lose some details. Statistical evaluation of average well colour development shows significant differences in community spread (Figure 5) for polluted and unpolluted regimes especially between communities in planted and unplanted soils. Marked differences were recorded

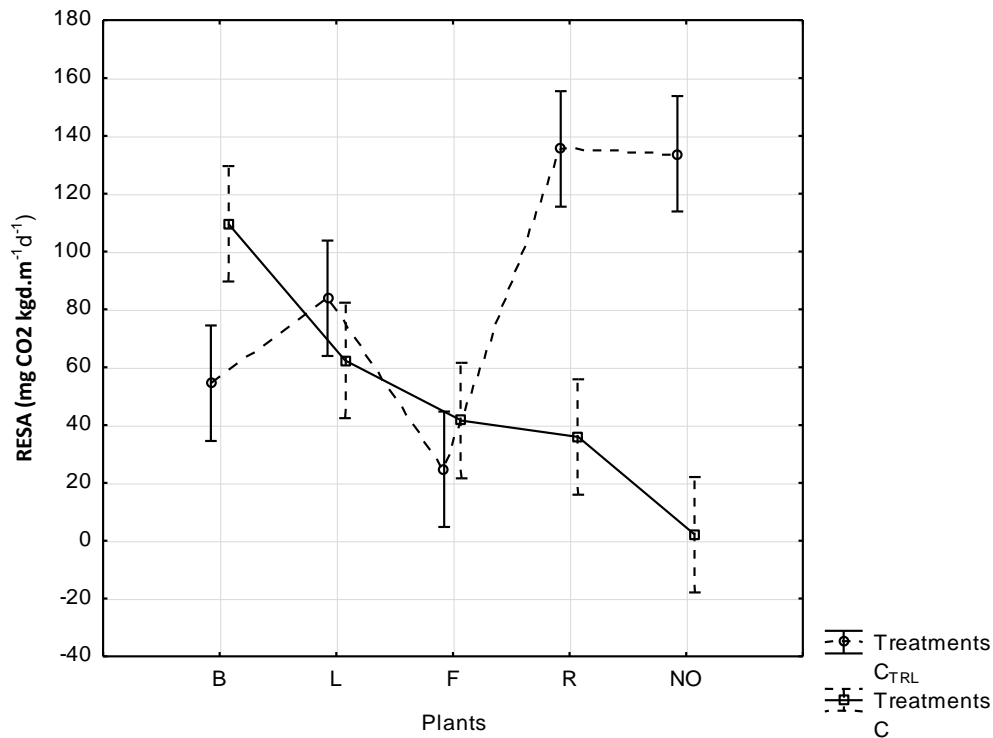


Figure 3. Respiratory activity among plants and between treatments. B, L, F, R, NO, and RESA denote Bambara, lemon grass, Fimbristylis, rubber, unplanted and respiratory activity.

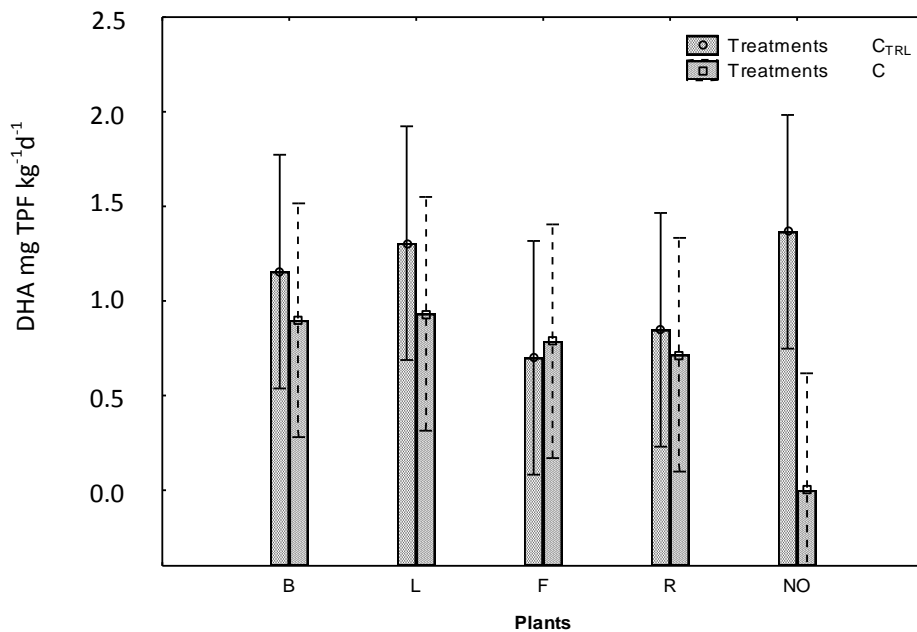


Figure 4. Dehydrogenase activity among plants and between treatments. B, L, F, R, NO, and DHA denote bambara, lemon grass, Fimbristylis, rubber, unplanted and dehydrogenases activity, respectively.

(Figure 6) among cultivated and uncultivated communities. The diversity (Figure 7) and evenness (not

shown) of species in this study showed wide distortions due to contamination and plant type and were higher in

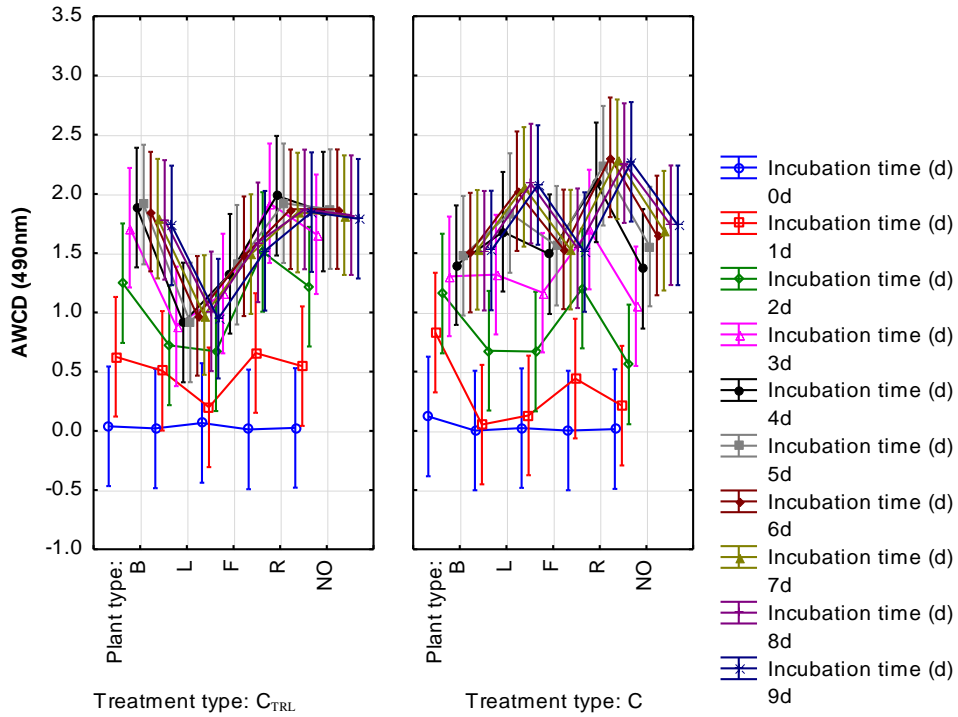


Figure 5. Variation of AWCD with time among plants and treatments. B, L, F, R, NO, and AWCD denote bambara, lemon grass, Fimbristylis, rubber, unplanted and average well colour development, respectively.

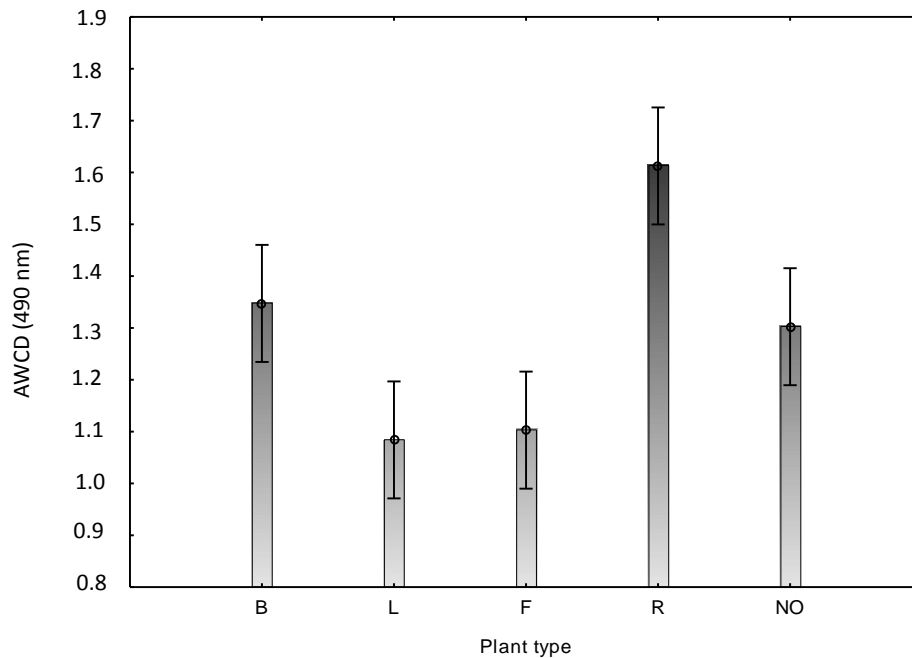


Figure 6. Colour development spread by plants. Vertical bars denote 0.95 confidence intervals. B, L, F, R, NO, and AWCD denote Bambara, Lemon grass, Fimbristylis, Rubber, unplanted and average well colour development, respectively.

communities from control planted soils closely followed by those of *H. brasiliensis* and *F. littoralis* in polluted soils.

It is worthy to mention that the two latter plants grow naturally in the polluted community, where test was

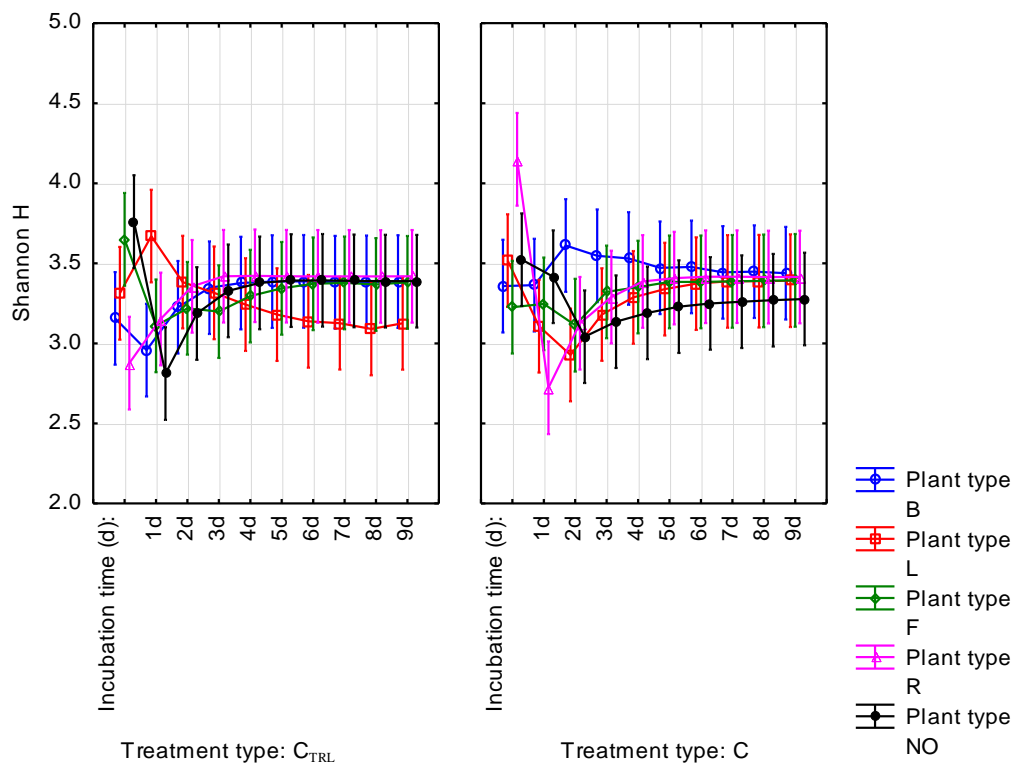


Figure 7. Species diversity characterization in an observed community. B, L, F, R, and NO denote bambara, lemon grass, Fimbristylis, Rubber, and unplanted regimes respectively.

sorced. Shape – wise, the dendrogram based on the result of the cluster analysis applied to the profiles of polluted soil and also appropriately reflects the difference in the origins of the samples (Figure 5). Near neutral buffered pH as reported by Liu et al. (2006), which is quite different from the acidic pH of study soils (Table 1) may limit some microorganisms that have adapted well to native soils. Such condition may have presented disadvantages in evaluation of soil microbial community structure. Although, retarded growth has been reported with crude oil pollution, Harper (1939) reported stimulated growth with low level of contamination. Given high percentage contaminant removal (data not shown) with phyto – assisted soil clean – up employed in this study, phenotypic growth, community increase and improved substrate consumption pattern in relatively cleaned soils corroborates this finding. These genomic data could be exploited to develop mass-target detection systems, that may enable identification of complete gamut of highly generalized biota in environmental biodiverse communities.

From data obtained from this study, there were marked shifts in the genotypic structure of soil microbial assemblage with crude oil pollution and diverse interactions were observed after a phytoremediation experiment. Community level physiological profiling generally, revealed significant changes due to contami-

Table 1. Chemical and physical characterization of control and polluted soils.

Identity	Polluted	Control
PAHs (mg/kg)	0.424	0.077
THC (mg/kg)	378.3	64.8
BTEX (mg/kg)	<0.001	<0.001
As (mg/kg)	1.25	0.49
Cd (mg/kg)	17.2	<0.001
Cr (mg/kg)	30.00	18.70
Fe (mg/kg)	20,642.50	16,657.50
Cu (mg/kg)	9.30	7.75
Pb (mg/kg)	806.20	400.30
TOC (%)	3.081	0.955
Cl ⁻	400	80
NO ₃ ⁻ (mg/kg)	7.75	26.25
SO ₄ ²⁻ (mg/kg)	215.00	305.00
N (%)	0.64	2.13
P (mg/kg)	0.67	26.30
K (cmol/kg)	1.612	0.068
Ca (cmol/kg)	<0.01	<0.01
Mg (cmol/kg)	4.721	0.682
Na (cmol/kg)	0.346	0.118
pH	3.45	5.45
Temp. (°C)	28.2	28.2

nation in metabolic diversity of mixed microbial communities. Limitations to this study may include PCR bias with DNA extractions and difficulty with quantifying some less dominant microorganisms in the community, as they might not be detected without fractionation. Also, the substrate concentration in the well of the BIOLOG plate may be much higher than that usually found in such parched environment.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Characterization of the dominant microorganisms responsible for the fermentation of dehulled maize grains into *nsiho* in Ghana

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Nsiho (white *kenkey*) is a type of *kenkey*, a sour stiff dumpling, produced from fermented maize meal in Ghana. The dominant microorganisms responsible for the fermentation of *nsiho* were characterized by analysing samples from four traditional production sites at Anum in the Eastern Region of Ghana. During 48 h of steeping dehulled maize grains, the pH values decreased from 6.05 to 5.93 to 3.59 to 3.55, whilst titratable acidity increased from 0.02 to 0.03 to 0.27 to 0.32%. In the subsequent 12 h dough fermentation, the pH decreased from 6.02 to 5.80 to 3.52 to 3.46, whilst titratable acidity increased from 0.25 to 0.27 to 0.35 to 0.38%. The lactic acid bacteria population increased by 2 to 5 log units to concentrations of 10^7 to 10^8 CFU/ml during steeping and by 2 to 3 log units from 10^5 to 10^6 CFU/g to 10^8 to 10^9 CFU/g during dough fermentation. Yeasts counts increased by 3 to 4 log units during steeping and by 2 to 4 log units during dough fermentation. The most frequently isolated lactic acid bacteria responsible for *nsiho* fermentation were identified as *Lactobacillus fermentum* (47.1%), *Lactobacillus brevis* (25%), *Lactobacillus plantarum* (14.42%), *Pediococcus pentosaceus* (8.65%) and *Pediococcus acidilactici*, (4.8%). The dominant yeasts species were *Saccharomyces cerevisiae* (47.6%), *Candida krusei* (29.1%), *Debaryomyces* spp., (15%) and *Trichosporon* spp., (8.3%). This is the first study to report on the microorganisms involved in *nsiho* fermentation.

Key words: *Nsiho*, dehulled maize, *kenkey*, lactic acid bacteria, indigenous African fermented foods.

INTRODUCTION

Traditional processing of maize into various fermented food products plays an important role in the food supply

system of Ghana, and contributes to curtailment of post-harvest losses and national food delivery (Sefa –Dedeh,

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Abbreviations: OGYEA, Oxytetracycline-glucose yeast extract agar; SPS, salt peptone solution.

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1993). Maize grains, by a variety of indigenous processes, are transformed into an intermediate or finished product with a stable shelf-life, improved digestibility and nutritive quality as well as desirable organoleptic properties. Processing of the grains may also include improvement of the protein quality of the product by fortification with legumes such as cowpeas and soybeans (Plahar and Leung, 1982; Nche et al., 1996; Obiri-Danso et al., 1997; Plahar et al., 1997; Sefa-Dedeh et al., 2000). One of the most common traditional products made from maize in Ghana is *kenkey*. Two main types of *kenkey* are known. *Ga kenkey* and *Fanti kenkey* have been the subject of much scientific investigation. A less common type of *kenkey* is produced from dehulled maize grains and is called *nsiho* or white *kenkey*. Production of *nsiho* involves dehulling or degerming maize grains which are then steeped in water for 48 h and milled into a meal. The meal is kneaded with water into a dough and left to ferment spontaneously for 12 to 24 h. Some producers however do not carry out dough fermentation. The dough is now pre-cooked, moulded into balls, wrapped in corn husks and steamed for 1 to 2 h. Some producers do not pre-cook the dough whilst others pre-cook part of the dough and mix it with the remaining dough before moulding and steaming into *nsiho*.

Production and vending of *nsiho* as well as the other types of *kenkey* is an important socio-economic activity in Ghana. This informal industry serves as a means of livelihood for numerous traditional food processors and their families. Presently, there is a pressing need to improve the informal traditional food processing sector as a whole in the advent of fast foods which could outcompete the indigenous foods. It is in this regard that *kenkey* and a lot of other indigenous African fermented foods have become subjects of intense scientific studies. Such investigations provide a basis for injection of suitable scientific and technological know-how to upgrade the traditional operations and the quality of the indigenous foods. With regards to *nsiho*, no detailed studies of its fermentation have been reported and there is very little information in the literature on the product. This work was carried out to characterize the dominant microorganisms involved in *nsiho* fermentation which could eventually lead to the development of a starter culture for its controlled fermentation during industrial production.

MATERIALS AND METHODS

Brief field study and sample collection

The brief study was carried out in three towns in the Asuogyaman District in the Eastern Region of Ghana, Anum, Senchi and Atimpoku. These towns are noted for the production of *nsiho* which is mainly produced in this part of the country. *Nsiho* producers were briefly interviewed to obtain information on their production practices

and collect samples for analysis. Samples were only collected from Anum where a two stage fermentation is carried out during *nsiho* production, that is, during steeping of dehulled maize grains and fermentation of dehulled maize dough. At Senchi and Atimpoku which are next to each other, fermentation is limited to steeping as no dough fermentation is carried out. Samples were aseptically collected from four production sites at Anum on three separate occasions for laboratory analysis. The samples were taken from various stages of production. They were dehulled maize grains, steep water at 0, 24 and 48 h, dough at 0, 4, 8 and 12 h of fermentation and the final product. The samples were transported in an ice chest to the Food Research Institute, CSIR, in Accra for analysis.

Chemical analysis

The pH of steep water was determined directly using a pH meter (Radiometer pHM 92. Radiometer Analytical A/S, Bagsvaerd, Denmark) after calibration using standard buffers. The pH of fermenting dough was determined after blending with distilled water in a ratio of 1:1. Titratable acidity was determined as described by Amoah-Awua et al. (1996). 80 ml of filtrate obtained from 10 g of dough dissolved in 200 ml distilled water was titrated against 0.1 N NaOH with 1% phenolphthalein. 1 ml of 0.1 N NaOH was taken as equivalent to 9.008×10^{-3} g lactic acid.

Microbiological analysis

Enumeration of microorganisms

For all solid samples, 10 g were added to 90.0 ml sterile salt peptone solution (SPS) containing 0.1% peptone and 0.8% NaCl, with pH adjusted to 7.2 and homogenized in a stomacher (Lad Blender, Model 4001, Seward Medical), for 30 s at normal speed. From appropriate ten-fold dilutions 1 ml aliquots of each dilution were plated on the appropriate media for enumeration and isolation of microorganisms. Aerobic mesophiles were enumerated by pour plate method on plate count agar (Oxoid CM325; Oxoid Ltd., Basingstoke, Hampshire, UK). Plates were incubated at 30°C for 72 h in accordance with the NMKL, No. 86 (2006). Lactic acid bacteria were enumerated by pour plate on deMan, Rogosa and Sharpe Agar (MRS, Oxoid CM361), pH 6.2, containing 0.1% cycloheximide to inhibit yeast growth and incubated anaerobically in an anaerobic jar with anaerocult A at 30°C for five days. Yeasts and moulds were enumerated by pour plate on oxytetracycline-glucose yeast extract agar (OGYEA), (Oxoid CM545) containing OGYEA supplement with pH adjusted to 7.0 and incubated at 25°C for 3 to 5 d in accordance with ISO No 21527-1 (2008).

Isolation and identification of lactic acid bacteria

About 20 colonies of LAB were selected from a segment of the highest dilution or suitable MRS plate and purified by plating repeatedly. The colonies were tested for Gram catalase and oxidase reaction and observed under phase contrast microscope. The colonies were tested for their ability to grow at different temperatures by inoculating them into MRS broth and incubating at either 10°C or 45°C for 72 to 96 h to observe growth as visual turbidity in the broth. Isolates were tested for growth at different pH in MRS broth (Oxoid CM359) with pH adjusted to 4.4 or 9.6 incubation at 30°C for 72 and observing for growth as visual turbidity in the broth. Isolates were tested for salt tolerance in MRS broth (Oxoid CM359) containing 6.5 and 18% (w/v) NaCl incubated

at 30°C for 5 days and observing for growth as visual turbidity. Isolates were tested for gas production from glucose in MRS basal medium to which glucose had been added. The medium was composed of peptone 10 g, yeast extract 5 g, tween 80 1 ml, dipotassium hydrogen phosphate 2 g, sodium acetate 5 g, triammonium citrate 2 g, MgSO₄·7H₂O 0.2 g, MnSO₄·4H₂O 0.05 g, 1 L distilled water, pH 6.5, but without glucose or meat extract. The basal medium was dispensed in 5 ml amounts into test tubes containing inverted Durham tubes and sterilized by autoclaving at 121°C for 15 min. Glucose was prepared as 10% solution and sterilized by filtration and added aseptically to the basal medium to give a final concentration of 2%. The inoculated tubes were examined for production of gas after 3 d incubation at 30°C. Isolates were tentatively identified by determining their pattern of carbohydrate fermentation using the API 50 CHL kit (BioMérieux, Marcy-l'Etoile, France) and comparing them to the API database.

Isolation and identification of yeasts

All colonies totaling 15 from a segment of the highest dilution or suitable OGYEA plate were selected and purified by successive sub-culturing in Malt Extract Broth (Oxoid CM57) and streaked repeatedly on OGYEA until pure colonies were obtained. The colonies were identified by carbohydrate fermentation and utilization patterns using ID 32 C kit (BioMérieux, Marcy-l'Etoile, France).

