African Journal of Biotechnology

Volume 15 Number 11, 16 March 2016

ISSN 1684-5315

Academic Journals

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Table of Content: Volume 15 Number 11 16 March, 2016

ARTICLES

African Journal of Biotechnology

Table of Content: Volume 15 Number 11 16 March, 2016

ARTICLES

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Vol. 15(11), pp. 376-383, 16 March, 2016
DOI: 10.5897/AJB2014.14307
Article Number: 4ECDE9A57526
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Glyoxalase I expression pattern in *Hevea brasiliensis* seedlings under varied stress conditions

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Received 7 November, 2014; Accepted 2 December, 2015

Drought is one of the most important stress factors which adversely affect plants' growth and productivity. Global climate change may make this situation more serious in the years ahead. Considering the long time span required for the generation of drought resistant genotypes in Rubber (*Hevea brasiliensis*) through conventional breeding, molecular interventions to engineer plants to have either drought responsive genes or genes expected to alter osmotic regulation would be very attractive. The glyoxalase pathway involving glyoxalase I and glyoxalase II enzymes is required for glutathione-based detoxification of methylglyoxal. In this study the effects of various abiotic stresses on the up-regulation of methylglyoxal levels and glyoxalase I activities in *Hevea brasiliensis* seedlings were investigated. Most of the stresses caused significant increase in methylglyoxal level and glyoxalase I activity, among which drought caused the highest induction of glx I followed by salinity, 2, 4-D, ABA, methylglyoxal, white light and $CdCl_2$. The stress-induced increases in methylglyoxal and glyoxalase I activity found in the present study suggest an important role of glyoxalase I in conferring drought tolerance. The up-regulation of glyoxalase I under drought stress indicates its future utility in developing tolerance to drought stress in *Hevea brasiliensis*. In the present study, a partial cDNA sequence coding for glyoxalase I was amplified by PCR using specific primers. The 440 bp cDNA amplicon obtained was sequenced and subjected to online BLAST analysis. The sequence of *Hevea brasiliensis* glyoxalase I (GenBank Acc. No: GU598520) had six open reading frames. The ORF finder revealed the longest ORF of 336 bp. Glyoxalase I from *Ricinus communis* had the highest nucleotide sequence homology (90%) compared to the amplified gene. BLASTP analysis also showed high homology between the deduced protein sequence of the amplified gene and glyoxalases from other species. Our results suggest that the multi-stress inducibility of glyoxalase I in the present study may be due to the fact that it might protect the plants against MG that is formed under various stresses including drought and confers tolerance by increasing the GSH-based detoxification system and decreasing lipid peroxidation

Key words: Glyoxalase I, *Hevea brasiliensis*, Methylglyoxal, drought tolerance, abiotic stress.

INTRODUCTION

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. *Hevea brasiliensis*, the Para rubber tree accounts for more than 99% of the world's natural rubber production. In recent

years, the global consumption of natural rubber is steadily increasing and the production has to be increased so as to meet the demand. Many of the recently developed high yielding *Hevea brasiliensis* clones including RRII 105 are susceptible to drought. One of the most severe environmental stresses, drought, is a major constraint for plant productivity worldwide. Thus, it is among the worst scourges of agriculture. The acclimation of plants to drought is often associated with increased levels of reactive oxygen species (ROS) such as superoxide radical. ROS attack the most sensitive biological macromolecules in cells and impair their functions. If drought stress is prolonged to a certain extent, ROS production will overwhelm the scavenging action of the antioxidant system, which results in extensive cellular damage and death.

The glyoxalase system comprises the enzymes glyoxalase-I (Gly I; lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase-II (Gly II; hydroxyacylglutathione hydrolase; EC 4.4.1.5). The two enzymes act co-coordinately to convert a variety of toxic-2-oxoaldehydes into less reactive 2-hyroxy acids, utilizing glutathione (GSH) as a cofactor. Methylglyoxal appears to be the primary physiological substrate for the glyoxalase system. Methylglyoxal is a potent cytotoxin found in all organisms, which is formed primarily as a byproduct of carbohydrate and lipid metabolism. Glyoxalase-I catalyzes the formation of S-D-lactoylglutathione (S-LG) from MG and GSH. S-LG is further metabolized to D-lactate and GSH by glyoxalase-II. A high level of MG accumulation is toxic to cells, since it inhibits cell proliferation and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system. Apart from MG, pathway intermediate S-LG (substrate for glyoxalase II) has also been found to be cytotoxic at high concentrations in that it inhibits DNA synthesis (Thornalley et al., 1996). The genes encoding glyoxalase I and glyoxalase II have been isolated and characterized from microbial and animal systems and found to have significant protein sequence homology (Rhee et al., 1987; Lu et al., 1990; Ray et al., 2001; Ranganathan et al., 1993).