RESULTS

Nsiho production at Senchi, Atimpoku and Anum

The brief field study confirmed *nsiho* or *white-kenkey* to be the most common type of *kenkey* produced in the Asuogyaman District in the Eastern Region of Ghana. It also showed that Senchi, Atimpoku and Anum were the most important towns in the production of *nsiho*. A previous extensive survey involving the current authors had shown *Ga-* and *Fanti-kenkey* to be the most common types of *kenkey* in Ghana, with *nsiho* being less common and confined to a few parts of the country (Obodai et al., 2014). All the *nsiho* producers interviewed in the present work were women and most of them had little formal education. They were engaged in *nsiho* production or vending as a family business in home-based operations with skills acquired within the family. A production unit usually involved 3 or 4 women who produced between 5 and 10 kg of *nsiho* per batch. The producers did not have any equipment of their own and used large utensils including plastic drums for their manual operations. For the mechanized operations, that is, dehulling and milling of maize grains, they used customer service mills available in the neighbourhood. *Nsiho* production was the main source of employment for the families concerned and it was considered a profitable business. Two different methods (variations) were observed for the production of *nsiho* as shown in Figure 1. Maize grains are cleaned by winnowing and sorting to remove, chaff, dust, stones and other debris. The cleaned maize is dehulled in a mill and

steeped in water for 48 h. The steeped grains are then milled in a plate mill into a meal. At Senchi and Atimpoku, the meal is pre-cooked for about 30 to 60 min into a thick gelatinous paste, *ohu*, which is then moulded into balls and wrapped in clean maize husks. The balls are packed into a pot lined with sticks and maize husks and containing a small amount of water. The balls are then steamed for 1 to 2 h into *nsiho*. At Anum, however, the meal is kneaded with a little water into stiff dough and fermented spontaneously for 6 to 12 h. Two-thirds of the dough is pre-cooked for about 30 to 60 min into *ohu* and mixed with the remaining uncooked dough. The mixture is then moulded into balls and wrapped in clean maize husks. The balls are packed into a perforated pan and placed over a pot of boiling water and steamed for 1 to 2 h.

Changes in pH and titratable acidity during steeping and dough fermentation

The results of pH and titratable acidity of steep water and fermenting dough from the four production sites at Anum are shown in Table 1. The pH values during 48 h of steeping decreased from 6.05 to 5.93 at the start of steeping to 3.59 to 3.55 by the end of steeping. During dough fermentation, the pH decreased from 6.02 to 5.80 for the freshly prepared dough to 3.52 to 3.46 at the end of the fermentation. The most pronounced drop in pH occurred within the first 24 h of steeping and in the dough between 4 and 8 h of fermentation. Percentage titratable acidity increased during steeping from 0.02 to 0.03 to 0.27 to 0.32% after 48 h of steeping. Similar results were observed for dough fermentation with titratable acidity changing from 0.25 to 0.27 to 0.35 to 0.38% at the end of the process. The drop in pH values and the corresponding increases in percentage titratable acidity during both steeping and dough fermentation indicate the occurrence of lactic acid fermentation, as has been reported during the production of *Ga* and *Fanti kenkey* from whole maize grains (Halm et al., 1993, 1996, 2004; Obiri-Danso et al., 1997; Amoa-Awua et al., 1998; 2006).

Changes in microbial population during steeping and dough fermentation

The population of aerobic mesophiles recorded during steeping and dough fermentation at the four production sites at Anum are shown in Table 2. The values represent mean counts for samples taken on three separate occasions. The counts were at concentrations of 10⁴ to 10⁶ CFU/ml at the start of steeping. These counts increased by 2 to 4 log units within the first 24 h to 10⁸ CFU/ml at all four production sites. Between 24 and 48 h of steeping the concentration of aerobic mesophiles

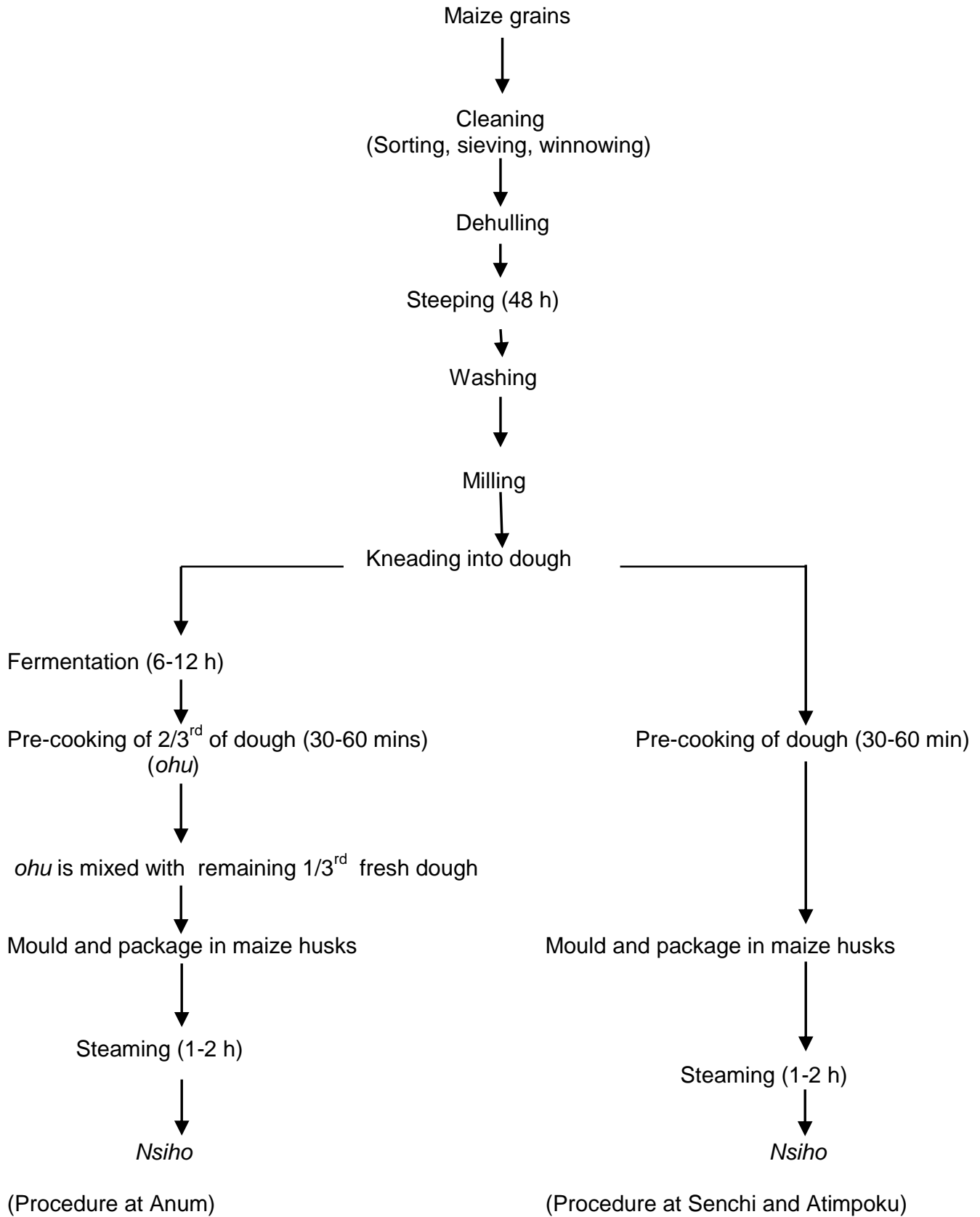


Figure 1. Flow diagram of the production of *Nsiho* (white-kenkey).

Table 1. Changes in pH and percentage titratable acidity during the fermentation of dehulled maize grains into *nsiho* (white *kenkey*) at Anum.

Sample	Production site 1	Production site 2	Production site 3	Production site 4
pH				
Steep water (h)				
0	5.98 ± 0.05	6.00 ± 0.03	6.05 ± 0.03	5.93 ± 0.01
24	4.01 ± 0.03	4.00 ± 0.01	3.81 ± 0.10	3.90 ± 0.01
48	3.59 ± 0.04	3.59 ± 0.03	3.57 ± 0.03	3.55 ± 0.02
Fermenting dough (h)				
0	5.98 ± 0.02	6.02 ± 0.01	5.98 ± 0.01	5.80 ± 0.02
4	5.44 ± 0.01	5.51 ± 0.01	5.51 ± 0.06	5.46 ± 0.01
8	3.55 ± 0.01	3.58 ± 0.03	3.76 ± 0.03	3.57 ± 0.04
12	3.51 ± 0.02	3.52 ± 0.04	3.49 ± 0.01	3.46 ± 0.01
Percentage titratable acidity				
Steep water (h)				
0	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
24	0.25 ± 0.01	0.25 ± 0.01	0.27 ± 0.01	0.28 ± 0.01
48	0.27 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.32 ± 0.01
Dough fermentation (h)				
0	0.27 ± 0.03	0.25 ± 0.02	0.26 ± 0.01	0.27 ± 0.04
4	0.28 ± 0.01	0.26 ± 0.03	0.28 ± 0.01	0.28 ± 0.03
8	0.31 ± 0.03	0.29 ± 0.03	0.31 ± 0.02	0.32 ± 0.05
12	0.36 ± 0.01	0.35 ± 0.04	0.35 ± 0.01	0.38 ± 0.01

Table 2. Changes in the population of aerobic mesophiles in CFU/ml or g during the fermentation of dehulled maize grains into *nsiho* (white *kenkey*) at Anum.

Sample	Production site 1	Production site 2	Production site 3	Production site 4
Steep water (h)				
0	(3.4 ± 1.6) 10 ⁵	(4.5 ± 0.3) 10 ⁴	(1.1 ± 0.2) 10 ⁶	(2.0 ± 1.2) 10 ⁶
24	(5.5 ± 2.4) 10 ⁸	(4.6 ± 1.7) 10 ⁸	(1.0 ± 0.2) 10 ⁸	(3.0 ± 0.4) 10 ⁸
48	(6.1 ± 1.3) 10 ⁸	(4.8 ± 1.6) 10 ⁸	(3.9 ± 0.9) 10 ⁸	(9.1 ± 0.4) 10 ⁸
Fermenting dough (h)				
0	(1.7 ± 0.6) 10 ⁶	(3.0 ± 1.4) 10 ⁶	(5.1 ± 0.4) 10 ⁷	(2.2 ± 1.5) 10 ⁶
4	(7.6 ± 0.8) 10 ⁶	(7.6 ± 0.8) 10 ⁶	(1.1 ± 0.2) 10 ⁸	(2.2 ± 0.6) 10 ⁷
8	(2.1 ± 0.6) 10 ⁷	(7.8 ± 0.7) 10 ⁷	(8.6 ± 0.4) 10 ⁸	(4.8 ± 0.6) 10 ⁸
12	(4.9 ± 0.9) 10 ⁸	(5.6 ± 0.5) 10 ⁸	(1.0 ± 0.5) 10 ⁹	(8.7 ± 1.0) 10 ⁸

remained at 10⁸ CFU/ml at all sites. For dough fermentation the aerobic mesophilic counts increased steadily over the 12 h of fermentation by 2 log units from 10⁶ to 10⁸ CFU/g at production sites 1, 2 and 4, and from 10⁷ to 10⁹ CFU/ml at production site 3 (Table 2). The aerobic mesophiles consisted of Gram positive catalase-negative rods and cocci, Gram positive catalase positive cocci and Gram negative bacteria. These isolates were

not characterized any further because it was assumed that they played no role in *nsiho* fermentation as reported for *kenkey* by Halm et al. (1993) and Olsen et al. (1995). Lactic acid bacteria were enumerated as Gram positive catalase negative rods, coccoids and cocci on MRS. They were present at the start of steeping at a mean concentration of 10⁵ CFU/ml at production site 1, 10⁴ CFU/ml at production sites 2 and 3, and at 10⁵ CFU/ml at

Table 3. Changes in the population of lactic acid bacteria in CFU/ml or g during the fermentation of dehulled maize grains into *nsiho* (white *kenkey*) at Anum.

Sample	Production site 1	Production site 2	Production site 3	Production site 4
Steep water (h)				
0	$(1.1 \pm 0.1) 10^5$	$(5.1 \pm 0.6) 10^4$	$(7.2 \pm 0.4) 10^4$	$(1.7 \pm 0.8) 10^3$
24	$(5.4 \pm 1.0) 10^8$	$(2.2 \pm 0.4) 10^8$	$(2.8 \pm 1.3) 10^8$	$(7.7 \pm 0.7) 10^6$
48	$(1.8 \pm 0.4) 10^7$	$(4.1 \pm 0.8) 10^7$	$(8.8 \pm 0.9) 10^7$	$(3.2 \pm 0.9) 10^8$
Fermenting dough (h)				
0	$(1.0 \pm 0.1) 10^5$	$(2.3 \pm 1.3) 10^6$	$(2.8 \pm 1.2) 10^6$	$(2.1 \pm 1.1) 10^6$
4	$(6.7 \pm 0.6) 10^6$	$(3.1 \pm 0.9) 10^6$	$(3.2 \pm 2.0) 10^7$	$(2.5 \pm 1.3) 10^7$
8	$(3.0 \pm 0.6) 10^7$	$(1.6 \pm 0.1) 10^7$	$(2.1 \pm 1.5) 10^9$	$(5.0 \pm 0.9) 10^7$
12	$(6.3 \pm 1.2) 10^8$	$(5.1 \pm 1.5) 10^8$	$(2.4 \pm 0.7) 10^9$	$(2.6 \pm 2.1) 10^8$

Table 4. Changes in yeast population in CFU/ml or g during the fermentation of dehulled maize grains into *nsiho* (white *kenkey*).

Sample	Production site 1	Production site 2	Production site 3	Production site 4
Steep water (h)				
0	$(4.1 \pm 0.5) 10^2$	$(2.8 \pm 0.6) 10^2$	$(1.6 \pm 0.8) 10^2$	$(7.5 \pm 0.6) 10^2$
24	$(4.9 \pm 0.3) 10^4$	$(6.9 \pm 0.4) 10^4$	$(1.9 \pm 1.1) 10^5$	$(9.7 \pm 0.8) 10^4$
48	$(1.8 \pm 0.5) 10^5$	$(9.8 \pm 0.1) 10^4$	$(9.1 \pm 0.4) 10^5$	$(1.9 \pm 0.9) 10^6$
Fermenting dough (h)				
0	$(5.7 \pm 0.8) 10^3$	$(5.9 \pm 0.1) 10^4$	$(7.8 \pm 1.1) 10^4$	$(9.1 \pm 0.7) 10^3$
4	$(2.5 \pm 0.6) 10^5$	$(4.9 \pm 0.5) 10^5$	$(1.8 \pm 0.1) 10^5$	$(5.9 \pm 0.5) 10^4$
8	$(6.0 \pm 0.3) 10^5$	$(1.0 \pm 0.1) 10^6$	$(1.0 \pm 0.4) 10^6$	$(4.0 \pm 0.6) 10^5$
12	$(1.8 \pm 0.7) 10^7$	$(7.8 \pm 0.6) 10^7$	$(2.7 \pm 0.6) 10^6$	$(7.8 \pm 0.5) 10^6$

production site 4 (Table 3). These are also mean values for samples taken on three separate occasions. At 24 h, the concentrations were at 10^8 CFU/ml at production sites 1, 2 and 3 but at 10^6 CFU/ml at production site 4. By the end of steeping at 48 h a 1 log unit drop in concentration was recorded at production sites 1, 2 and 3 to 10^7 CFU/ml, whilst a 2 log unit increase occurred at production site 4 to 10^8 CFU/ml. In the 12 h dough fermentation, LAB counts increased by 2 log units from 10^6 to 10^8 CFU/g at production sites 2 and 4 and by 3 log units at production site 1 (10^5 to 10^8 CFU/g) and production site 3 (10^6 to 10^9 CFU/g).

Yeasts counts at the start of steeping at all production sites were at concentrations of 10^2 CFU/ml representing mean values for sampling on the three separate occasions (Table 4). Within 24 h, the counts increased by 2 log units at production sites 1, 2 and 4, and by 3 log units at production site 3. At 48 h no further increase was recorded at production sites 2 and 3, whilst an increase by 1 log unit was recorded at production site 1 and by 2 log units at production site 4. During 12 h dough fermentation yeast count increased by 4 log units from

10^3 and 10^4 CFU/g at production sites 1 and 2, respectively. At production site 3 the yeast population increased from 10^4 to 10^6 CFU/g and at production site 4 from 10^3 to 10^6 CFU/g respectively.

Tentative identification of lactic acid bacteria and yeasts

Only lactic acid bacteria and yeasts were tentatively identified in the present work because they have consistently been shown to be responsible for the fermentation of *kenkey* (Halm et al., 1993; Hayford and Jespersen, 1999; Hayford and Jakobsen, 1999; Hayford et al., 1999). A total number of 208 LAB were isolated from steep water and fermenting dough samples. They were isolated as Gram positive catalase negative rods, cocobacilli or cocci on MRS. They were assumed to be lactic acid bacteria belonging to the genera *Lactobacillus* and *Lactococci*. After grouping based on biochemical characterization, the most frequently occurring species was found to account for 47.1% of the total number of

LAB isolates. They were heterofermentative based on their ability to produce CO₂ from glucose. They also grew at pH 4.4 and 9.6 and at 45°C, but not at 10°C nor in 18% NaCl. They mostly fermented galactose, D-glucose, D-fructose, D-mannose, ribose, melibiose, saccharose, gluconate, maltose, D-raffinose, 5-ketogluconate, D-xylose, lactose, cellobiose, esculin, trehalose, β-gentobiose, salin, amygdalin, l-arabinose, galactose and mannitol in the API 50 CHL galleries. Based on this pattern of carbohydrate fermentation they were tentatively identified as *Lactobacillus fermentum*.

The second most frequently occurring species accounted for 25% of the isolates. They were very short rods or coccobacilli and grew at pH 4.4 and 9.6 and at 45°C but not at 10°C and 18% NaCl. They were able to ferment L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, amygdaline, maltose, melibiose, saccharose, gluconate and 2 keto-gluconate but did not utilize glycerol, erythritol, sorbose, or rhamnose. They were identified as *Lactobacillus brevis*.

The third most dominant species which accounted for 14.42% of the LAB isolates were rods which were identified as *Lactobacillus plantarum*. This was because they grew at pH 4.4 and 9.6, but not in 6.5 and 18% NaCl. They were also able to ferment arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, D-turanose, mannitol, esculin, salicin, sorbitol, maltose, lactose, cellobiose and gluconate.

Other LAB species which were cocci and appeared as tetrads were identified based on their carbohydrate fermentation profiles as *Pediococcus pentosaceus* (8.65%) and *Pediococcus acidilactici* (4.81%). *P. pentosaceus* isolates mainly fermented L-arabinose, ribose, galactose, D-xylose, D-fructose, D-glucose, D-mannose, salicin, cellobiose, esculin, lactose, melibiose, saccharose and β-gentobiose. *P. acidilactici* isolates mainly fermented ribose, D-xylose, L-xylose, D-fructose, D-glucose, D-mannose but not melibiose and saccharose.

A total of 185 yeasts were isolated from steep water and fermenting dough samples from the four production sites. The isolates were characterized by colony and cell morphology as well as by their pattern of carbohydrate fermentation and utilization in ID 32C galleries. The most frequently occurring species accounted for 47.6% of all the yeast isolates. They utilized galactose, glucose, sucrose, raffinose, maltose, DL-lactate, trehalose, α-methyl-D-glucoside, melibiose but could not assimilate lactose. They were identified as *Saccharomyces cerevisiae*. The second dominant yeast which made up 29.1% of the yeasts, utilized glucose, N-acetylglucosamide and DL-lactate out of the 32 carbohydrate tested. They were identified as *Candida krusei*. The third species constituted 15% of all the total yeast isolates and utilized D-melibiose, D-glucose. They were identified as *Debaryomyces* spp. and *Trichosporon* spp.,

was the least frequently isolated yeast (8.3%). It utilized only D-melibiose and D-glucose. *Debaryomyces* spp. and *Trichosporon* spp occurred mainly at the initial stages of steeping, whilst the others occurred at all the various stages of processing.

DISCUSSION

Souring of *nsiho*

In this work samples were only collected for analysis from *nsiho* production sites at Anum. In the process at Anum fermentation occurs at two different stages, during steeping and during dough fermentation. At Senchi and Atimpoku fermentation occurs only during steeping since no dough fermentation is carried out. Therefore, by studying the process at Anum information was obtained on both steeping and dough fermentation. The current study has shown that a steady increase in titratable acidity with a corresponding decrease in pH occurred during steeping of dehulled maize grains and *nsiho* dough fermentation. This was expected since previous studies have shown that other types of kenkey, notably *Ga-* and *Fanti-kenkey*, undergo lactic acid fermentation (Halm et al., 1993; Obiri-Danso et al., 1997; Hayford and Jakobsen, 1999). LAB counts in the present work increased during both steeping and dough fermentation and was responsible for the reduction in pH and increase in titratable acidity. Homofermentative lactic acid bacteria metabolize glucose to lactic acid by the Embden Meyerhof pathway. Heterofermentative lactic acid bacteria on the other hand metabolize glucose through the phosphoketolase pathways. This yields lactic acid and acetic acid if the bifidus pathway is used or lactic acid, acetic acid, ethanol and CO₂ through the 6P-gluconate pathway (Kandler, 1983). Several authors have reported a decrease in pH and an increase in titratable acidity during steeping of whole maize grains and fermentation of maize dough in *kenkey* production. According to Plahar and Leug (1982) the main carboxylic acids produced in maize dough fermentation are D+L-lactic acid and acetic acid in concentrations of 0.8 to 1.4% and 0.1 to 0.16%, respectively. Other acids produced are propionic and butyric acids with values of 30 and 40 mg/kg (Plahar and Leung, 1982; Halm et al., 1993; 1996; 2004). The present work has shown that dehulling or degerming maize grains does not change the trends in acidification or souring of *kenkey* during production.

Role of lactic acid bacteria in *nsiho* fermentation

Wide variations, 2 to 5 log unit increases, were recorded in the lactic acid bacteria population during steeping at

the four different production sites at Anum. This could be attributed to wide variations in the lactic acid bacteria population at the start of steeping, that is, mean counts of 10^5 , 10^4 , 10^4 , 10^3 CFU/ml at the different production sites. This initial LAB population was dependent on the conditions at the different production sites. The important factors included the population of LAB on the dehulled grains, on utensils and containers used, in the steeping tanks, etc. These are the sources of LAB for the spontaneous fermentation of the grains during steeping. By the end of steeping, there were very little differences in the mean LAB population at the different production sites; 10^7 and 10^8 CFU/ml. In steeping of whole maize grains in *kenkey* production Halm et al. (1993) recorded a LAB population of 1.7×10^8 CFU/ml at the end of steeping from the initial concentration of 8.2×10^6 CFU/ml. Increases in LAB population during 12 h of dough fermentation were by 2 and 3 log units at the different production sites. The LAB population as well as titratable acidity at the start of dough fermentation was lower than at the end of steeping which was the first fermentation stage. This could be attributed to loss of cells and acids in the steep water which was decanted off and also addition of water (dilution) to the milled meal to form the dough. This will also explain the changes in pH at these stages. The LAB counts at the end of 12 h dough fermentation were between 10^8 and 10^9 CFU/g. In whole maize dough fermentation Halm et al. (1993) reported LAB population of 10^9 CFU/g at the end of fermentation. The dominant lactic acid bacteria identified in the present work to be responsible for *nsiho* fermentation was *L. fermentum* which accounted for nearly half of the lactic acid bacteria population. This result is in agreement with the work of Halm et al. (1993) who found fermentation of whole maize meal in *Ga-* and *Fanti-* *kenkey* production to be dominated by a group of obligately heterofermentative lactobacilli consistent with *L. fermentum* and *Lactobacillus reuteri* in their patterns of carbohydrate fermentation. Hayford et al. (1999) later confirmed the dominant species to be *L. fermentum* using molecular characterization. It is therefore not surprising that *L. fermentum* has been found in the present work to be responsible for the fermentation of *nsiho*, a different type of *kenkey*. This study therefore shows that polishing of maize grains by removal of the testa and germ has little effect on the composition of the LAB which ferments the cereal. In Benin, Hounhouigan et al. (1993) also reported *L. fermentum* to be the dominant lactic acid bacteria responsible for the fermentation of maize into *mawe* which involves fermentation of partially dehulled maize grains.

In this study, *L. plantarum* and *L. brevis* were also isolated in high numbers during steeping and dough fermentation. The presence of *L. plantarum* in maize dough fermentation has been reported. Nche et al. (1996) identified *L. plantarum*, *L. brevis*, *Lactobacillus confuses*

and *Pediococcus* species as the main lactic acid bacteria present in fermenting maize and maize cowpea dough. Olasupo et al. (1997) in their studies on selected African fermented foods obtained 48 lactobacillus isolates from *kenkey* which they identified as *L. plantarum*, *L. fermentum*, *L. brevis*, *Lactobacillus delbrueckii* and *L. acidophilus*. Olsen et al. (1995) found *L. plantarum* at the initial stage of *kenkey* fermentation where it dominated the heterofermentative lactic acid bacteria present. In whole maize *kenkey* production, Olsen et al. (1995) showed that about half of all *L. plantarum* and practically all *L. fermentum* isolates inhibited all other Gram positive and Gram negative bacteria and explained the elimination of these organisms during the initial stages of *kenkey* production.

The presence of *P. pentosaceus* and *P. acidilactici* which were identified in the lactic acid bacteria composition in *nsiho* in the current work have also been reported in *kenkey* by Halm et al. (1993). Their presence can be linked to production of propionic acid which both Plahar and Leung (1982) and Halm et al. (1993) have reported to be one of the main organic acids present in *kenkey*. These organisms may also ferment lactic acid and do so as a primary end-product of CHO catabolism.

Role of yeasts in *nsiho* fermentation

The dominant yeasts identified in the current work to be involved in the *nsiho* fermentation were *S. cerevisiae* and *C. krusei*. The other yeasts species which occurred only at the initial stages of steeping were *Debaryomyces* and *Trichosporon* species. In whole maize *kenkey* production, Hayford and Jespersen (1999) and Hayford and Jakobsen (1999) confirmed the dominant yeast species during steeping and dough fermentation to be *S. cerevisiae* and *C. krusei* by molecular methods. Obiri-Danso et al. (1997) had previously reported *S. cerevisiae* and *C. krusei* as the yeasts species involved in *kenkey* fermentation. Jespersen et al. (1994) isolated *S. cerevisiae* and *C. krusei* as the dominant yeast in maize dough fermentation and suggested that since yeast are known to produce a wide range of aromatic compounds including organic acids, esters, aldehydes, alcohols, lactones and terpenes, they are likely to influence the organoleptic and structural quality of fermented maize dough. Jespersen et al. (1994) also identified *Debaryomyces* and *Trichosporon* species in the yeast population during *kenkey* production. The present work has shown that *L. fermentum* and *S. cerevisiae* are the predominant microbial species responsible for the fermentation of dehulled maize grains into *nsiho*. They accounted for nearly half of the lactic acid bacteria and yeast populations. Thus, the same predominant organisms responsible for the fermentation of whole maize grains into *Ga-* and *Fante-**kenkey* are also respon-

sible for fermentation of dehulled maize grains into *nsiho*. In 1996, Halm et al. successfully developed and tested a mixed starter culture containing a strain each of *L. fermentum* and *S. cerevisiae* for the production of *Ga* and *Fanti kenkey*.

Conclusion

The fermentation of dehulled maize grains in *nsiho* production is similar in character to fermentation of whole maize grains in *Ga* and *Fanti-kenkey* production. This is with respect to the microbiological and biochemical changes which take place during fermentation. In *nsiho* production, lactic acid fermentation occurs during both steeping of maize grains and dough fermentation. This results in a sour product with a low pH and high percentage titratable acidity. The lactic acid population is dominated by *L. fermentum* and also includes *P. pentosaceus*, *P. acidilactici*, *L. plantarum* and *L. brevis*. Yeasts are also involved in these fermentations with *S. cerevisiae* and *C. krusei* being dominant.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

The nutritional quality of *Spirulina platensis* of Tamenrasset, Algeria

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***Spirulina platensis*, a blue green microalga, has been used since ancient times as a source of food because of its high protein content (65%) and nutritional value. Lipids isolated from *S. platensis* have been shown to contain high levels of polyunsaturated fatty acids, including linolenic acid which is a precursor of arachidonic acid; this cyanobacteria contains, also, several kinds of sterols. The aim of this study is to evaluate the nutritional quality of *S. platensis* of Tamenrasset, Algeria. This study shows the analysis of nutritional quality of *S. platensis* of Tamenrasset, Algeria, to know nutritional value of our local strain. Biochemical analysis was performed for moisture, protein, glucose, lipid and minerals content. The chemical composition is based on the identification of fatty acids, using gas chromatography and quantification of the mineral elements by using the atomic absorption spectrometry (AAS). The results showed that *S. platensis* of Algeria has an important nutritional quality.**

Key words: *Spirulina platensis*, nutritional, quality, proteins, fatty acids.

INTRODUCTION

Spirulina platensis is a cyanobacterium which acquired the ability for photosynthesis before any other organism and is considered to be the ancestor from which the higher plants evolved. The use of *S. platensis* as food by indigenous populations in different parts of the world is well documented. *S. platensis* was rediscovered in the mid-1960s (Subramanian, 2004).

Nowadays, this organism is used as a food supplement and is marketed in the form of pills, capsules and powder or incorporated into various types of food like cakes, biscuits, noodles, health drinks, etc. Various countries are developing strategic programs for the production and use of *S. platensis* (Subramanian, 2004).

Microalgae have received increased attention due to

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the fact that they represent one of the most promising sources of biological activity compounds that could be used as functional ingredients (Pulz and Gross, 2004). Their balanced chemical composition (good quality proteins, balanced fatty acid profiles, vitamins, antioxidants and minerals) and their interesting attributes can be applied in the formulation of novel food products (Spolaore et al., 2006).

S. platensis, a filamentous blue-green (cyanobacteria) alga, attracted the interest of researchers. The biochemical components provide the marketing value to *S. platensis*, it is one of the most promising microalgae for culture due to its high nutritional values (Baylan et al., 2012). It is also well known as a source of protein (60-70 g/100 g) of high biological value, since it is a rich source of vitamins, mainly vitamin B12 and pro-vitamin A, minerals, especially iron and γ -linolenic acid, an essential fatty acid precursor for prostaglandins (Belay et al., 1993; Sempore et al., 2006; Habib et al., 2008).

The concentrated nutritional profile of *S. platensis* occurs naturally, so it is ideal for those preferring a whole food supplement to artificial nutrient sources. *S. platensis*, the blue-green alga, has a unique blend of nutrients that no single source can provide. Moreover, most research has focused on the health effects of *S. platensis* as a dietary supplement for humans and animals. Many studies have shown the effects of these microalgae that may result in significant therapeutic applications: an anti-cancer effect (Hirahashi et al., 2012; Mao et al., 2005; Khan et al., 2005; Basha et al., 2008), a hypolipidemic effect (Narmadha et al. 2012) and a protective effect against diabetes and obesity. These advantages make *S. platensis* a good raw material for the healthy food (Anitha and Chandralekh, 2010).

The production of this small alga is based on environmental conditions (water, climate, salinity, etc.) and its trade and economic interest were highlighted by the Algerian researchers to glimpse the culture of *S. platensis* in southern Algeria. The aim of this study is to analyze *S. platensis* grown in southern Algeria, Tamenrasset and demonstrate its nutritional quality.

MATERIALS AND METHODS

Source of *S. platensis*

The strain of *S. platensis* powder used was obtained from Tamanrasset (South of Algeria). The location in the Tamanrasset region is at the Guelta of Palm located at 1824 m altitude.

Physicochemical analysis of *S. platensis*

A pH meter was used to determine the pH of a solution at 4% *S. platensis* (4 g *S. platensis* powder diluted in 100 mL distilled water). Determination of the humidity content was done according to the official method of American Oil Chemist's Society (AOCS) in 1990. The results were expressed in percentage weight of water relative to the initial weight, by the following equation:

$$H = \frac{m_1 - m_2}{m_1 - m_0} \times 100$$

H is the humidity content expressed as percentage (%) by mass; m_0 is the mass in grams of the empty capsule; m_1 is the mass in grams of the dish and the sample; m_2 is the mass in grams of the dish and the dry residue.

It should be noted that this method does not only measure the water content. We recommend using the term "mass loss", as a correct term instead of the one already used "humidity", because the determination of loss is not only water, but any volatile compounds in the operational conditions of drying (Le Meste, 2002).

The protein content was determined using Kjeldahl method, the results were expressed using the following equation:

$$P = \frac{1.4 \times N \times (V_1 - V_0)}{m} \times 6.25$$

P is the protein ratio expressed as a percentage (%) by weight; N is the normal hydrochloric acid; V_1 , V_0 is the titration volumes of the sample and blank; m is the mass in grams of the initial sample; 6.25 is the conversion factor of *S. platensis* protein.

The anthrone colorimetric method described in research method was applied to measure the total soluble sugar content. One-tenth of a dried gram sample (shattered, fineness: passed through 100-mesh) was weighed in a 10 mL centrifuge tube, and 6-7 mL of 80% ethanol was added to it. The sample was heated in an 80°C water bath for 30 min, then centrifuged (3000 rpm/min) for 5 min. The supernatant was collected, and the extraction was repeated twice (3000 tr/min for 10 min each) (Xinglu and Qiufeng, 2011).

The total supernatant was collected into a flask, and 80% ethanol was added to total volume of 50 mL. Then, 1 mL of solution was taken, and 1.5 mL of water was added, followed by 6.5 mL of anthrone reagent. The sample was mixed and incubated at room temperature (18-30°C) for 15 min to allow color developing. The absorbency at 620 nm wavelength was measured after the sample was cooled down.

$$\text{Content of total soluble sugar \%} = \left\{ \left[\frac{C \times (V/a)}{W \times 10^6} \right] \times 100 \right\}$$

Where, C is the glucose content obtained by referring to the standard curve (μg); V is the total volume of the extracted solution (mL); a is the volume of sample solution for color developing (mL); W is the weight of sample (g).

The fat content is determined by the Soxhlet extraction method by using hexane as solvent (NF V 03-905). 50 g of sample was placed in the Soxhlet and added to 500 ml of hexane in the flask, and the temperature was set to 60°C. Thereafter, most of the solvent were removed using the rotary evaporator. The flask containing the lipid was placed in an oven for 30 min at 103°C, then in a desiccator for 30 min. The lipid weight was obtained by the difference between the final weight and the initial weight of the flask. The results are given by the following formula:

$$\text{Fat content (\% MS)} = (A - B) \times \frac{100}{C} \times \frac{\text{MS}}{100}$$

Where, A is the weight of the ball + extract in grams; B is the weight of the empty flask in grams; C is the weight of the sample in grams; MS is the dry matter percentage.

The ash content (mineral) was estimated to incineration in a muffle furnace to 550°C so as to obtain all of the cations in the form of carbonate and other anhydrous inorganic salts (AOCS, 1990). The results expressed in percentage by weight of ash to the initial weight ratios were obtained from the following expression:

Table 1. Results of physicochemical analysis of *S. platensis*

Parameter	<i>S. platensis</i>
Potential of hydrogen (pH)	7.81 ±0.05
Humidity (%)	5.42 ± 0.031
Protein (%)	60.32± 0.15
Lipid (%)	7.28 ±0.021
Rate of total sugars (%)	17.63 ±0.133
Rate ash (%)	6.88 ± 0.05
Caloric intake (kcal)	369.28

$$C = \frac{m_3 - m_0}{m_1 - m_0} \times 100$$

C is the ash, expressed as a percentage (%) by weight; m_0 is the mass in grams of the empty crucible; m_1 is the mass in grams of the crucible and the sample; m_2 is the mass in grams of the crucible and its contents (ash) after incineration.

Determination of mineral contents by atomic absorption spectroscopy

Atomic absorption spectrometry is a method of elemental analysis which uses the property of atoms excitation by the addition of an external energy as a defined electromagnetic radiation (photon) frequency, ν (Lynch, 2001).

The mineral elements (Mg, Cu, Fe, ZN, K and Na) were determined by atomic absorption spectrometry type (Varian AA 240) related to the flame atomizer (GTA 120). Indeed, the concentration of minerals in ppm was determined using standard prepared curves.

The chemical composition of fatty acids

The fatty acid composition of *S. platensis* was determined by gas chromatography (GC). The fat tested was obtained by esterification by soxhlet of *S. platensis*. 0.35 g of *S. platensis* in a flask of 100 mL was added to 6 mL of a methanol solution (2 g NaOH in 100 mL methanol) for 10 min at 70°C; was added after 7 mL of BF₃ (2 min at 70°C), then 5 mL N-heptane (1 min), and adjusted to 100 mL with a saturated NaCl solution. After decanting the solution, the lipid phase was recovered.

The condition for the GPC analysis included: instruments, Chromatography Chrompack with detector CP9002, injector FID SPLIT 1/100, carrier gas: Azote, column DB23 (column length: 30 m; column diameter: 0.32 mm; film thickness: 0.25 μ m); temperatures: injection, 250°C; detector, 250°C; temperature programming in column, starting temperature was 150°C and final temperature, was 220°C; rate of temperature increase was 4°C/min, amount injected is 0.2 μ L; paper speed was 0.5 Cm/mN

Statistical analysis

The results were analyzed using one-way analysis of variance (STATISTICA), (Version 10). A p value < 0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

Physicochemical analysis of *S. platensis*

The results of physicochemical analysis performed on *S. platensis* are shown in Table 1. The composition variety depends on growing conditions and production techniques of *S. platensis*; some differences were observed. It was noticed that the pH of *S. platensis* was 7.81 ± 0.05, a slightly basic pH. Moisture (water content) is the water content of the *S. platensis* powder, measured as a percentage of water relative to its dry weight. It is 5.42 ±0.031% for our test; this value is similar to that found by previous work: 4-6% by Espiard (2002) and 4-7% by Pierlovisi (2007).

S. platensis is rich in protein because they represent 50-70% of its dry matter (Clément, 1975; Fox, 1999). The highest values are obtained when the harvest takes place at the beginning of the light period. On the other hand, in comparison with other vegetable protein sources which are less rich, *S. platensis* is consumable as a whole (Dillon et al., 1995). This value (60.32 ±0.15%) showed great value when compared with the average protein content of some legume seeds: bean (22%), peas (22%) and even soybeans (38%). *S. platensis* appears as one of the greatest protein-rich plant species (Léonard and Compère, 1967).

The incorporation of *S. platensis* powder resulted in considerable improvements of the contents of protein in the product of foods (Rodríguez De Marco et al., 2014). Protein contents of *S. platensis* show very high digestibility (83-90% as compared to 95.1% for pure casein) due to lack of cellulose walls. Hence, cooking is not necessary to increase the proteins availability (Hoseini et al., 2013). The major protein constituents with significant beneficial health effects are the phycobiliproteins, phycocyanin C and allophycocyanin (at approximately 10:1 ratio), which have linear tetrapyrrole prosthetic groups (phycocyanobilin) covalently linked to specific cysteine residues of the proteins. Phycocyanins constitute about 15-25% of the dry weight of the microalgae (Bermejo et al., 1997; Romay et al., 2003). Phycocyanins can be considered as a safe natural food colorant in non-acidic foodstuffs such as chewing gum, confectionaries and dairy products (Downham and Collins, 2000)

The carbohydrate content was 17.63 ± 0.133%, and this value is similar to the values of other researchers which represented 13.6 to 25% carbohydrates of the dry matter of *S. platensis*. The wall of *S. platensis* as Gram-negative bacteria, is composed of glucosamine and muramic acid associated with peptides (Quillet, 1975).

The cell wall of *S. platensis* has an approximate 0.5% of its dry weight glycogen content (Quillet, 1975; Fox, 1999). The major polymeric component of *S. platensis* is a branched polysaccharide, structurally similar to glycogen. High molecular weight anionic polysaccharides with antiviral and immunomodulating activities have been isolated from *S. platensis* (Parages et al., 2012).

Table 2. Composition of the inorganic powder of *S. platensis* (mg/g).

Inorganic powder	Fe	Zn	Ca	Na	K
<i>S. platensis</i> (this study)	0.88	0.009	0.22	27	20
<i>S. platensis</i> (Johnson and Shubert, 1986)	0.58-1.8	0.021-0.040	1.3-14	4.5	6.4- 15.4

According to Hayashi (1996) and Lee and Coll (1998), the antiviral and immunomodulating activities of polysaccharides of *S. platensis* are discussed in the related sections. A sulphated polysaccharide fraction with antiviral property (calcium spirulan) has been extensively purified and shown to be composed of rhamnose, 3-O-methylrhamnose (acofriose), 2,3-di-O-methylrhamnose, 3-O-methylxylose, uronic acids and sulfate.

The fat content is 7.28% dry weight; *S. platensis* can be considered to have very high protein content and less fat sources. This feature gives it the advantage of being relatively easy to be kept away from lipid oxidation and rancidity phenomena. Lipids generally represent 6 to 8% of the dry weight of *S. platensis* but this percentage may reach 11%. The total lipid composition is characterized by a balance between saturated fatty acids and polyunsaturated fatty acids (Hudson and Karis, 1974).

Lipids contents of *S. platensis*, are separated into a saponifiable fraction (83%) and a non-saponifiable fraction (17%), containing essential pigments, paraffin, sterols and terpene alcohol. Half of the total lipids are fatty acids and cholesterol (< 0.1 mg/100 g of *S. platensis* dry mass) (Gershwin and Belay, 2008), which is a component of *S. platensis* sterol fraction (Clement, 1975).

S. platensis is rich in mineral ($6.88 \pm 0.05\%$) in our study; *S. platensis* contains all essential minerals for the body: iron, magnesium, manganese, potassium, calcium, phosphorus, zinc and selenium (uncommon). Meanwhile, *S. platensis* is one of the best natural sources of iron according to Fox (1999). The calorific value of *S. platensis* is not very high (369.28 kcal/g), it is easily restored by its protein and vitamin value, when compared with other energy foods such as cereals.

Determination of mineral contents by atomic absorption spectroscopy (NF V05-113 1972)

Analytical results of the mineral composition of the powder of *S. platensis* are shown in Table 2. *S. platensis* is rich in iron bioavailability which is two to three times higher than that of meat, and proves very useful in improving iron deficiency-anemia associated with protein-energy malnutrition (Pierlovisi, 2007).

S. platensis is also a good source of magnesium bioavailable in humans. Potassium is richly represented in *S. platensis*; interesting asset in industrialized countries where the sodium/potassium ratio is often too high.

Finally, it is possible to enrich *S. platensis* strains with some trace elements (zinc, selenium, etc.) (Falquet, 2012).

The high levels of several micronutrients, especially minerals (iron 0.58-1.8, calcium 1.3-14, phosphorus 6.7-9.0 and potassium 6.4-15.4 g/kg) in *S. platensis*, which have made it suitable nutritional supplement for vegetarians, are due to absorption of these elements while growing. Consequently, mineral content of *S. platensis* depends on source and culture conditions. Calcium, phosphorus and magnesium are present in quantities comparable to those found in milk. *S. platensis* is considered to be an iron rich food, with an iron content ten times higher than in common iron rich foods. Absorption of *S. platensis* iron is 60% more than ferrous sulphate (present in iron supplements) (Falquet, 2012).

The chemical composition of fatty acids

Figure 1 illustrates the profile and the percentages of the components identified by CPG as shown in Table 3.

The fatty acid profile of *S. platensis* varies depending on the strain studied. *S. platensis* contains mainly polyunsaturated fatty acids essential for 18 carbon atoms, in particular the omega-6 (ω_6). It is indeed one of the best sources of gamma linolenic acid (18: 3 ω_6) after human milk and some expensive vegetable oils according to Pierlovisi (2007).

The presence of gamma-linolenic acid, C18: 3 omega - 6 was noted because of its scarcity in common foods and its presumed high food value (Kay, 1991; Cohen and Voushak, 1991; Otles and Pire, 2001).

The omega-3 and omega-6 fatty acids in *S. platensis* would prevent the accumulation of cholesterol in the body. This may partly explain the decrease in cholesterol and triglycerides observed in experiments for Ramamoorthy and Premakumari (1996) and Samuels et al. (2002).

S. maxima and *S. platensis* contain γ linolenic acid (GLA), which comprises 10-20 and 49% of their fatty acids, respectively. *S. platensis* can be considered as a good source of GLA. *S. maxima* also contains unsaturated oleic and linoleic acids as well as saturated palmitic acid, which constitute more than 60% of its lipids. Monogalactosyl- and sulfoquinovosyl-diacylglycerol as well as phosphatidylglycerol are the major *S.* lipids (20-25% each) (Petkov, 1988; Toyub et al., 2011).

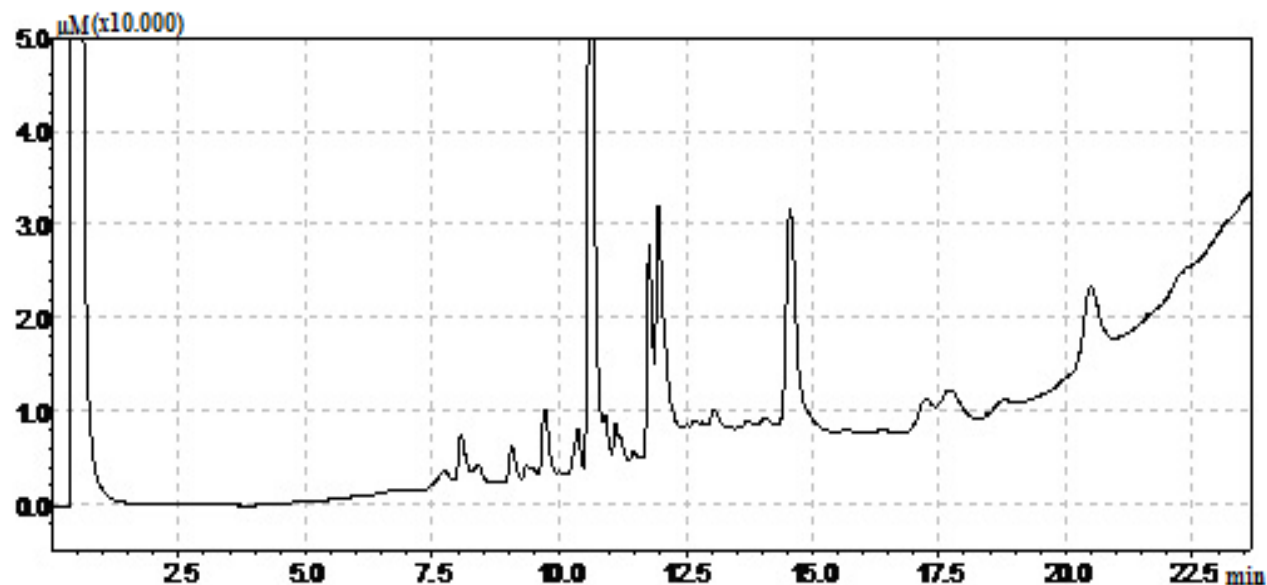


Figure 1. Fatty acid profile of *S. platensis*. Retention time (min) = 8.066: lauric acid; 9.718: myristic acid; 10.615: palmitic acid; 10.909: palmitoleic acid (omega 6); 11.111: stearic acid; 11.473: oleic acid (omega 6); 11.781: linoleic acid (omega 6); 11.961: gamma linolenic acid (omega 6); 14.567: behenic acid.

Table 3. Fatty acid composition of *S. platensis*.

Fatty acids	Nomenclature	Contents (%)	Contents (%) (Pascaud, 1993)
Lauric acid	C 12:0	3.10	-
Myristic acid	C 14:0	3.60	0.2-0.5
Palmitic acid	C 16:0	42.79	25
Palmitoleic acid (Omega 6)	C 16:1	0.52	3.8
Stearic acid	C 18:0	1.81	1.7
Oleic acid (Omega 6)	C 18:1	0.33	16.6
Linoleic acid (Omega 6)	C 18:2	9.43	12
Gamma linolenic acid (Omega 6)	C 18:3	18.41	40.1
Behenic acid	C 22:0	20.01	traces

Conclusion

S. platensis represents a source of important natural compounds for human nutrition; its nutritional quality fits the standard measurements, when compared with other searches. We can conclude that *S. platensis* cultivated is characterized by a high nutritional quality. This *S. platensis* is characterized by a high protein content of up to $60.32 \pm 0.15\%$ of the dry weight. This is the richest food known today because the protein content is twice that of soybeans and more than three of meat or fish. Carbohydrate represents $17.63 \pm 0.133\%$ of the dry weight. It is a low calorie food. Total lipid varies between $7.28 \pm 0.021\%$ of dry weight. It provides minerals and trace elements such as iron, magnesium, manganese, phosphorus, selenium and zinc. *S. platensis* contains essential polyunsaturated fatty acids with 18 carbon

atoms, in particular, the omega-6 (ω_6).

Conflict of Interest

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Inclusion of sweet sorghum flour in bread formulations

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Sweet sorghum (*Sorghum bicolor* L. Moench) has been studied as an additional source of raw material for production or partial replacement of foods due to its high fiber concentration. Its consumption is associated with the prevention of some diseases and nutritional benefits. The aim of this study was to evaluate the partial replacement of wheat flour by sweet sorghum flour in bread formulations in order to characterize the nutritional and physical profile of the flour. Four bread formulations were prepared and evaluated for sensory and textural profile. The composition of sweet sorghum flour showed high fiber content and relative protein value, and moisture showed average value, in accordance with limits established by legislation. The addition of 3% sweet sorghum flour to bread was shown to be technically feasible, with great acceptance by consumers, being a nutritious and tasty option.

Key words: Functional food, fiber, baking, texture.

INTRODUCTION

Bread, one of the most consumed foods worldwide, has high energy value and low cost. It is used as food for different social classes. In addition to its good flavor, bread has important nutritional value, being also a source of protein, fiber and minerals (Almeida et al., 2008). Bread is one of the main foods consumed daily in all parts of the world, although there is a wide variety of different types, the term generally refers to fermented products containing wheat (Hager et al., 2012). According to Santos et al. (2012), the use of mixed flour is aimed at

the partial replacement of the raw material used in order to introduce fibers and increase the nutritional value of the product. Ingredients for baking are best used for the inclusion of fibers, due to the large consumption in the habitual diet of the population. Thus, bread enriched with fibers can be of great significance for those who need a higher intake of this food due to its protective effect against cardiovascular diseases (Justo et al., 2007). The replacement of wheat flour by gluten free flour are used because increasingly these substitutions can provide

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Abbreviations: SRW, Solubility rate in water; SRM, solubility rate in milk; ARO, absorption rate in oil; FSS, flour of sweet sorghum.

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breads with different grain characteristics technologically feasible and acceptable with higher nutritional value compared to the wheat flour (Collar et al., 2014). Thus, functional foods not only satisfy hunger but provide the necessary nutrients, prevent diseases and increase physical and mental well-being of consumers (Wansink et al., 2005).

Sweet sorghum is an excellent ingredient of renewable, the foods contains high amount of soluble and insoluble carbohydrates, that presents fast growth and has high resistance to harsh climate conditions (like drought) and requires low fertilization and irrigation (Matsakas and Christakopoulos, 2013). Souza et al. (2005) evaluated the quality of sweet sorghum products formulated alone or combined with sugarcane juice and reported that the flour obtained from three types of sorghum grains showed significant contents of ash and total sugars, and can be used as additional raw material in the production of brown sugar and grains in the preparation of flour, featuring sweet sorghum as a valid alternative for use as food. However, studies involving sweet sorghum (bagasse) in food formulations aiming at increasing the nutritional properties have not yet been carried out. Sweet sorghum is a high biomass- and sugar-yielding gramineous crop whose origin is in Africa (Chohnan et al., 2011), is a C4 crop possessing high photosynthetic efficiency and can grow in geographical areas with a temperate climate, it is the only crop that provides grain and stem that can be used for sugar, alcohol, syrup, jaggery, fodder, fuel, bedding, roofing, fencing, for industry of paper or simply to chew because is sweet, having so great versatility (Ratnavathi et al., 2011).

This study aimed the partial replacement of wheat flour by sweet sorghum flour in the preparation of bread in order to assess and physical of the flour, as well as the sensory characteristics of bread enriched with sweet sorghum flour. The inclusion of sweet sorghum fiber might be used to develop new types of bread.

MATERIALS AND METHODS

Sweet sorghum

Sweet sorghum was obtained from the Experimental Farm at the Federal Institute of Goiás - Rio Verde Campus, Rio Verde - GO, Brazil, where the weather was classified as Tropical wet (UR). Sowing was held on January 20, 2013 and manual harvested on May 20, 2013. Sweet sorghum flour was processed at the Laboratory of Fruits and Vegetables - Federal Institute of Goiás. Initially, the sweet sorghum flour were cleaned to remove sheaths and sanitized with chlorinated water (10 ml water sanitary / liter of water), and then with the help of sugarcane grinder, juice was extracted. Bagasse was stored in plastic bags and frozen at -18°C until time of drying. Drying was performed in a forced air circulation oven at 75°C up to constant weight. Then, the dried were submitted to milling in Willey type mill, aseptically packed in polyethylene bags and stored at room temperature until time of analysis and processing of breads. Physicochemical analyses were performed at

the Laboratory of Food Sciences, Food Engineering Unit - Federal Institute of Goiás. Crude protein, ash, ether extract and crude fiber values were expressed on a dry basis with an average of five replicates and solubility rate in water (SRW), solubility rate in milk (SRM) and absorption rate in oil (ARO) results with average of six replicates.

Crude fiber

For analysis of crude fiber, 200 ml of sulfuric acid and sodium hydroxide solution and 1.0 g of Celite were used, according to Silva and Queiroz (2002).

Ash

The ash content was calculated from the ratio between the amount of incinerated ash and the sample mass (AOAC, 1995) (AOAC, 1995), being expressed as percentage (%) using the following equation:

$$\text{Ash (\%)} = \frac{\text{g of ash}}{\text{g of sample}} \times 100$$

Ether extract

Ether extract was determined by extraction of oils and greases (Marconi, MA 044/8/50) using petroleum ether as solvent according to official method No. 032 / IV (IAL, 2005). Lipid content (%) was obtained using the following formula:

$$100 \times \frac{N}{P}$$

Where, N = grams of lipids, P = grams of sample.

Moisture

The moisture content was determined using a forced air drying oven for 24 h at 105°C according to methodology proposed by AOAC (1995).

Protein

Protein was determined with the aid of a Kjeldahl digester at temperature of 400°C. The results were expressed in percentage (AOAC, 1995).

Solubility rate in water, milk and absorption rate in oil

The absorption rate in oil (ARO) was determined with the aid of a centrifuge. ARO calculation used the following equation:

$$\text{ARO} = \frac{mh}{md}$$

Where, mh = mass of hydrated sample, md = mass of dried sample.

Solubility rate in water (SRW) and solubility rate in milk (SRM) were obtained by the same methodology used to obtain absorption rate

Table 1. Ingredients used in the formulations of breads fortified with sweet sorghum flour (FSS).

Ingredients	Formulation (g)			
	1	2	3	4
Wheat flour	515	510	505	500
FSS	0	5	10	15
Yeast	60	60	60	60
Water	85	85	85	85
Milk	85	85	85	85
Sugar	70	70	70	70
Fat	65	65	65	65
Egg	50	50	50	50

in oil. Results are expressed by the following equation:

$$\text{SRW and SRM} = \frac{\text{msd}}{\text{ma}} \times 100$$

Where, msd is the mass of dehydrated solid and m is the mass of sample.

Scanning electron microscopy

Flour of sweet sorghum (FSS) microscopy was performed at the High-Resolution Microscopy Multiuser Laboratory at the Institute of Physics, Federal University of Goiás. Scanning Electron Microscope, Jeol, JSM - 6610, equipped with EDS, thermoscientific NSS spectral imaging was used.

Breads enriched with sweet sorghum flour

Four bread formulations were processed (treatments) with addition of 0% (control), 1, 2 and 3% sweet sorghum flour. Breads were produced using bread molder G. PANIZ, cabinets for the storage of dough during fermentation, cylinder and Tedesco kiln. The amount of ingredients to be used in formulations was also calculated (Table 1). Solid and liquid ingredients were mixed in the container, placed in the bread molder where they were mixed and homogenized, and after forming the dough, it was divided and rounded. Then, the dough was placed in appropriate storage facilities for growth and fermentation room temperature, and finally placed in 180°C industrial kiln for 8 min.

Physical and sensory analysis of breads enriched with sweet sorghum flour

Texture

Bread firmness was determined at the Laboratory of Post-Harvest Vegetable Products Federal Institute of Goiás, Rio Verde Campus, using the Brookfield LFRA texturometer applying loads between 4500-0 g and 100-0 g, used to compress 30 mm of bread thickness.

Weight and length

The length of breads was evaluated before and after baking

through DIGIMESS caliper with results expressed in mm. Weight was assessed by analytical scales, with results in grams.

Color

Instrumental color parameters (L*, a*, b*) of breads were determined in Color Flex EZ colorimeter at the Laboratory of Post-Harvest Vegetable Products, Federal Institute of Goiás, Rio Verde Campus. Sensory and visual characteristics were determined in order to quantify the consumer preference for different types of bread with addition of FSS and purchase intent. Instrumental color parameters (L*, a*, b*) of breads were determined in Color Flex EZ colorimeter at the Laboratory of Post-Harvest Vegetable Products, Federal Institute of Goiás, Rio Verde Campus (Minolta, 1994).

Sensory analysis

Sensory and visual characteristics were determined in order to quantify the consumer preference for different types of bread with addition of FSS and purchase intent. Sensory analysis was performed at the Laboratory of Sensory Analysis, Federal Institute of Goiás, Rio Verde Campus. Analyses were performed with 50 untrained panelists in individual booth (IAL, 2005). Sensory analysis was performed in four formulations: 0% (control), 1, 2 and 3% sweet sorghum flour. Breads were served in white plastic cups (50 mL capacity), accompanied with a glass of mineral water at room temperature (to be drunk between samples). Regarding the purchase intent, questions were asked where panelists chose to buy or not to buy.

Statistical analyses

The results obtained were analyzed in a completely randomized design with the use of the SISVAR software (Ferreira, 2003). Physicochemical analyzes was done with three replicates per treatment using the Tukey test (0.05) to compare means.

RESULTS AND DISCUSSION

Chemical composition, solubility rate in water and milk and absorption rate in oil of sweet sorghum flour are

Table 2. Moisture, protein, ash, ether extract, crude fiber and SRW, SRM and ARO values of sweet sorghum flour (FSS).

Parameter	Mean value
Moisture (%)	4.95 ± 1.18
Crude protein (%)	3.24 ± 0.78
Ash (%)	6.33 ± 1.14
Ether extract (%)	3.77 ± 1.77
Crude fiber (%)	78.62 ± 28.40
SRW (%)	17.99 ± 0.51
SRM (%)	9.64 ± 2.87
ARO (g of oil/g of FSS)	3.48 ± 0.15

Chemical composition, moisture, crude protein, ash, ether extract, crude fiber, SRW, SRM and ARO values followed by standard deviation. The physical-chemical parameters were expressed on a wet basis.

shown in Table 2. The high fiber, ash and ether extract contents of sweet sorghum flour may be related characteristics intrinsic in plants monocots, because, Oliveira et al. (2012) characterize chemically elephant grass and reported similar values to the sorghum saccharine flour. This vicinity of values may be related because both species belong to the family *Poaceae*. The moisture content of flour of 4.95% was in accordance with limits established by Brazilian legislation (maximum 14%). The moisture 4.95% content of flour of sweet sorghum was in accordance with limits established by Brazilian legislation (Brazil, 1978). The low moisture content reduces probability of microbial growth in the product due to the low water activity.

The protein value of FSS of this study was higher than the protein content (2.32%) reported by Freitas et al. (2008) for hydrolyzed sugarcane bagasse. The high ash content of sweet sorghum flour may be related to intrinsic plant characteristics. However, the ash content (6.33%) was higher than the limit set by law (maximum of 2.0%) (Brazil, 1978). Martino et al. (2012) to evaluate in eight sorghum genotypes for human consumption had relatively higher protein values than those found in sweet sorghum flour of the present study. This reduction may have occurred during processing of sorghum flour. Processes involving heat as drying may cause loss of nutritional value and protein denaturation (Fellows, 2006). The effect of fiber on digestion is based on physical properties such as water absorption, gel filtration, ion exchange and organic absorption. SRW, SRM and ARO resulted in average values of 17.99%; 9.64% to 3.48 g oil / g FSS, respectively. According to Fernandes et al. (2002), increased solubility rate in water is probably due to starch fragmentation, increasing the amount of soluble solids. The determination of solubility and absorption rates aims to determine the hygroscopic properties of flour. These analyses reveal the technological quality of

flour to be incorporated into food products. Scanning electron microscopy images (Figure 1) show the external morphology of sweet sorghum flour. The figure with 30 times magnification shows an overview of FSS with uneven surface and heterogeneous constitution full of multiform structures. SEM showed that sweet sorghum flour is a rich source of fibers, and its inclusion together with wheat flour is an alternative to the addition of fibers to bakery products. Images with 3000 and 10,000 times magnification show the presence of starch granules adhered to the fibrous structures.

Fiber as seen in the image above magnified 200 times comprises the cell wall components of plants that are not digested by the human body but play a vital role in stimulating peristalsis, bowel movements that determine higher or lower rate of passage of food through the gastrointestinal tract, the effect of fiber on digestion is based on physical properties such as water absorption, gel filtration, ion exchange and organic absorption (Derivi et al., 2002). Individuals with low daily intake of dietary fiber are prone to a number of problems ranging from discomfort caused by intestinal gases, intestinal cancer and cardiovascular problems (BOAS, 2001). Figure 2 shows the texture of bread, according to the load required for compressing 30 mm of bread. The graph simulates the load in grams required to compress the thickness of 30 mm of bread enriched with FSS. It was observed that for compressing 30 mm of thickness among different treatments, breads with the addition of FSS required larger load because the texture of bread with higher FSS content is more consistent for not forming the gluten network required for expansion. Formulation containing 3% FSS required greater compressive load compared to control formulation. However, there was no interference in the texture analysis. The curves were ascending for all treatments. The addition of fiber increases firmness, the amylopectin

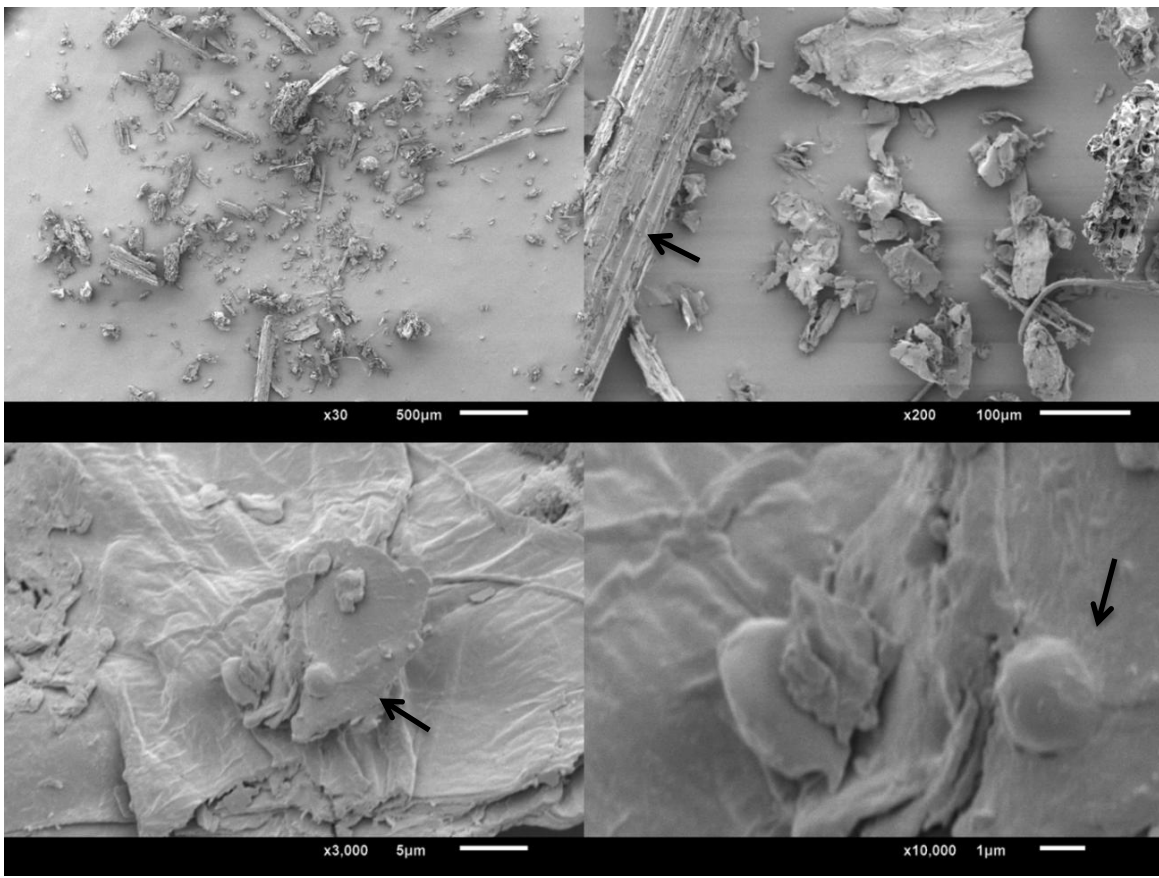


Figure 1. Scanning electron microscopy of sweet sorghum flour.

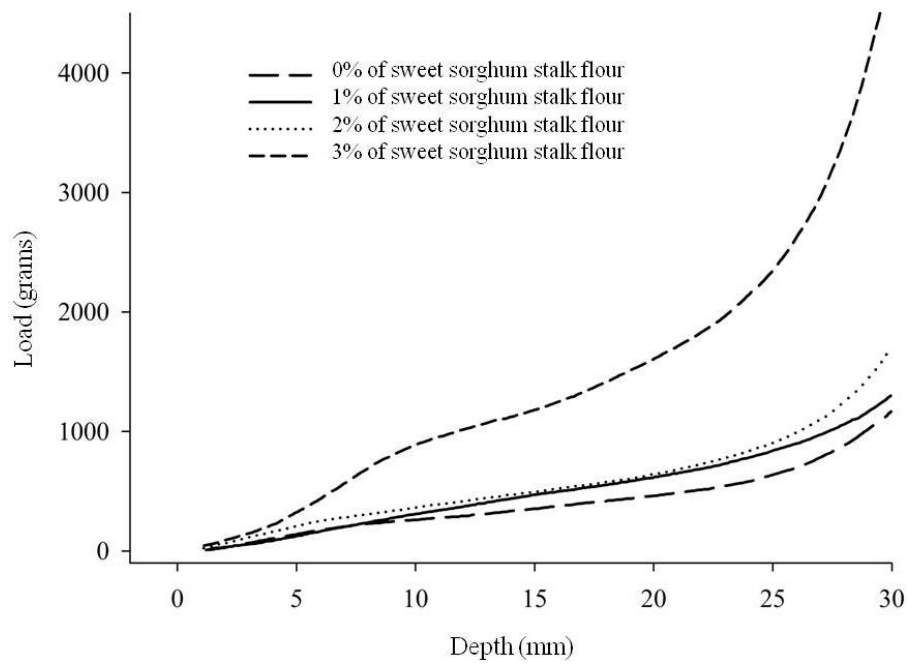


Figure 2. Texture of breads fortified with sweet sorghum flour.

Table 3. Average values for parameters weight loss, length and standard deviation of breads fortified with sweet sorghum flour (FSS).

FSS (%)	Weight loss (%)	Length (mm)
0	7.12 ± 2.99 ^a	24.62 ± 3.93 ^a
1	8.73 ± 3.43 ^a	15.25 ± 3.56 ^b
2	6.56 ± 3.94 ^a	24.20 ± 7.37 ^a
3	8.24 ± 4.28 ^a	18.34 ± 7.29 ^b
VC (%)	48.26	28.28

Different letters in the column significantly differ at 5% probability.

Table 4. Mean values for instrumental color parameters (L *, a * and b *) and standard deviation of breads fortified with sweet sorghum flour (FSS).

FSS (%)	Parameter		
	L*	a*	b*
0	44.81 ± 3.92 ^c	13.28 ± 2.58 ^a	29.53 ± 2.26 ^c
1	45.90 ± 5.25 ^c	16.93 ± 0.72 ^a	30.13 ± 2.41 ^c
2	49.07 ± 5.27 ^b	18.18 ± 2.74 ^a	32.79 ± 2.40 ^b
3	58.47 ± 5.11 ^a	60.31 ± 3.14 ^a	34.79 ± 1.24 ^a
VC (%)	9.82	15.60	6.73

L * ranging from 0 (black) to 100 (white); a * ranging from red (+ a *) to green (-a *); and b * ranging from yellow (+ b *) to blue (-b *). Different letters in the column significantly differ at 5% probability.

downgrading, and increase the bread moisture content (Skendi et al., 2010).

Table 3 shows the weight loss values, and when the dough is baked, the measure shows the loss of moisture that causes weight loss and values did not differ significantly from each other, with mean value of 7.66%. Table 3 shows the weight loss values, and when the dough is roast occurs the loss of moisture consequently weight loss and values did the breads fortified with sweet sorghum flour (FSS) not differ significantly from each other ($p < 0.05$). Table 4 presents the average values of lightness parameters (L *), a * and b * chromaticity determined in the four types of breads fortified with sweet sorghum flour, where L * ranges from 0 (black) to 100 (white), a * from red (+ a *) to green (-a *); and b * from yellow (+ b *) to blue (-b *). The addition of 3% FSS to the dough resulted in greater lightness value (L *), with significant difference ($p < 0.05$) from the others, and breads containing 1 and 0% showed no significant difference from each other ($p > 0.05$). In assessing the chromaticity coordinate (a *), it was observed that there was no significant difference ($p < 0.05$); in treatments 0, 1, 2, 3, respectively, the average results were: 13.28; 16.93; 18.18 and 60.31. However, there is an increase in red intensity in breads enriched with sweet sorghum flour. Observing values of chromaticity coordinate (b *), the control treatment (0% FSS) showed the lowest value (±

2.26) along with treatment with addition of 1% FSS, which showed value equal to 30.13, giving less tendency to yellow, with no significant difference ($p < 0.05$) between each other. Bread with the highest FSS percentage (3%) showed the highest b * value (more yellow), 34.79, significantly deferring ($p < 0.05$) from each other.

According to results obtained, it could be inferred that the addition of FSS to bread formulations can contribute to better acceptability by consumers due to significant variations in instrumental color parameters, L * and b *. At the time of purchase, consumers can be influenced by the bread color due to attractive characteristics related to senses. At the time of purchase, consumers can be influenced by the bread color due to attractive characteristics related to senses the red and yellow colors attract the attention of consumers (Toledo et al., 2014). Sensory parameters such as color, aroma, flavor, texture and appearance did not differ significantly ($p > 0.05$) by comparing with the Tukey test at 5% probability (Table 5), but when observing the average note is the preference of the panelists for the product obtained starting from the formulation containing 1% of sorghum flour. Regarding the purchase intent, 96% of panelists would buy the product if they were for sale and 4% of them would not. Of the 50 panelists, 64% consumed bread every day; 4.16% every 15 days; 27.08% once a week and 6.25% once a month. These

Table 5. Average values and standard deviation of the sensory analysis of breads fortified with sweet sorghum flour (FSS).

FSS (%)	Sensory parameter				
	Color	Aroma	Flavor	Texture	Appearance
0	7.58 ± 1.44 ^a	7.40 ± 1.41 ^a	7.24 ± 1.55 ^a	7.52 ± 1.52 ^a	7.66 ± 1.55 ^a
1	7.72 ± 1.70 ^a	7.18 ± 1.68 ^a	7.36 ± 1.45 ^a	7.60 ± 1.60 ^a	7.72 ± 1.53 ^a
2	7.64 ± 1.80 ^a	7.08 ± 1.71 ^a	7.02 ± 1.74 ^a	7.08 ± 1.96 ^a	7.60 ± 1.67 ^a
3	7.28 ± 2.05 ^a	6.78 ± 1.83 ^a	6.74 ± 1.83 ^a	6.74 ± 1.71 ^a	7.22 ± 1.60 ^a
VC (%)	23.35	23.47	23.35	23.65	21.15

Different letters in column differ by the Tukey test at 5% probability.

results indicate that bread is part of the diet of consumers and studies aiming to improve formulations by enriching with alternative sources of flour can contribute to increase bread consumption.

Conclusion

The chemical composition of sweet sorghum flour showed high fiber content and relative protein content. The 5% moisture content provides a long-term storage, provided that flour is kept at appropriate places.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Critical evaluation of proteomic protocols for passion fruit (*Passiflora edulis* Sims) leaves, a crop with juice market benefits

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Passion fruit grows practically all over Brazilian territory; its production is largely destined to juice industry and expanding to overseas markets. The suitability of four protein extraction protocols for plant proteome was investigated to determine the best choice for studies concerning passion fruit leaf proteins. Trichloroacetic acid (TCA)/acetone extraction; isoelectric focusing (IEF) buffer extraction; phenol (Phe) extraction and Phe-SDS extraction were tested. The Phe method produced the best results, showing higher reproducibility of resolved protein spots and clearer 2D gel background staining. In comparison, the Phe-SDS method presented fewer spots and lower reproducibility. The TCA/acetone method produced the fewest identifiable spots and the IEF buffer produced the poorest results, displaying fewer reproducibly detected spots, more vertical streaks and darker 2D staining. Selected spots, obtained with Phe method, were identified by spectrometric analysis (MALDI-TOF-TOF) to exemplify the viability to perform more comprehensive proteomic studies with passion fruit leaves and, therefore increase information about stress-related and developmental responses in this fruit crop.

Key words: Passion fruit, proteomic, protein extraction, juice industry.

INTRODUCTION

Currently, proteomics approach is acknowledged as a powerful strategy to analyze protein complexity and therefore, gain a better understanding of physiological

responses to developmental and environmental cues in a target living organism. As emphasized by Remmerie et al. (2011) advances in bioinformatics are contributing to

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proteomic studies in non-genomic model species. On the other hand, more information are becoming available about passion fruit for instance, a floral expressed sequence tag (EST) sequence data base was reported (Cutri and Dornelas, 2012) and more recently, efforts were made to have its genome elucidated (Santos et al., 2014). Nevertheless, sample extraction and preparation is of pivotal importance in any proteomic research. However, as “plant scientists” already know, proteomic analysis of vegetal tissues and organs can be very frustrating. The obstacles come mainly from the severe interference of intrinsic molecules such as, pigments, cell wall compounds, carbohydrates, lipids, (poly)phenolic compounds and a myriad of secondary metabolites (Wang et al., 2003; Jamet et al., 2008). Thus, the removal of these contaminants in order to increase the resolution of protein spots and to obtain reproducibility between 2D gels patterns from independent extraction experiments by reducing artifacts and minimizing protein losses remains a challenge.

To this end, a number of protein extraction protocols were developed or modified on the basis of the tissue sample peculiarities (Saravanan and Rose, 2004; Rodrigues et al., 2009; Lee et al., 2010). The Trichloroacetic acid (TCA)/acetone method is largely, if not most, used method in plant proteomic studies and has been reported for several plant species, that is, *Arabidopsis*, barley, Mexican lime, *Withania somnifera*, *Populus cathayana*, common bean and wheat (Guo et al., 2012; Fatehi et al., 2012; Taheri et al., 2011; Dhar et al., 2012; Zhang et al., 2010; Salvati et al., 2012; Xu et al., 2013). A different type of extraction is accomplished by direct solubilization of proteins with IEF buffer (Kang et al., 2007; Afroz et al., 2010), once it contains the detergent CHAPS and chaotropic agents (for example, urea and thiourea). The attractiveness of this method relies on the reduction of protein losses due to the absence of precipitations and washing steps, as well as its simplicity and speed of operation.

Another extraction method originally described by Hurkman and Tanaka (1986), is based on the solubilization of proteins in Phe and subsequent precipitation with methanol and ammonium acetate, followed by resolubilization in IEF buffer. Although being applied to proteomic studies with model plants, as *Arabidopsis*, (Mooney et al., 2006) this technique is frequently the choice for resistant tissues. For instance, seeds (Hajduch et al., 2005; Hajduch et al., 2006; Hajduch et al., 2007; Houston et al., 2009), autumn olive fruit (Wu et al., 2011), *Vitis vinifera* and *Gmelina arborea* Linn. Roxb leaves (Jellouli et al., 2010; Rasinemi et al., 2010) as well as rice seedlings (Chi et al., 2010). The inclusion of SDS in phenol (Phe) based extraction procedure was positively correlated with 2D gel quality (Wang et al., 2003).

Passion fruit is a tropical crop that presents great potential for industrialized juice production, based on its distinctive and exotic aroma, with Brazil being one of the

most prominent producers, having plantations spread over practically the entire territory. The pattern of juice production principally for domestic consumption is changing, and expanding into international markets (Bernacci et al., 2008; Oliveira et al., 2012). In spite of its prospect as a tropical fruit crop, there is a lack of information regarding proteomic approaches using passion fruit tissues. Therefore, this study compared the effectiveness of four distinct protein extraction methods for passion fruit leaves suitable for proteomic studies. A methodology was sought that combined reproducibility between several experiments with a high number of well resolved protein spots.

MATERIALS AND METHODS

Plant material

Passion fruits (*Passiflora edulis* Sims) were purchased at the local market of Campos dos Goytacazes, a city located in the northern region of Rio de Janeiro State, Brazil. The seeds were collected, dried at room temperature, and stored at 4°C in the dark. Plants were grown in vermiculite pots and maintained in environmental chambers for 17 h under 300 mE m⁻² s⁻¹ light at 28°C and for 7 h in the dark at 18°C and 62% relative humidity. Four-week-old plants (with 3-4 developed leaves) were used for all experiments. Three different plants had their leaves collected and subsequently ground into fine powder in liquid nitrogen using a pre-cooled mortar and pestle. For all samples, protein extraction (with individual buffers) was performed in a cold room at 4°C to avoid protease degradation by intrinsic protease activity, as described below.

TCA/acetone extraction

This method is based on precipitation of proteins by TCA/acetone according to Hajheidari et al. (2005), and subsequent resolubilization in IEF buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT and 2% v/v ampholytes, pH 4-7, Pharmalyte, Amersham). Leaves were ground to a fine powder in liquid N₂, 10% (w/w) insoluble polyvinylpyrrolidone (PVPP) was added and proteins extracted in an ice-bath with 5 mL of ice-cold extraction solution (10% (w/v) TCA and 0.07% (v/v) β-mercaptoethanol in acetone) for each g of powdered leaves. After 1 h incubation at -20°C, samples were centrifuged at 10,000 g (4°C) for 15 min. The pellets were incubated again in 10% w/v TCA/acetone with 0.07% β-mercaptoethanol v/v at -20°C for 1 h and then centrifuged at 10,000 g (4°C) for 15 min. The washing step was performed twice with ice-cold acetone with 0.07% β-mercaptoethanol. The pellets were air dried and resolubilized in IEF buffer, while insoluble material was removed by centrifugation as describe above. The supernatant was stored at -20°C until analysis.

IEF buffer extraction

Based on the work of Kang et al. (2007), leaves were ground to a fine powder in liquid N₂, 10% (w/w) insoluble PVPP was added and proteins extracted in an ice-bath with 2 mL of ice-cold IEF buffer for each gram of powdered leaves. After centrifugation at 10,000g (4°C) for 15 min, the resulting supernatant was ready for protein quantification and 2D electrophoresis.

Phe extraction

In this protocol, leaves were ground to a fine powder in liquid N₂, 10% (w/w) PVPP was added and proteins extracted in an ice-bath with 3 mL of ice-cold extraction buffer (0.5 M Tris-HCl pH 7.5, 50 mM EDTA, 2% β-mercaptoethanol and 0.7 M sucrose) for each gram of powdered leaves. After centrifugation at 10,000g (4°C) for 15 min the supernatant was collected and an equal volume of water-saturated phenol was added. After homogenization, samples were centrifuged at 10,000 g (4°C) and the upper phenol layer was collected. Next, the Phe phase was re-extracted twice with extraction buffer as above. The protein precipitation was achieved by adding 5 volumes (v/v) of 0.1 M ammonium acetate in methanol and 16 h incubation at -20°C. After centrifugation at 10,000 g (4°C) for 5 min, pellets were washed three times with 0.1 M ammonium acetate in methanol and once with acetone 100%, (Schuster and Davies, 1983; Wu et al., 2011). Pellets were air dried and proteins resolubilized in IEF buffer, while insoluble material removed by centrifugation. The supernatant was collected for protein quantification and 2D analysis.

Phe-SDS extraction

This methodology (Wang et al., 2003) is similar to the Phe extraction with the main difference being the inclusion of SDS (2%) in the extraction buffer. Protein purification was carried out as described for Phe procedure.

Protein quantification

Protein concentration in all extracts was determined using 2-D Quant kit (GE Healthcare) following manufacturer's instruction.

Gel electrophoresis analysis, staining and image analysis

To compare the effectiveness of all extraction methods by 2D analysis, equal amount of protein (500 µg) were loaded on the first dimension. IEF (IPG strips, 18 cm length, pH 4-7, Immobililine™ DryStrip GE Healthcare) was performed using IPGfor (GE Healthcare) as follow: (1) step to 50 V (600 Vh); (2) step to 200 V (200 Vh); (3) 500 V (500 Vh); (4) step to 1000 V (1000 Vh); (5) gradient to 4000 V (5000 Vh); (6) gradient to 8000 V (6000 Vh); (7) Step to 8000 V (54000 Vh); (8) step to 100 V (600 Vh). After focusing, proteins were reduced with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of equilibration buffer (6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCl, pH 8.8) for 15 min. The strips were transferred to 10% SDS-PAGE gels for second dimension electrophoresis with the Protean II xi Cell (Bio-rad, Hercules, CA, USA), using SDS electrophoresis buffer (250 mM Tris pH 8.4, 1.92 M glycine and 1% SDS) with 25 mA per gel for 6 h. The gels were stained with Colloidal Coomassie Blue (Neuhoff et al., 1985). 2D gels were scanned with ImageScanner (GE Healthcare) and the data were analyzed using the ImageMaster 2D-Platinum, Version 7.0 software (GE Healthcare) to discriminate the protein spots. The mean ± SD of 3 independent extraction experiments for each methodology was used to produce the results. To check for reproducibility of tested protein extraction methodologies, a coefficient of variation (sd/mean)*100 was calculated. For analytical replicates to be reliable, a coefficient of variation below 10% is expected. 1D analysis from proteins extracted by Phe methodology were analyzed by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue R-250.

Protein identification by MALDI-TOF-TOF

The spots were collected and incubated in 25 mM ammonium

bicarbonate containing 50% (v/v) acetonitrile for 16 h. After discoloration, the spots were washed with deionized water and covered with acetonitrile 100%; subsequently they were dried in speed-vac for 15 min. Later, the spots were rehydrated in 50 mM ammonium bicarbonate with trypsin 33 ng/µL for 1 h in ice. Excess of protease solution was removed and the samples were incubated at 58°C for 30 min. The reaction was stopped by addition of 1 µL of formic acid 5%. Afterward, peptides were extracted with 30 µL of formic acid 5%, acetonitrile 50% solution and sonicated for 10 min. This process was repeated twice, and the samples were concentrated in a SpeedVac (Savant Instruments, Inc., Farmingdale, NY) to about 10 µL and desalted using Zip-Tip (C18 resin:P10 Millipore Corporation, Bedford, MA). Peptides were eluted from the column with 60% acetonitrile 0.1% trifluoroacetic acid. The sample solution (0.3 µL) was mixed with an equal volume of a matrix solution (R-cyano- 4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in 50% acetonitrile 0.1% trifluoroacetic acid) on the target plate and allowed to dry at room temperature. The MS/MS data were acquired with a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1600 shots were accumulated for spectra in the S mode, while 2400 shots were accumulated for spectra in the MS/MS mode. Six of the most intense ion signals with a signal-to-noise ratio above 30 were selected as precursors for MS/MS acquisition, with the exclusion of common trypsin autolysis peaks and matrix ion signals. External calibration in MS mode was performed using a mixture of four peptides: des-Arg1-Bradykinin (m/z 904.468); angiotensin I (m/z 1,296.685); Glu1-fibrinopeptide B (m/z 1,570.677); and ACTH (18_39) (m/z 2,465.199). MS/MS spectra were externally calibrated using known fragment ion masses observed in the MS/MS spectrum of angiotensin I. The Mascot MS/MS Ion Search (www.matrixscience.com) was used to blast sequences against the NCBI nr databank. Combined MS-MS/MS searches were conducted with parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification) and methionine oxidation (variable modification). According to MASCOT probability analysis, only hits significant at P<0.05 were accepted.

RESULTS AND DISCUSSION

Figure 1A shows the 2D protein profile when leaf proteins were obtained by TCA/acetone extraction. Protein yield produced by this method was 21±1.6 mg/g of total fresh leaves. In this case, despite a reasonable stained background only 249 protein spots were resolved, with considerable variation in recognizable proteins between the independent experiments (±44). When leaf proteins were directly extracted with IEF buffer (Figure 1B), the 2D protein profile quality was significantly reduced due to vertical streaks, darker stained background, and above all the highest variation of discerned protein spots between the individual experiments was observed, that is, 478±138, while protein yield for this method was 8.7±2.3 mg/g.

Figure 1C shows the protein profile obtained with Phe extraction method, given a protein yield of 9.7±0.2 mg/g, of which appears to be the best option to study passion fruit leaf proteins. This conclusion was not only due to the number of resolved protein spots (~400) and clearer stained background, but most importantly because of the highest reproducibility of observed spots between independent extractions (393±14). Even though Phe-SDS

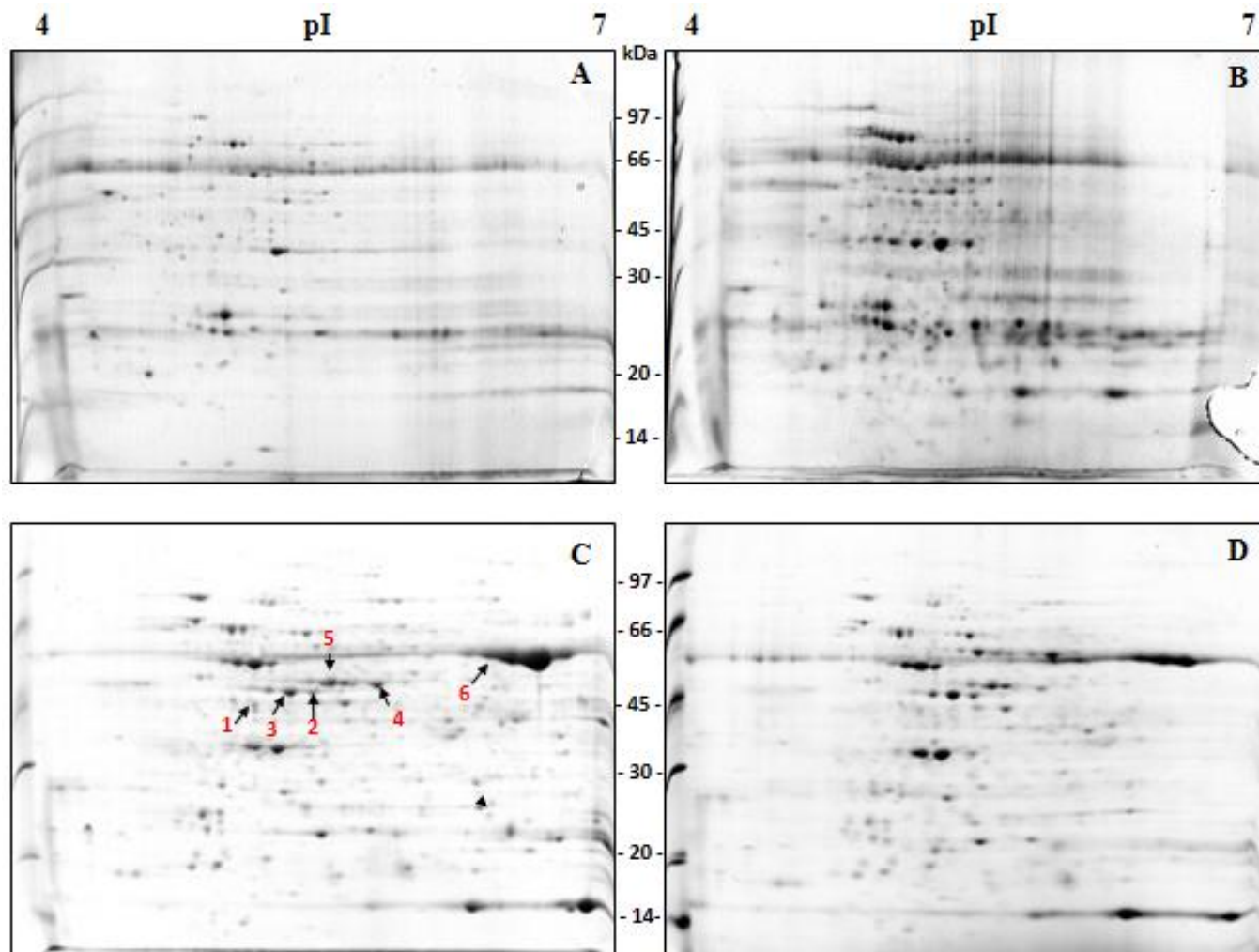


Figure 1. Representative 2D gel analysis of proteins extracted from passion fruit leaves. **A**, TCA/acetone method; **B**, IEF buffer method; **C**, Phe method. **D**, Phe-SDS method. IEF was carried on 18 cm strips pH 4-7 (500 μ g of protein/strip). Protein spots were visualized in 2D gels by Colloidal Coomassie Blue and molecular weight markers are in kDa. In all cases, leaves from 3 plants were pooled for each extraction procedure to obtain an average pattern.

methodology (rendering protein yield of 6.6 ± 2.4 mg/g) presented a quite clean staining background, in comparison with the Phe method it produced considerably fewer protein spots and a higher variation between the individual experiments (338 ± 67 ; Figure 1D).

Extreme care was taken to optimize each extraction procedure and standardize the electrophoretic and staining conditions throughout the entire comparative study. Thus, it was reasoned that differences among the 2D gels patterns (compare Figures 1A-D) might be reflecting artifacts provoked by the interference of substances remaining from each extraction method. With regards to protein yield among different extraction procedures, the TCA/acetone methodology provided higher amounts of extracted proteins while the other 3 tested methods produced quite similar protein yields. Evaluation of sample preparation protocols for plant

tissues suitable for 2D gel presented clear variation on protein yielding among tested procedures. For instance, in the work described by Saravanan and Rose (2004), it was the Phe extraction procedure that gave higher protein yield (in a similar range to our data) over methods based on TCA extraction using tomato green fruit and tomato root. While Jellouli et al. (2010) showed much higher variation among tested protocols using roots from grapevine when comparing with what has been obtained with passion fruit leaves.

In this work, it is believed that the improvements in the 2D gel quality offered by the Phe protocol, which allowed more protein spots to be unambiguously noted, compensates for the choice of a laborious extraction method. Moreover, it was the only protocol rendering identified protein spots with a coefficient of variation below 10% (data not shown), reinforcing its suitability for

Table 1. Examples of proteins identified from *Passiflora edulis Sims* leaf by MALDI-TOF-TOF.

Spot n°	Protein name [species]	Th. Mr/pl	Ex. Mr/pl	Score	PM	AccN
1	Chloroplast sedoheptulose-1,7-bisphosphatase [<i>Solanum lycopersicum</i>]	43,017/6.07	42,000/5.14	89	1	gi 350538149
2	Rubisco activase precursor [<i>Spinacia oleracea</i>]	51,737/6.28	44,666/5.44	272	2	gi 170129
3	Rubisco activase precursor [<i>Spinacia oleracea</i>]	51,737/6.28	44,666/5.32	303	2	gi 170129
4	Hypothetical protein Osl_20474 [<i>Oryza sativa Indica Group</i>]	30,521/6.86	45,666/5.77	391	3	gi 125552851
5	Hypothetical protein VITISV_014296 [<i>Vitis vinifera</i>]	49,185/6.54	46,666/5.48	59	1	gi 147784261
6	Ribulose 1,5-bisphosphate carboxylase [<i>Canarium ovatum</i>]	52,695/5.86	53,000/6.33	429	4	gi 7259805

Th. Mr/pl, theoretical; Ex. Mr/pl, experimental; Score, more than 50; PM, the number of unique peptides matched; AccN, accession number. The assigned protein that best matched has been given with the species in which it has been identified and its accession number.

2D gel analysis. Table 1 shows the identification 6 protein spots selected from leaf proteins extracted with Phe procedure (Figure 1C) by MALDI-TOF-TOF analysis. Such results illustrate the appropriateness for further research, such as studies of stress-related responses (or even developmental) in passion fruit by combining 2D analysis and mass spectrometry techniques to identify differentially regulated/expressed proteins. Moreover, as leaf proteins extracted by Phe procedure also rendered good quality 1D gels, that is, SDS-PAGE analysis (Figure 2), additional proteomic strategy, namely shotgun (Mirzaei et al., 2012; Monavarfeshani et al., 2013) is potentially promising. The results described here can certainly facilitate and stimulate proteomic studies with passion fruit in Brazil and internationally, especially with advances of ESTs, genomic and suppression subtractive hybridization strategies (Cutri and Dornelas, 2012; Santos et al., 2014; Munhoz et al., 2015).

Conclusions

The Phe extraction procedure provided the best results to analyze passion fruit leaf proteins via 2D gels for proteomic analyzes. Therefore, our results provide framework for more comprehensive studies on this model plant addressing responses to different stimulus, in order to better understand the physiology of a tropical crop having strong possibility in the fruit juice industry, not only in Brazil but also abroad. Additionally, with the wider recognition of its potential for processed juice market, this crop can become an attractive option for even small farmers.

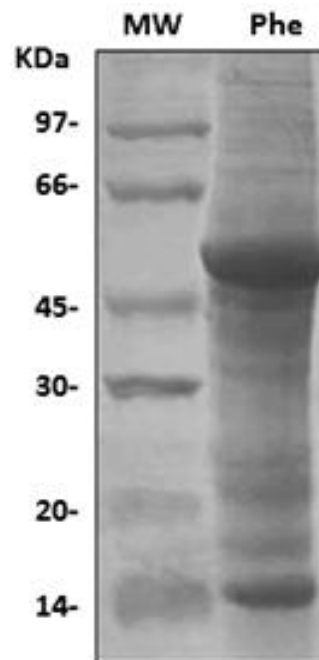


Figure 2. Electrophoretic analysis (10%) SDS-PAGE. MW: Molecular weight markers; Phe, leaf proteins extracted by Phe procedure (60 µg). The gel was stained with Coomassie Brilliant Blue R-250.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Analysis of alkaloid phytochemical compounds in the ethanolic extract of *Datura stramonium* and evaluation of antimicrobial activity

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The aim of this study was to assess the compounds of alkaloids extracts from the leaves of *Datura stramonium*, which can be the basis for the synthesis of new antibiotics. In this study, the alkaloid compounds of *D. stramonium* have been evaluated. The chemical compositions of the leaves of ethanolic extract of *D. stramonium* were investigated using gas chromatography-mass spectroscopy. Gc-MS analysis of alkaloid leaves ethanolic extract of *D. stramonium* revealed the existence of the Ethyl iso-allochololate, D-asycarpidan-1-methanol, acetate (ester), 3-(1,5-dimethyl-hexyl)3a,10,10,12b-tetramethyl1,2,3,3a,4,6,8,9,10,10a,11,12,12a,12b-tetradec-ahydro-benzo[4,5]cyclohept,2,7-Diphenyl-1,6-dioxopyridazino [4,5:2,3] pyrrolo[4,5-d] pyridazine, 3,8,8-Trimethoxy-3-piperidyl-2,2-benaphthalene-1,1,4,4-tetrone, [5 β] Pregnane3, 20 β -diol,14 α ,18 α -[4-methyl,3-oxo-[1-oxa-4-azabutane-1,4-diyl], diacetate, 1-monolinoleoylglycerol trimethylsilyl ether and 17-[1,5-dimethylhexyl]-10,13-dimethyl-3sstyrylhexadecahydrocyclopenta[a]phenathren-2-one. Alkaloids extract from leaves of *D. stramonium* were assayed for *in vitro* antibacterial activity against *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aerogenosa* and *Klebsiella pneumonia* by using the diffusion method in agar. The zone of inhibition was compared with different standard antibiotics. The diameters of inhibition zones ranged from 0.8 to 2.01 mm for all treatments.

Key words: Alkaloids, antibacterial activity, *Datura stramonium*, gas chromatography-mass spectroscopy.

INTRODUCTION

Datura stramonium is an annual herb, with stem erect and spreading branches above. It is common in the waste land, fields and gardens in Baghdad district (Figure 1). Leaves, seeds and roots contain the alkaloid daturine (a mixture of the two alkaloids hyoscyamine and atropine)

and also contain scopolamine alkaloid (Hyosine) acids, tannin and fatty oil. Plants are rich source of secondary metabolites with interesting biological activities (Palombo and Semple, 2001; Koduru et al., 2006). Several plant products have been shown to exert a protective role

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Abbreviation: GC-MS, Gas chromatography-mass spectroscopy.

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Figure 1. Leaves of *Datura stramonium*.

against the formation of free radicals and playing a beneficial role in maintaining disease condition (Ajitha and Rajanarayana, 2001). Very few of these chemicals are toxic also (Haraguchi et al., 1999; Sashikumar et al., 2003). The phytochemicals with adequate antibacterial activity will be used for the treatment of bacterial infections (Iwu et al., 1999; Purohit and Vyas, 2004; Krishnaraju et al., 2005). *Datura stramonium* is poisonous to cattle, horses, sheep, and children and causes the following symptoms: headache, nausea, vertigo, extreme thirsty, dry burning sensation in the skin, and in extreme cases death.

The main toxic alkaloids in *D. stramonium* are the tropane alkaloids which are the atropines (dl-hyoscyamine) and scopolamine (l-hyoscyne) (Friedman, 2004; Steenkamp et al., 2004). Atropine and scopolamine are competitive antagonists of muscarinic cholinergic receptors and are central nervous system depressants (Halpern, 2004). Intentional poisoning with *D. stramonium* has also been reported in several cases, namely a fatal poisoning with *D. stramonium* for its mind altering properties and the eating and chewing of *Datura* in a suicides attempt (Klein and Odera, 1984; Forrester, 2006; Monteriol et al., 2007). The toxicity of *D. stramonium* in grazing animals have been suspected by livestock owners and field veterinarians especially at time of drought

or after ingesting freshly harvested maize that will be used for ensiling and heavily contaminated with young *D. stramonium*.

Successful extraction is largely dependent on the type of solvent used in the extraction procedure. The most often tested extracts are: water extract as a sample of extract that are primarily used in traditional medicine and extracts from organic solvents such as methanol, ethanol as well as ethyl acetate, acetone, chloroform, dichloromethane (Alves et al., 2000; Palombo and Semple, 2001; Uzun et al., 2004; Cos et al., 2006; Ncube et al., 2008; Stanojević et al., 2010). Considering the high economical and pharmacological importance of secondary plant metabolites, industries are deeply interested in utilizing plant tissue culture technique for large scale production of these substances.

MATERIALS AND METHODS

Collection and preparation of plant material

In this research, the leaves were dried at room temperature for ten days and when properly dried the leaves were powdered using clean pestle and mortar, and the powdered plant was size reduced with a sieve. The fine powder was then packed in airtight container to avoid the effect of humidity and then stored at room temperature (Imad et al., 2015).

Extraction and identification of alkaloids

The powdered leaves (2 g) were boiled in a water bath with 20 ml of 5% sulphuric acid in 50% ethanol. The mixture was cooled and filtered. A portion was reserved. Another portion of the filtrate was put in 100 ml of separating funnel and the solution was made alkaline by adding two drops of concentrated ammonia solution. Equal volume of chloroform was added and shaken gently to allow the layer to separate. The lower chloroform layer was run off into a second separating funnel. The ammoniacal layer was reserved. The chloroform layer was extracted with two quantities each of 5 ml of dilute sulphuric acid. The various extracts were then used for the following test:

Wagner's test

To the filtrate in test tube III, 1 ml of Wagner's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

Dragendoff's test

To the filtrate in test tube II, 1 ml of Dragendoff's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

Mayer's test

To the filtrate in test tube I, 1 ml of mayer's reagent was added drop by drop. Formation of a greenish coloured or cream precipitate indicates the presence of alkaloids (Evans, 2002).

Table 1. Compounds present in the alkaloid extract of *Datura stramonium* using GC-MS analysis.

S/N	Alkaloid compound	Formula	Molecular Weight	Structure
1	Ethyl iso-allocholate	C ₂₆ H ₄₄ O ₅	436	Figure 2
2	D-asycarpidan-1-methanol, acetate (ester)	C ₂₀ H ₂₆ N ₂ O ₂	326	Figure 3
3	3-(1,5-Dimethyl-hexyl) 3a,10,10,12b-tetramethyl 1,2,3,3a,4,6,8,9,10,10a,11,12,12a, 12b-tetradecahydro-benzo [4,5] cyclohept	C ₃₀ H ₅₀	410	Figure 4
4	2,7-Diphenyl-1,6-dioxopyridazino[4,5:2,3] pyrrolo[4,5-d]pyridazine	C ₂₀ H ₁₃ N ₅ O ₂	355	Figure 5
5	3,8,8-Trimethoxy-3-piperidyl-2,2-benaphthalene-1,1,4,4-tetrone	C ₂₈ H ₂₅ N ₃ O ₇	487	Figure 6
6	[5β]Pregnane-3,20 β-diol,14α,18α-[4-methyl,3-oxo-[1-oxa-4-azabutane-1,4-diyl], diacetate	C ₂₈ H ₄₃ N ₃ O ₆	489	Figure 7
7	1-Monolinoleoylglycerol trimethylsilyl ether	C ₂₇ H ₅₄ O ₄ Si ₂	498	Figure 8
8	17-[1,5-Dimethylhexyl]-10,13-dimethyl-3-sstyrylhexadecahydrocyclopenta[a]phenathren-2-one	C ₃₅ H ₅₂ O	488	Figure 9

Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis of the ethanol extract of *D. stramonium* was carried out using a Clarus 500 Perkin – elmer (Auto system XL) gas chromatograph equipped and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% dimethyl poly siloxane), 30 m × 0.25 mm ID × 1 μm of capillary column. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization system operated in electron impact mode with ionization energy of 70 ev. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature rose to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Helium gas (99.999%) was used as carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 ml was employed (split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed at 110°C (isothermal for 2 min), with an increase of 100°C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 ev; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min and the total GC-MS running time was 36 min. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45 to 450 (m/z). The mass detector used in this analysis was Turbo-Mass Gold-Perkin Elmer and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver 5.2 (Ameera et al., 2015; Huda et al., 2015).

Measurement of antibacterial activity

The antibacterial activity of alkaloids was determined by using agar well diffusion method. Wells of 5 mm diameter were punched in the agar medium with sterile cork borer and filled with plant alkaloid extract. Standard antibiotics, penicillin, kanamycin, cefotaxime, streptomycin and rifampin (1 mg/ml) were also tested for their antibacterial activity. The plates were incubated at 37°C for 24 h. The negative control was added without adding the cultures to know the sterile conditions. The Petri dishes were placed in the

refrigerator at 4°C or at room temperature for 1 h for diffusion, incubated at 37°C for 24 h, then the zone of inhibition produced by different antibiotics was observed. Measure it using a scale and record the average of two diameters of each zone of inhibition.

RESULTS AND DISCUSSION

GC-MS analysis of alkaloid compound clearly showed the presence of eight compounds. The alkaloid compound, formula, molecular weight and exact mass are presented in Table 1. Chromatogram GC-MS analysis of the ethanol extract of *D. stramonium* showed the presence of eight major peaks and the components corresponding to the peaks were determined as follows. The first set up peaks was determined to be ethyl iso-allocholate (Figure 2). The second peak was indicated to be D-asycarpidan-1-methanol, acetate (ester) (Figure 3). The next peaks was considered to be 3-(1,5-dimethyl-hexyl)3a,10,10,12b-tetramethyl1,2,3,3a,4,6,8,9,10,10a,11,12,12a,12b-tetra decahydro-benzo[4,5]cyclohept, 2,7-diphenyl-1,6-dioxopyrid-azino[4,5:2,3] pyrrolo[4,5-d]pyridazine, 3,8,8-trimethoxy-3-piperidyl-2,2-benaphthalene-1,1,4,4-tetrone, [5β]Pregnane-3,20 β-diol,14α,18α-[4-methyl,3-oxo-[1-oxa-4-azabutane-1,4-diyl], diacetate, 1-monolinoleoylglycerol trimethylsilyl ether, and 17-[1,5-dimethylhexyl]-10,13-dimethyl-3-sstyrylhexadecahydrocyclopenta [a]phenathren -2-one (Figures 4 to 9). Among the identified phyto-compounds have the property of anti-oxidant and antimicrobial activities (Singh et al., 1998; Kumar et al., 2001; John and Senthilkumar, 2005; Venkatesan et al., 2005; Santh, 2006; Sazada et al., 2009). Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects.

Continued further exploration of plant derived

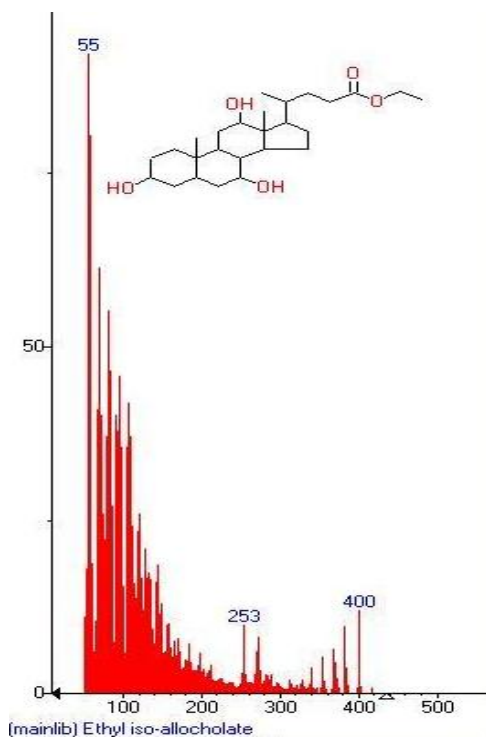


Figure 2. Structure of Ethyl iso-allocholate present in the leaves extract of *Datura stramonium* using GC-MS analysis.

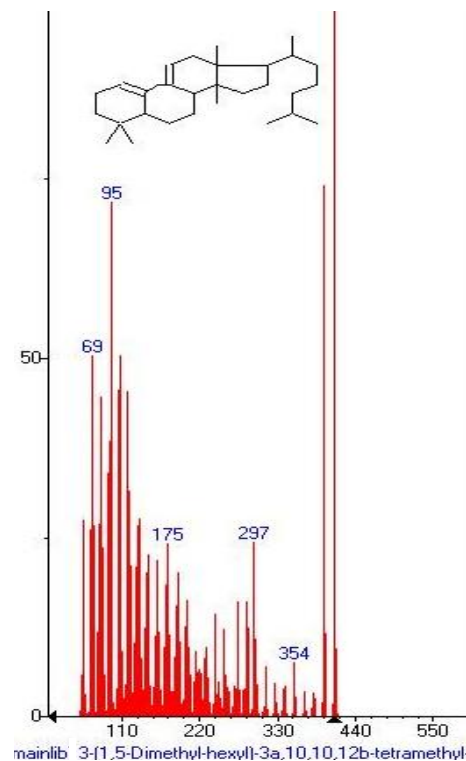


Figure 4. Structure of 3-(1,5-Dimethyl-hexyl)3a,10,10,12b tetramethyl 1, 2, 3, 3a, 4, 6, 8, 9, 10, 10a, 11, 12, 12a, 12b-tetradecahydro-benzo[4,5]cyclohept present in the leaves extract of *Datura stramonium* using GC-MS analysis.

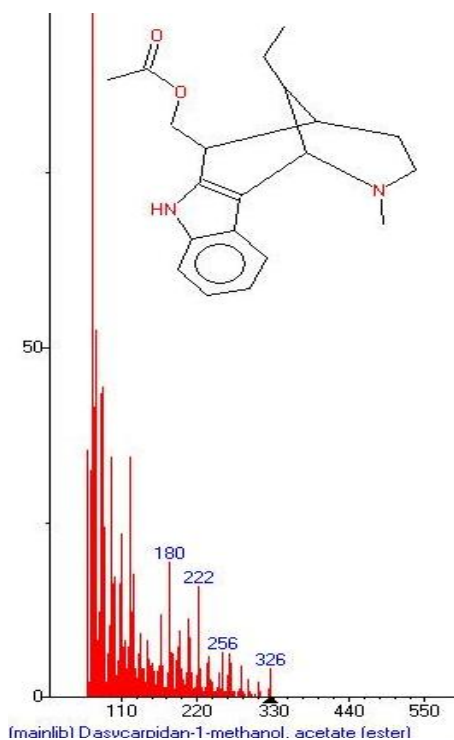


Figure 3. Structure of D-asycarpidan-1-methanol, acetate (ester) present in the leaves extract of *Datura stramonium* using GC-MS analysis.

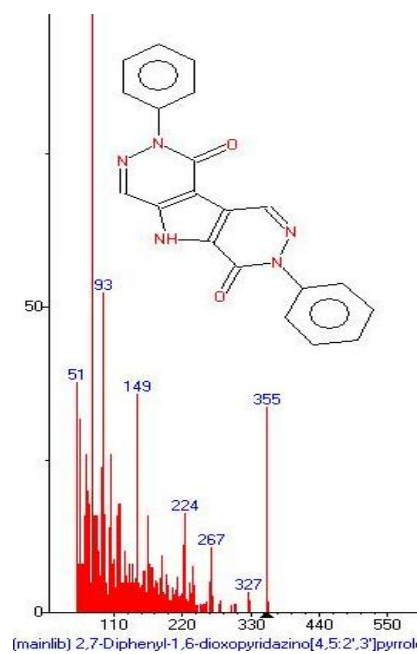


Figure 5. Structure of 2,7-Diphenyl-1,6-dioxypyridazino[4,5:2,3] pyrrolo[4,5-d]pyridazine present in the leaves extract of *Datura stramonium* using GC-MS analysis.

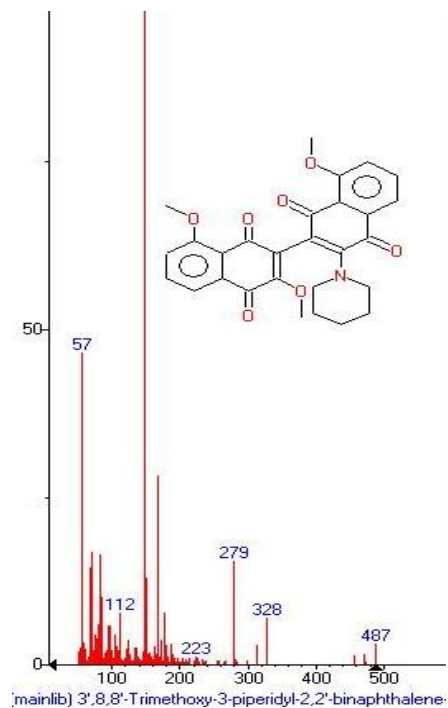


Figure 6. Structure of 3,8,8-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1,4,4-tetrone present in the leaves extract of *Datura stramonium* using GC-MS analysis.

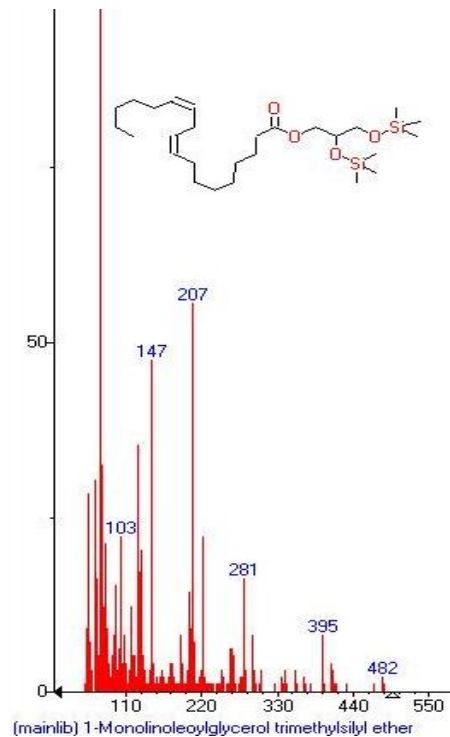


Figure 8. Structure of 1-monolinoleoylglycerol trimethylsilyl ether present in the leaves extract of *Datura stramonium* using GC-MS analysis.

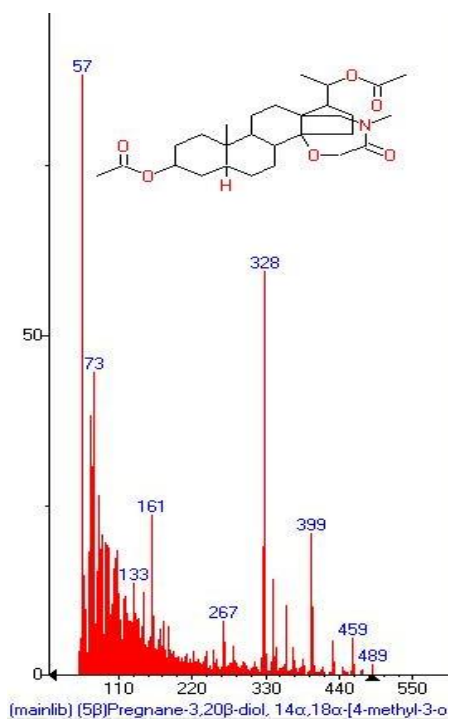


Figure 7. Structure of [5β]Pregnane-3,20 β-diol, 14α,18α-[4-methyl,3-oxo-[1-oxa-4-azabutane-1,4-diy]], diacetate present in the leaves extract of *Datura stramonium* using GC-MS analysis.

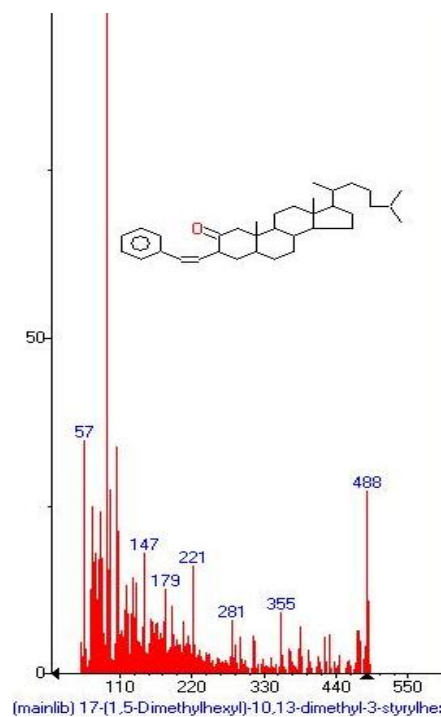


Figure 9. Structure of 17- [1,5-dimethylhexyl]-10,13-dimethyl-3-sstyrylhexadecahydro-cyclopenta[a]phenanthren-2-one present in the leaves extract of *Datura stramonium* using GC-MS analysis.

Table 2. Zone of inhibition (mm) of test bacterial strains to alkaloid leaf extracts of *Datura stramonium* (L.) and standard antibiotics.

Alkaloid/Antibiotics	<i>Escherichia coli</i>	<i>Pseudomonas eurogenosa</i>	<i>Staphylococcus aureus</i>	<i>Proteus mirabilis</i>	<i>Klebsiella pneumonia</i>
Alkaloid	1.8±0.42	1.4±0.59	1.3±0.5	2.01±0.51	1.7±0.62
Kanamycin	0.9±0.1	0.5±0.4	0.6±0.2	0.4±0.1	0.8±0.3
Cefotaxime	1.1±0.3	1.5±0.1	1.2±0.1	1.2±0.3	1.3±0.5
Penicillin	1.6±0.1	1±0.5	1±0.4	1±0.2	1.1±0.2
Streptomycin	1.2±0.3	1.3±0.6	1.9±0.61	1.2±0.6	1.7±0.2
Rifampin	1.2±0.5	1.1±0.1	0.8±0.2	1.1±0.1	0.6±0.1

antimicrobials is needed today.

The results of the antimicrobial activity of extracts of leaves of *D. stramonium* are presented in Table 2. We observe that the sensitivity tests show the effect of crude extracted alkaloids from seeds and roots of different bacterial strains, giving varying diameters depending on the tested strains. The clear zone of growth inhibition was noted around the well due to diffusion of alkaloid compound. The diameter of the zone denotes the relative susceptibility of the test microorganism to a particular antimicrobial. The obtained results of the crude extracts were compared with the standard antibiotics such as penicillin, kanamycin, cefotaxime, Streptomycin and Rifampin. All the tested organisms are highly sensitive to the ethanol leaf extract (1.4 to 2 mm) than the standard antibiotics which showed more or less activity (0.4 to 1.7 mm). The presence of antimicrobial substances in the higher plants is well established. Plants have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health. However, further studies are needed, including toxicity evaluation and purification of active antibacterial constituents from *D. stramonium* extracts looking toward a pharmaceutical use.

Conclusion

Eight chemical alkaloids constituents have been identified from ethanolic extract of the *D. stramonium* by gas chromatogram mass spectrometry (GC-MS). *In vitro* antibacterial evaluation of *D. stramonium* forms a primary platform for further phytochemical and pharmacological investigation for the development of new potential antimicrobial compounds.

Conflict of Interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of manganese, 2,5-xylidine, veratryl alcohol and tween 80 on the production of ligninolytic enzymes by *Ceriporiopsis subvermispota*

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The effects of adding manganese, 2,5-xylidine, veratryl alcohol and Tween 80 in a culture medium used for the production of ligninolytic enzymes by polyurethane foam-immobilized *Ceriporiopsis subvermispota* were studied. While 11 ppm Mn²⁺ promoted the highest maximum activity of manganese peroxidase (108.0 ± 43.3 U/L, in the 6th day of cultivation), the medium without manganese led to the highest maximum activity of laccase (15.5 ± 2.1 U/L, in the 12th day of cultivation). By supplementing the medium containing 11 ppm Mn²⁺ with 1.0 mM 2,5-xylidine, it was possible to improve the maximum activity of laccase to 21.5 ± 4.9 U/L. The supplementation of the medium containing 11 ppm Mn²⁺ with 1.0 mM veratryl alcohol, in turn, led to an apparent second peak of MnP activity (110.0 ± 1.4 U/L, in the 24th day of cultivation; compared to 147.5 ± 60.1 U/L, in the 9th day of cultivation). When the medium containing 11 ppm Mn²⁺ and 1.0 mM 2,5-xylidine was supplemented with 0.05% (v/v) Tween 80, the maximum activities of Lac and MnP reached 53.3 ± 17.7 U/L (21st day of cultivation) and 174.8 ± 1.4 U/L (9th day of cultivation), respectively. During the cultivations, the exhaustion of glucose in the medium promoted nutritional stress, which, in turn, led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium. The concentrations of extracellular proteins increased throughout the cultivations; such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

Key words: *Ceriporiopsis subvermispota*, manganese, 2,5-xylidine, veratryl alcohol, Tween 80; manganese peroxidase, laccase.

INTRODUCTION

Ceriporiopsis subvermispota, a fungus that has already been used for biopulping in industrial scale (Akhtar et al., 2000), is known for its selectivity in lignin degradation,

due to its inefficiency in degrading wood polysaccharides, mainly cellulose, and to its efficiency in degrading lignin (Fernandez-Fueyo et al., 2012a; Fernandez-Fueyo et al.,

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Abbreviations: Lac, Laccase; MnP, manganese peroxidase; DNS, 3,5-dinitrosalicylic acid.

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2012b). The degradation of lignin by *C. subvermispora* is related to its non-specific extracellular enzymatic system composed by laccases (Lac) and manganese peroxidases (MnP) (Carvalho et al., 2008), which can perform their action in association with low molecular weight compounds (Aguar and Ferraz, 2008). The production of ligninolytic enzymes by filamentous fungi is regulated by several factors. In this context, the sources of carbon, nitrogen and inorganic ions available in the cultivation medium, as well as the presence of inducing compounds, like aromatics, are mostly important (Bonnarme and Jeffries, 1990; Buswell et al., 1995; Arora and Gill, 2000). For example, the addition of veratryl alcohol in cultures of *Botryosphaeria sp.* increased the production of constitutive Lac (PPO-I and PPO-II) in 100 and 25-fold (Dekker and Barbosa, 2001). The supplementation with 2,5-xylydine, in turn, has proved very effective in inducing the activity of Lac in cultures of *Trametes versicolor* (Rancaño et al., 2003).

Moreover, Mn^{2+} plays a central role in ligninolysis by many fungi, including *C. subvermispora*; stimulating the secretion of and acting as a substrate for the manganese peroxidases (Hofrichter, 2002).

With regard to the non-ionic surfactant Tween 80, many authors, including Ürek and Pazarlioglu (2005) and Dekker et al. (2007), have observed an increase in the excretion of enzymes when cultures of ligninolytic fungi were performed in the presence of this surfactant. Previously, Asther et al. (1987) have suggested that Tween 80 transforms the structure of the cell membrane, favoring the excretion of enzymes into the medium.

To evaluate the effects of such additives in the production of MnP and Lac by *C. subvermispora* immobilized in polyurethane foam, a culture medium with defined composition, supplemented or not with manganese, 2,5-xylydine, veratryl alcohol and Tween 80, was used for cultivations under fixed environmental conditions. By following the concentrations of biomass, reducing sugars, ammonium and proteins, as well as the values of pH and conductivity, during the cultivations, it was possible to establish the metabolic behavior exhibited by the fungus in selected media.

MATERIALS AND METHODS

Fungus and inoculum preparation

C. subvermispora, from a stock culture, was initially activated in agar plates, using a medium composed by 2% (m/v) malt extract and 2% (m/v) agar, at 27°C for 7 days. Then, the inoculum was prepared in 2 L Erlenmeyer flasks containing 200 mL of liquid medium composed by 2.4% (m/v) potato/dextrose extract and 0.7% (m/v) yeast extract; sterilized at 121°C for 15 min, and inoculated with 20 pellets (8 mm diameter) taken from a recently activated culture. After 12 days of static incubation at 27°C, the grown mycelium was recovered by filtration, washed (300 mL sterile water), and macerated (100 mL sterile water) using an aluminum blender. An aliquot of 20 mL was taken from this suspension and used to determine the dry mass of mycelium contained in the

suspension, by oven-drying at 105°C until constant mass. Based on this determination, the volume of suspension needed to inoculate each cultivation flask with an initial concentration of 500 mg of mycelium per liter of medium was determined.

Basal medium

The basal medium (Ruttiman-Johnson et al., 1993) was composed, per liter of solution, by: 10.0 g glucose, 10.0 mmol ammonium tartrate, 10.0 mmol trans-aconitic acid, 2.0 g KH_2PO_4 , 0.5 g $MgSO_4 \times 7 H_2O$, 0.1 g $CaCl_2 \times 2 H_2O$, 1.0 mg thiamine chlorhydrate, and 7.0 mL of a solution of trace elements. The solution of trace elements was composed, per liter of solution, by: 15.0 g nitrilotriacetic acid, 1.0 g $FeSO_4 \times 7H_2O$, 1.8 g $CoCl \times 6H_2O$, 1.0 g $ZnSO_4 \times 7H_2O$, 0.07 g $Al_2(SO_4)_3 \times 18 H_2O$, 1.0 g $CuSO_4 \times 5 H_2O$, 0.1 g H_3BO_3 , 0.1 g $NaMoO_4 \times 2 H_2O$, 30.0 g $MgSO_4 \times 7 H_2O$, 10.0 g NaCl, 0.82 g $CaCl_2$. The component solutions were autoclaved separately, at 121°C for 15 min, prior to the formulation of the culture medium; with exception of the thiamine chlorhydrate solution, which was sterilized by filtration (syringe filter, membrane with 22 μm).

Cultivation media and conditions

The cultivation of the immobilized cells was performed as follows: 12 cubes of 1.5 cm³ of polyurethane foam, previously washed, dried and weighted, were added in 125 mL Erlenmeyer flasks. The flasks were autoclaved at 121°C for 15 min. After cooling, 30 mL of basal medium and 15 mg (dry basis) of homogenized mycelium were added in each flask. The cultures were incubated statically at 27°C for 30 days, with samples (1 flask = 1 sample) taken every three days. Four sets of cultivations were performed: In the first, the concentration of manganese ions (Mn^{2+}) was adjusted to 0, 11 or 40 ppm of Mn^{2+} ; added or not as $MnSO_4 \times H_2O$. In the second, the medium containing 11 ppm of Mn^{2+} was supplemented with either 0.5 or 1.0 mM of 2,5-xylydine. In the third, the medium containing 11 ppm of Mn^{2+} was supplemented or not with either 1.0 or 2.0 mM of veratryl alcohol. In the last, the medium containing 11 ppm of Mn^{2+} and 1.0 mM of 2,5-xylydine was supplemented or not with either 0.05 or 0.50% (v/v) of Tween 80. The additives were sterilized by filtration (syringe filter, membrane with 22 μm) and added into the culture medium at the beginning of the cultivations, prior to inoculation; with exception of the manganese sulfate, which was added or not in the solution of trace elements. The cultivation using the basal medium containing 11 ppm Mn^{2+} was carried out in sextuplicates; the others, in duplicates.

Recovery of mycelium and determination of biomass concentration

The mycelium was recovered by filtration. The content of each Erlenmeyer flask was quantitatively transferred into a stainless steel funnel adapted to a glass filter (AP40, Millipore), previously dried and weighted, and coupled to a Kitasato flask maintained under vacuum. The material retained in the filter (mycelia and sponge cubes) was washed with 30 mL of distilled water, transferred into weighing bottles, and oven-dried at 105°C until constant mass; which was used to determine biomass concentrations, after discounting the foam dry weights.

Determination of glucose concentration

The concentration of glucose in the samples was determined by using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). In a

Table 1. Maximum activities of manganese peroxidase and laccase (average \pm standard deviation) determined during the cultivations performed in the different media.

Cultivation medium	MnP (U/L)	Lac (U/L)
Mn 0 ppm	37.0 \pm 2.8 ^a ($p < 0.20$)	15.5 \pm 2.1 ^a ($p < 0.05$)
Mn 11 ppm	108.0 \pm 43.3 ^b	5.3 \pm 2.9 ^b
Mn 40 ppm	55.5 \pm 26.2 ^{a,b}	7.0 \pm 1.4 ^b
Mn 11 ppm	108.0 \pm 43.3 ^a ($p < 0.20$)	5.3 \pm 2.9 ^a ($p < 0.05$)
Mn 11 ppm + X 0.5 mM	91.0 \pm 15.6 ^a	14.0 \pm 1.4 ^b
Mn 11 ppm + X 1.0 mM	99.5 \pm 19.1 ^a	21.5 \pm 4.9 ^b
Mn 11 ppm	108.0 \pm 43.3 ^a ($p < 0.20$)	5.3 \pm 2.9 ^a ($p < 0.20$)
Mn 11 ppm + VA 1.0 mM	147.5 \pm 60.1 ^a	4.0 \pm 0.0 ^a
Mn 11 ppm + VA 2.0 mM	100.0 \pm 19.8 ^a	8.5 \pm 2.1 ^a
Mn 11 ppm + X 1.0 mM	99.5 \pm 19.1 ^a ($p < 0.20$)	21.5 \pm 4.9 ^a ($p < 0.05$)
Mn 11 ppm + X 1.0 mM + T80 0.05% v/v	174.8 \pm 1.4 ^b	53.3 \pm 17.7 ^b
Mn 11 ppm + X 1.0 mM + T80 0.50% v/v	181.8 \pm 23.3 ^b	60.6 \pm 5.7 ^b

For each set of cultivations, maximum enzyme activities denoted with different superscript letters represent statistically different values.

tube, 0.5 mL of sample, 1.0 mL of deionized water and 3.0 mL of DNS were added. After heating the mixture at 100°C for 5 min in a water bath, and cooling, 20 mL of deionized water was added. After mixing, the absorbance at 540 nm was measured. The value was converted in glucose concentration, by using an appropriate calibration curve.

Determination of ammonium concentration

The concentration of ammonium in the samples was determined by using the phenol-hypochlorite method (Weatherburn, 1967). Two reagent solutions were prepared before each analysis: Solution A, composed by 5.0 g of phenol and 25.0 mg of sodium nitroprusside, dissolved in 500 mL of deionized water, and Solution B, composed by 2.5 g of NaOH and 4.2 mL of sodium hypochlorite (5% active chlorine), dissolved in 500 mL of deionized water. In a tube, 20 μ L of sample, 5.0 mL of solution A and 5.0 mL of solution B were added. The mixture was homogenized and left under room temperature for 30 min, then, had its absorbance at 625 nm determined. The value was converted in ammonium concentration, by using a calibration curve prepared with ammonium sulfate.

Determination of protein concentration

The concentration of extracellular proteins in the samples was determined by using the Bradford's micro-method (Bradford, 1976). In a tube, 0.1 mL of sample and 1.0 mL of Bradford's reagent were added. After homogenization, the mixture was kept under room temperature for 15 min. Afterwards, the absorbance at 595 nm was measured. The value was converted in protein concentration, by using a calibration curve prepared with bovine serum albumin.

Determination of enzyme activities

The activity of Lac was determined using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] as substrate. The oxidation reaction was carried out in 0.3 mL of 50 mM citrate-phosphate buffer pH 5.0, 0.1 mL of deionized water, 0.5 mL of sample and 0.1 mL of 1 mM ABTS. The substrate oxidation was monitored at 420 nm, considering the value of 36,000 M⁻¹ cm⁻¹ (Bourbonnais and Paice, 1990) as the absorptivity of the oxidized product.

The activity of MnP was determined using phenol red as substrate. The reaction mixture was composed by 1.5 mL of sodium succinate buffer (50 mM, pH 3.2), 1.5 mL of 50 mM sodium lactate, 0.5 mL of 0.1% phenol red, 0.5 mL of 1 mM manganese sulfate, 0.25 mL of 1.8% bovine albumin, 0.25 mL of 2 mM hydrogen peroxide, and 0.5 mL of sample. After the beginning of the reaction, fractions of 1.0 mL were removed from the tube containing the reaction mixture every 1 min and transferred into cuvettes containing 65 μ L of 6.5 M sodium hydroxide, prior to reading the absorbance at 610 nm. The value of 22,000 M⁻¹ cm⁻¹ (Khindaria et al., 1994) was considered as the absorptivity of the oxidized product.

Determination of pH and conductivity

The pH was determined by using a pH meter; the conductivity, by using a conductivimeter. The equipments were calibrated before the determinations.

Statistical analysis

One way analysis of variance was utilized for multiple sample comparisons with respect to maximum enzyme activities determined during each set of cultivations. The mean values were then compared to each other, by means of Tukey's or Kruskal-Wallis's tests, in order to identify statistically significant differences.

RESULTS AND DISCUSSION

Effects of the manganese concentration on the productions of MnP and Lac

In the first set of experiments, the effect of manganese concentration on the production of MnP and Lac was evaluated by varying the concentration of Mn²⁺ ions in the culture medium, from 0 to 40 ppm. As can be seen in Table 1, the medium absent in Mn²⁺ promoted a maximum MnP activity of 37.0 \pm 2.8 U/L. As shown in

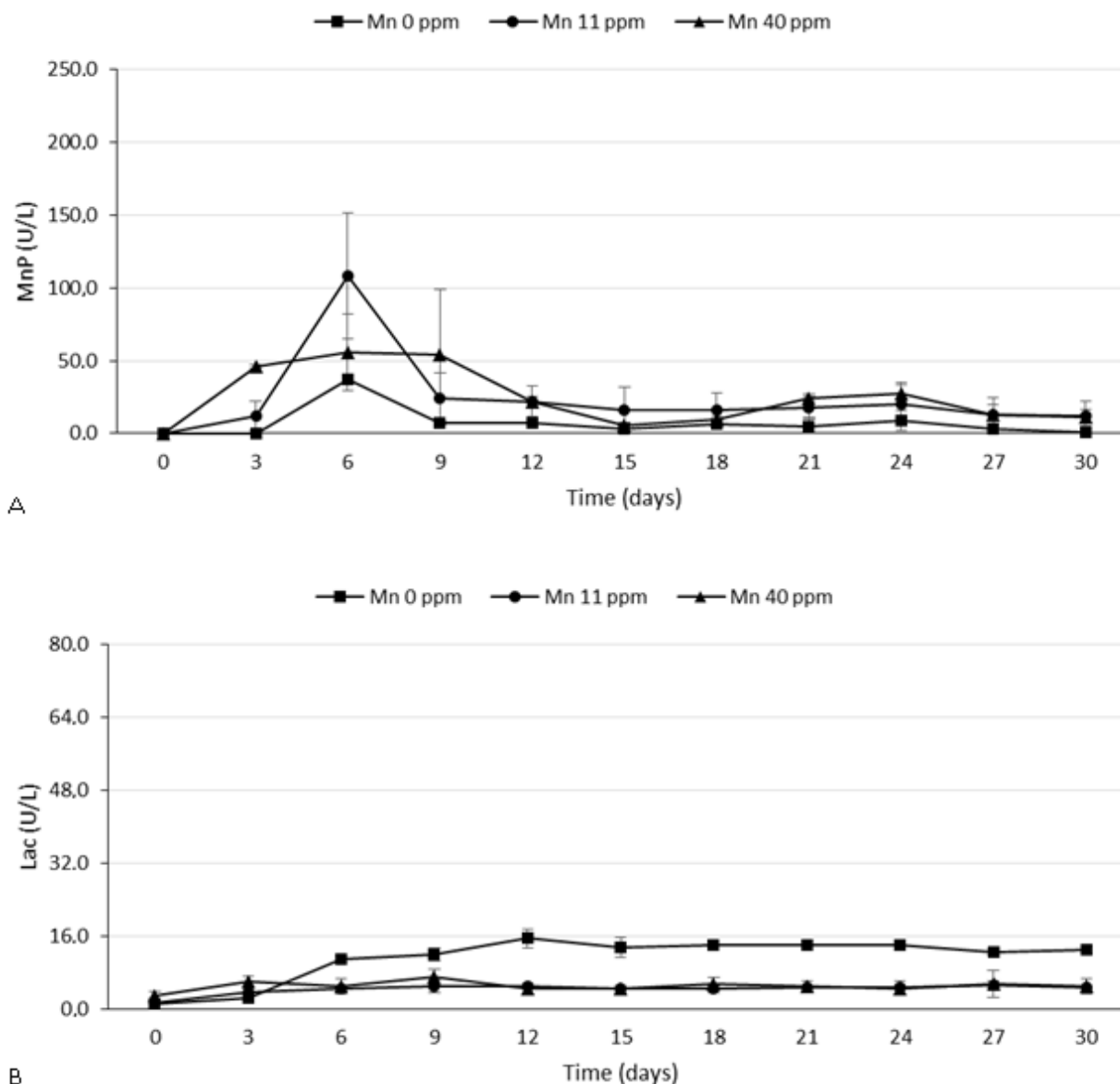


Figure 1. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of Mn^{2+} . Error bars represent standard deviations, calculated from sextuplicates.

Figure 1, this peak of activity was determined in the 6th day of cultivation; a decrease of activity being observed thereafter. On the other hand, two peaks of MnP activity, 55.5 ± 26.2 and 27.5 ± 7.8 U/L, in the 6th and 24th days of cultivation, respectively, were observed when supplementing the medium with 40 ppm of Mn^{2+} . As presented in Table 1, the statistical analysis of the data indicated that the supplementation of the medium with Mn^{2+} led to higher maximum MnP activities.

Ruttimann et al. (1992) reported that the addition of 11 ppm Mn^{2+} into the culture medium led to the highest production of MnP in submerged cultures of *C. subvermispora*. Manubens et al. (2003), in a further

study, demonstrated that the addition of manganese into the medium not only affected the levels of transcription of *mnp* genes, but also was essential for the detection of extracellular MnP activity. Later, Gutierrez et al. (2008) proposed the existence of a robust homeostatic machinery to deal with the regulation of Mn^{2+} metabolism in this fungus; although upregulation in the expression of *mnp* genes had not been observed after the submerged cultivations in media containing from 0 to 5 mM Mn^{2+} .

Table 1 also shows that the media supplemented with Mn^{2+} promoted maximum Lac activities of 5.3 ± 2.9 (11 ppm) and 7.0 ± 1.4 U/L (40 ppm). The cultivation not supplemented with $MnSO_4$, however, led to the highest

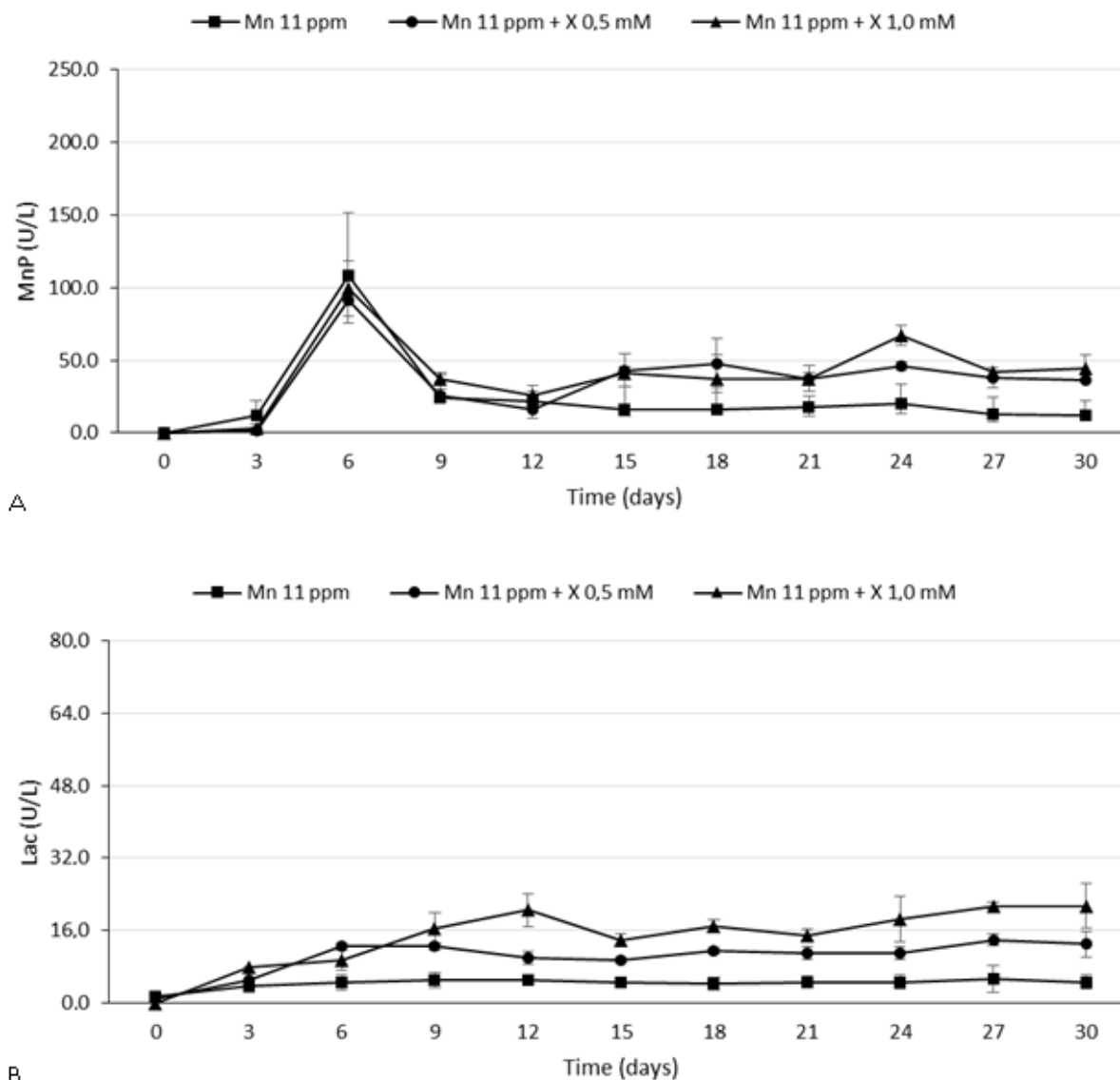


Figure 2. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of 2,5-xylydine, in presence of Mn^{2+} (11 ppm). Error bars represent standard deviations, calculated from duplicates.

maximum activity (15.5 ± 2.1 U/L); in the 12th day of cultivation (Figure 1).

Daina et al. (2002), studying the degradation of β -5 lignin model dimers by *C. subvermisporea*, however, did not observe variation in Lac titers when the fungus was grown in the absence or presence of 0.2 mM Mn^{2+} ; although the production of MnP had been stimulated in the presence of this metal.

Effects of the addition of inducers on the productions of MnP and Lac

To evaluate a possible stimulation in the production of

Lac and MnP, *C. subvermisporea* was grown in the presence of 2,5-xylydine (0.5 and 1.0 mM) or veratryl alcohol (1.0 and 2.0 mM), using the basal medium supplemented with 11 ppm Mn^{2+} . The maximum activities of MnP and Lac are presented in Table 1; the time profiles of enzyme activities are shown in Figures 2 and 3.

As can be seen in Table 1, the maximum activity of MnP was not favored by the addition of veratryl alcohol nor by the addition of 2,5-xylydine, independently of the concentrations added into the medium. In spite of this, second peaks of MnP activity were apparently observed in the presence, but not in the absence, of the inducers. In the presence of 1.0 mM 2,5-xylydine, a MnP activity of

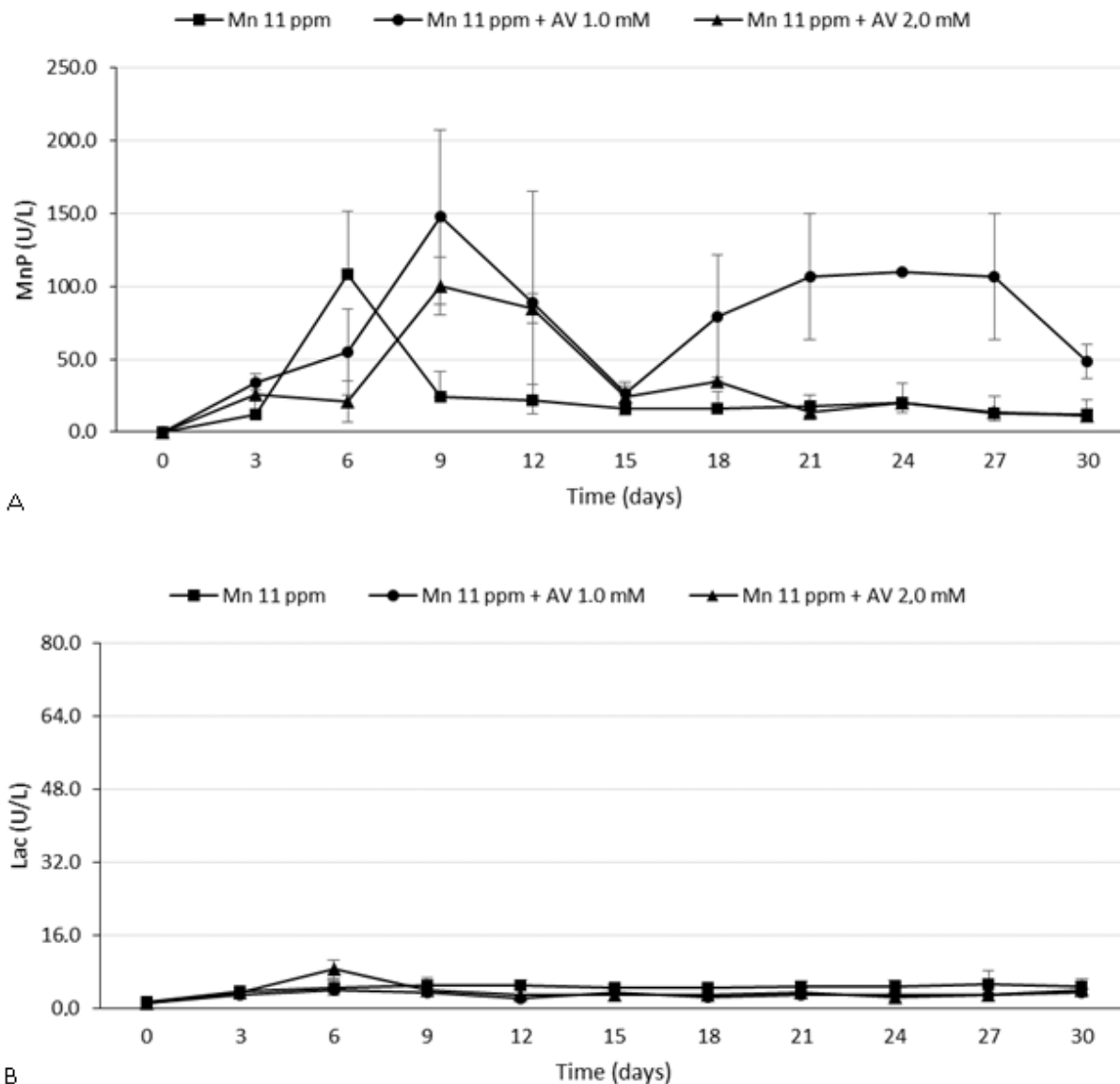


Figure 3. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of veratryl alcohol, in presence of Mn^{2+} (11 ppm). Error bars represent standard deviations, calculated from duplicates.

67.0 ± 7.1 U/L was determined in the 24th day of cultivation, compared to 99.5 ± 19.1 U/L (6th day of cultivation) (Figure 2). In the presence of 1.0 mM veratryl alcohol, a MnP activity of 110.0 ± 1.4 U/L was determined in the 24th day of cultivation, compared to 147.5 ± 60.1 U/L (9th day of cultivation) (Figure 3).

With regard to Lac production, the addition of veratryl alcohol into the cultivation medium did not significantly improve the maximum activity of this enzyme. The supplementation with increasing concentrations of 2,5-xylidine, on the other hand, did; from 5.3 ± 2.9 U/L, in the medium without inducer, to 21.5 ± 4.9 U/L, in the medium supplemented with 1.0 mM of inducer (Table 1).

Such results confirm the inductive effect of 2,5-xylidine in stimulating the production of Lac by different fungi. For instance: *C. subvermispora* (Fukushima and Kirk, 1995), *Dichomitus squalens* (Perie et al., 1998), *Panus tigrinus* (Quarantino et al., 2008), *Pleurotus dryinus* (Elisashvili et al., 2006) and *T. versicolor* (Rancaño et al., 2003). According to Eggert et al. (1996), one of the functions of Lac is the detoxification of aromatic compounds that are highly reactive, promoting the formation of free radicals and their concomitant polymerization; being postulated that the induction of Lac by 2,5-xylidine is due to a mechanism of defense developed by fungi to eliminate the toxic effects of this compound.

Effects of the addition of surfactant on the productions of MnP and Lac

Tween 80 is a surfactant that favors the excretion of extracellular enzymes by filamentous fungi. In order to evaluate the effect of this compound in the production of MnP and Lac, cultivations of *C. subvermispora* in medium supplemented with 11 ppm of Mn^{2+} and 1.0 mM of 2,5-xylidine, in the presence (0.05 or 0.50% v/v) or absence of Tween 80, were performed.

As can be seen in Table 1, the supplementation of the medium with Tween 80, in both concentrations, significantly improved the activities of both MnP and Lac. The maximum activities of MnP determined in the cultures supplemented with 0.05 and 0.50% (v/v) of Tween 80 were 174.8 ± 1.4 and 181.8 ± 23.3 U/L, respectively, compared to 99.5 ± 19.1 U/L (medium not supplemented with surfactant). The maximum activities of Lac determined in the cultures supplemented with 0.05 and 0.50% (v/v) of Tween 80, on the other hand, were 53.3 ± 17.7 and 60.6 ± 5.7 U/L, respectively, compared to 21.5 ± 4.9 U/L (medium not supplemented with surfactant).

While, in the medium supplemented with 11 ppm Mn^{2+} and 1.0 mM 2,5-xylidine, the activity of MnP was relatively small and unstable, decreasing sharply after the 6th day of cultivation, in the media supplemented with Tween 80, the activities of MnP reached considerably higher values that were maintained for relatively longer periods. Qualitatively, the same behavior was observed for Lac activities (Figure 4).

Couto et al. (2001), for example, also reported that the supplementation of a defined medium with 0.05% (v/v) Tween 80, in addition to 2 mM veratryl alcohol, improved the production of extracellular ligninolytic enzymes by immobilized *Phanerochaete chrysosporium*.

Metabolic behavior exhibited by the fungus during selected cultivations

As can be seen in Figure 5, in all the three selected cultivations, namely in the media supplemented with 11 ppm Mn^{2+} , with 11 ppm Mn^{2+} and 1.0 mM 2,5 xylidine, and with 11 ppm Mn^{2+} , 1.0 mM 2,5 xylidine and 0.05% v/v Tween 80, the fungus completely consumed the available glucose in the first 6 to 9 days of cultivation. Although there was considerable variation in the measurement of biomass concentrations, the growth of mycelium appears to have occurred mainly during this time, together with the consumption of ammonium ions. Afterwards, increases in the concentrations of ammonium were detected in all the three cultivations, coinciding with increases in the conductivities and pHs of the culture media.

From the above mentioned results, it can be inferred that the exhaustion of glucose in the medium promoted

nutritional stress, which, in turn, led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium in the medium. According to White et al. (2002), the phenomenon of cell autolysis is frequent during cultivations of filamentous fungi. Bainbridge et al. (1971), for example, reported the occurrence of cell autolysis, simultaneous to an increase in the concentration of ammonium in the medium, when continuous cultures of *Aspergillus nidulans* were submitted to limitation in the carbon source; but not when the fungus was grown in excess of this nutrient.

According to Eden and Eden (1984), the conductivity of fermentation media is related to the production and consumption of electrolytes due to the microbial metabolism. Colombie et al. (2007), for example, observed that the assimilation of ammoniacal nitrogen during cultivations of a wine making yeast led to the decrease in the conductivity of the fermentation medium. In the present study, as already mentioned, the initial decreases in the conductivities (and also in the pHs) of the fermentation media were followed by a period of increases in these parameters; which is compatible with an initial consumption of the ammonium available in the media, followed by the occurrence of cell autolysis due to glucose limitation.

It is worth to mention, as well, that the basal medium used in the present study was buffered, by the addition of trans-aconitic acid. This, however, did not prevent variations in the pH of the medium during the cultivations.

Last, it was observed that the concentrations of extracellular proteins increased throughout the cultivations (Figure 5). Maximum contents of 137.4 ± 12.2 , 89.2 ± 13.3 and 52.9 ± 31.1 mg/L were achieved for the media supplemented with Tween 80, 2,5-xylidine and manganese, respectively. Such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

No activities of lignin peroxidase (LiP) and cellobiose dehydrogenase (CDH) were detected during the cultivations (data not shown); in spite of the existence of studies reporting the production of LiP (Tanaka et al., 2009) and CDH (Harreither et al., 2009) by *C. subvermispora*. Moreover, it was not possible to establish a correlation between the concentrations of extracellular proteins determined by Bradford's method with those determined by direct light absorption at 280 nm (data not shown); probably due to the presence of interfering compounds (Zaia et al., 1998). Similarly, it was not possible to establish a correlation between the absorbances determined at 405 and 610 nm with the respective activities of MnP and Lac (data not shown); in spite of the existence of studies reporting the use of such spectral analyses in both qualitative and quantitative characterizations (Rubia et al., 2002; Cambria et al., 2000).

The ability of accumulating considerable amounts of

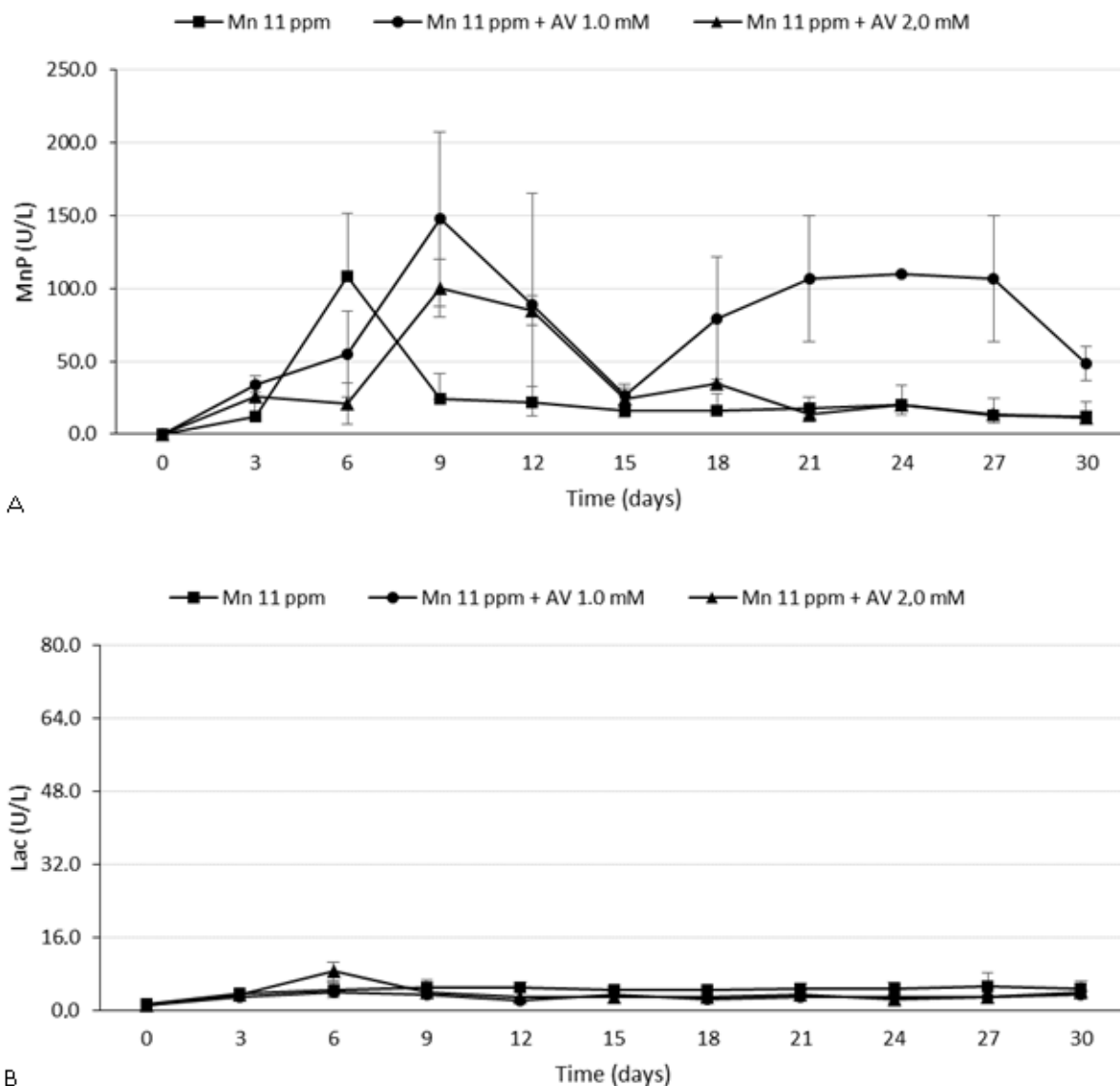


Figure 4. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of Tween 80, in presence of Mn^{2+} (11 ppm) and 2,5-xylydine (1,0 mM). Error bars represent standard deviations, calculated from duplicates.

extracellular proteins, exhibited by ligninolytic fungi, is well reported in the literature. Galhaup et al. (2002), for example, reported that the concentration of extracellular proteins reached 700 mg/L at the end of a fed-batch cultivation of *Trametes pubescens* in a reactor of 20 L. The medium used for the submerged cultivation was composed by glucose (40 g/L), meat peptone (10 g/L), $MgSO_4 \cdot H_2O$ (1 g/L) and $CuSO_4 \cdot 5 H_2O$ (2 mM); the feed, by a solution of glucose (320 g/L).

Conclusions

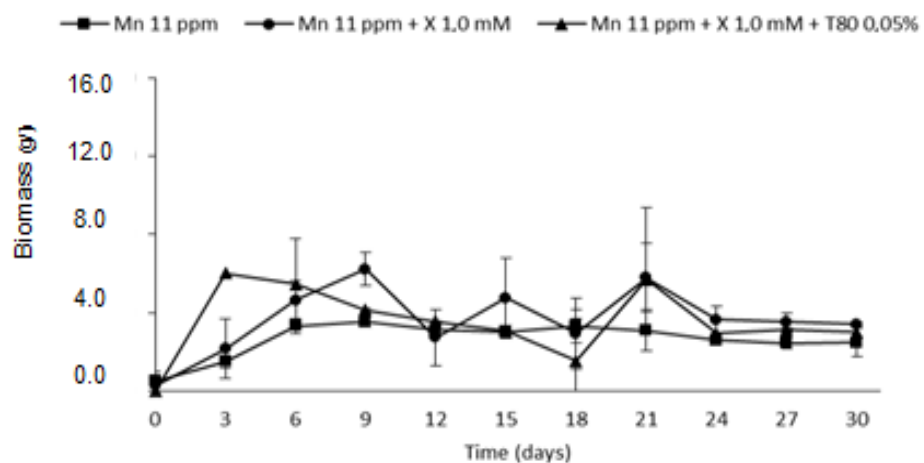
While the supplementation of the basal medium with

manganese stimulated the production of MnP, the suppression of this metal in the medium led to a higher production of Lac.

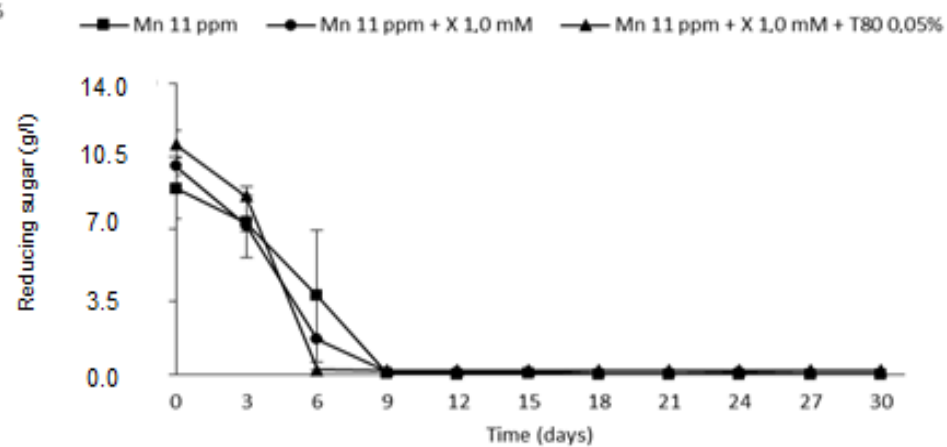
The supplementation of the medium containing 11 ppm Mn^{2+} with 1.0 mM 2,5-xylydine stimulated a higher production of Lac; the supplementation of the same medium with 1.0 mM veratryl alcohol, generated a second peak of MnP activity.

Tween 80 improved the activities of both MnP and Lac determined during the cultivations; the maximum values were significantly higher than that determined in the medium without surfactant, and were also maintained for relatively longer periods.

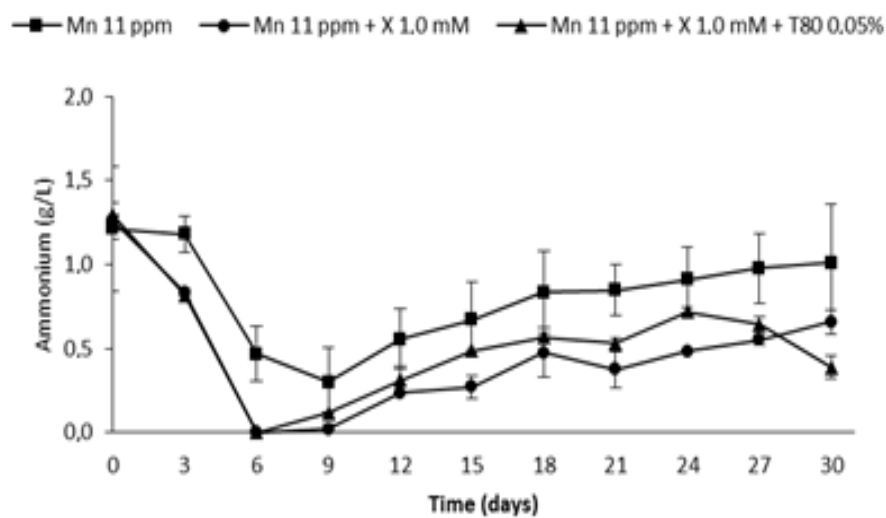
The exhaustion of the glucose available in the culture



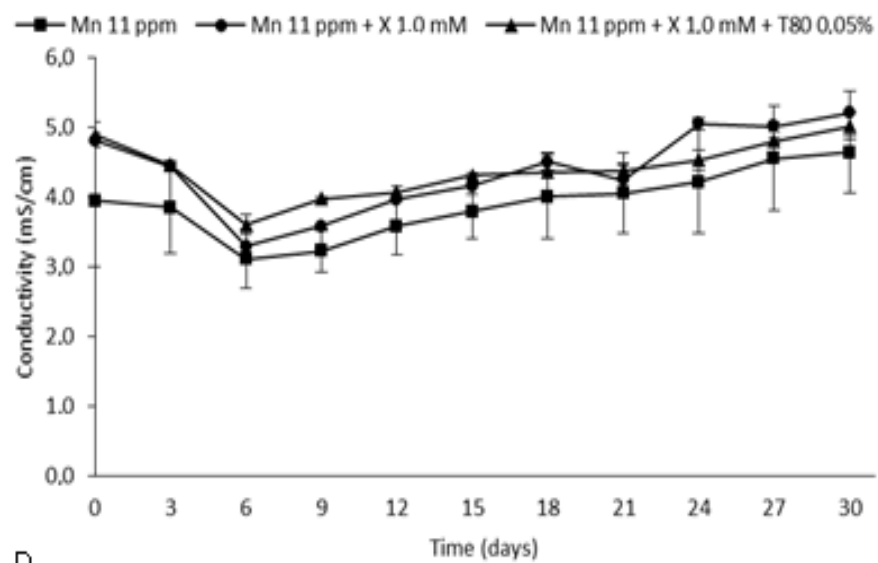
A



B



C



D

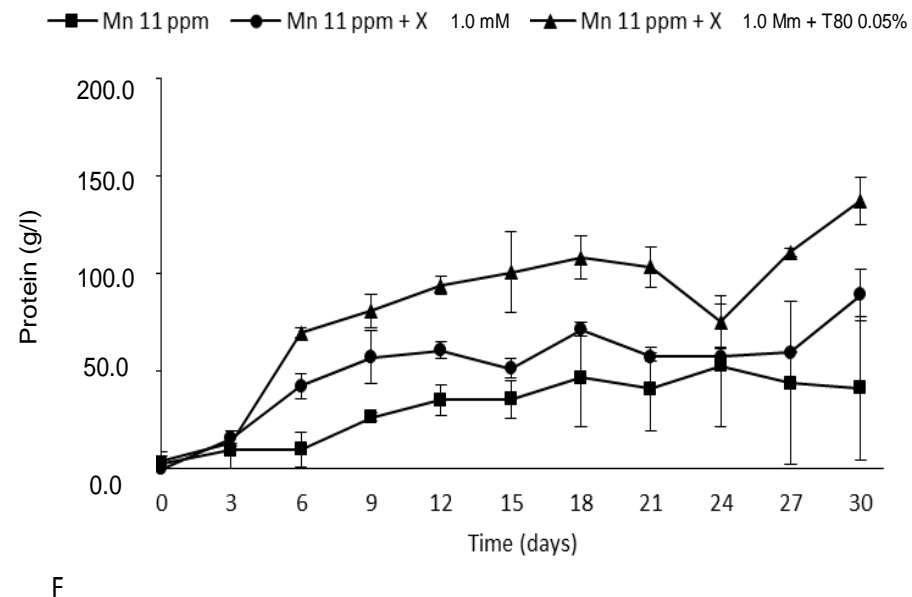
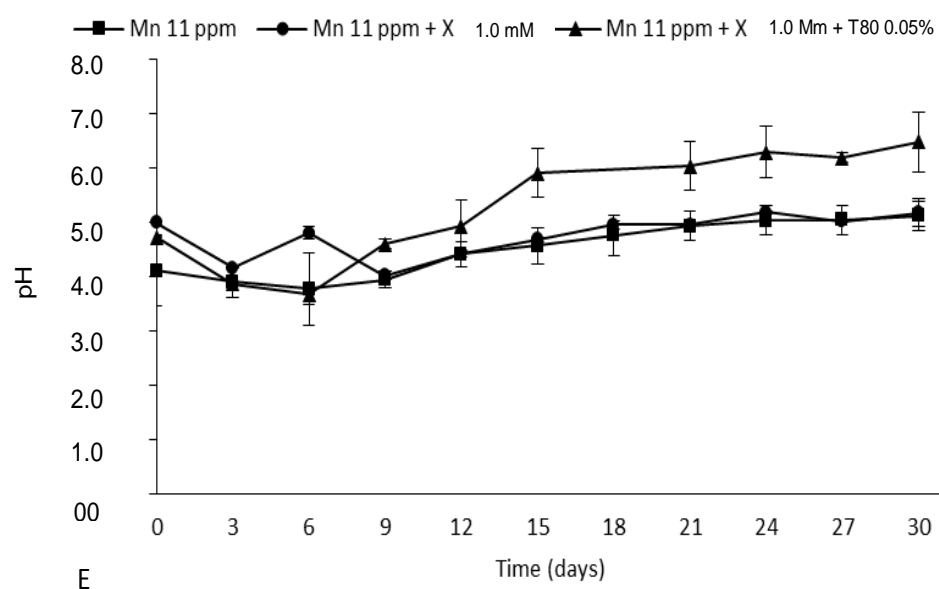


Figure 5. Concentrations of biomass (A), reducing sugars (B), ammonium ions (C) and proteins (F), and values of conductivity (D) and pH (E) determined during the cultivations in selected media. Error bars represent standard deviations, as described in Figures 1, 2 and 4. Mn – Mn²⁺; X – 2,5-xylidine; T80 – Tween 80.

medium led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium in the medium.

The concentrations of extracellular proteins increased throughout the cultivations; such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

Conflict of interests

The authors did not declare any conflict of interest.

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