Glyoxalase I activity has been studied in several higher plant species, and in some cases the enzyme has been further characterized (Deswal et al., 1993; Paulus et al., 1993; Umeda et al., 1994). In tomato (*Lycopersicon esculentum*), an 848-bp cDNA clone was identified by differential screening of salt induced genes, and glyoxalase I activity was confirmed by expression in yeast (Gody et al., 1990; Espartero et al., 1995). Using a similar approach in the resurrection grass *Sporobolus staphianus*, a 1.2-kb cDNA clone was found in desiccated plants (Blomstedt et al., 1998). In addition, a cDNA clone encoding a 186-residue short glyoxalase I has been isolated from epicotyls of *Cicer arietinum* grown under osmotic stress conditions (Romo et al., 1998), but glyoxalase I cDNA from *Brassica juncea* was cloned and found to confer resistance to stress when expressed in *E.coli* and tobacco. Over-expression of glyoxalase I resulted not only in improved tolerance against MG, but interestingly, the transgenic plants tolerated higher levels of salinity as compared with the non-transgenic plants. Over-expression of glyoxalase I and glyoxalase II together conferred improved salinity tolerance, thus offering another effective strategy for manipulating stress tolerance in crop plants. Recent investigations in plants have brought new developments in the involvement of the glyoxalase system in stress tolerance and its involvement with oxidative defense systems (Hossain et al., 2009; Yadav et al., 2005; Bhomkar et al., 2008). Glyoxalase I has also been found to be one of the several genes induced in response to drought and cold stresses in *Arabidopsis* (Seki et al., 2001).

The present study investigates the methylglyoxal levels and glyoxalase I activity under different abiotic stresses in *Hevea brasiliensis,* and provides sequence information on glyoxalase I from rubber.

MATERIALS AND METHODS

Plant material

Young, healthy, 20 days old seedlings of *Hevea brasiliensis* clone RRII 105 were used for various stress treatments. Before use, the seedlings were removed from soil and thoroughly washed with deionized water.

Stress treatments

The seedlings were subjected to different stress treatments for the estimation of methylglyoxal and Glyoxalase I levels. For drought stress treatment, seedlings were placed in a pot without water and kept at 25°C. Seedlings were placed in 20 ml of 300 mM NaCl solution for salt stress. 1 mM $CdCl_2$ solution was also used as heavy metal stress. To study the effect of white light, seedlings were placed in a beaker containing 20 ml of distilled water and exposed to white light (60 µmol photon $m^{-2}s^{-1}$) and illuminated for 18 and 24 h at 25°C. To observe hormonal effects on *Hevea brasiliensis* glyoxalase I, 50 µM ABA and 50 µM 2, 4-D solutions were used. Four seedlings were used in each treatment and were incubated for 24 h in the dark. 25 mM MG solution was used for chemical stress. Seedlings incubated in 20 ml of distilled water in the dark at 25°C were used as controls.

Sample preparation for MG estimation

Methylglyoxal was estimated according to the method of Hossain et

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al., (2009). In brief about 0.5 g hypocotyl tissue was homogenized in 3 ml of 0.5 M perchloric acid. After incubating 15 min on ice, the mixture was centrifuged at 4°C for 10 min at 11,000 g. The supernatant was decolorized by adding charcoal (10 mg/ml), kept for 15 min at room temperature, and centrifuged at 11,000 g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000 g for 10 min. The neutralized supernatant was used for MG estimation.

Methylglyoxal assay

Methylglyoxal assay was carried out according to the method of yadav et al. (2005). In a total volume of 1 ml, 250 µl of 7.2 mM 1, 2-diaminobenzene, 100 µl of 5 M perchloric acid, and 650 µl of the neutralized supernatant were added in that order. The absorbance at the 335 nm of the derivatized MG was read after 25 min in a spectrophotometer. The final concentration of the MG was calculated from the standard curve and expressed in terms of µg/ml.

Preparation of crude enzyme solution

The cotyledon and roots were removed from the stress treated seedlings, and hypocotyls were homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10% (w/v) glycerol. The homogenates were centrifuged at 11, 500 g for 10 min and the supernatant was used as a crude enzyme solution. For glyoxalase I assay, proteins were precipitated by ammonium sulphate at 65% saturation from the crude enzyme solution and centrifuged at 11,500 g for 10 min. The precipitate was dissolved in a minimum volume of buffer and transferred to a dialyzed membrane, dialyzed against 10 mM potassium phosphate buffer (pH 7.0) overnight, and then used for glyoxalase I assay.

Glyoxalase I enzyme assay

Glyoxalase I assay was carried out according to Hossain et al. (2009). Briefly, the assay mixture contained 100 Mm potassium phosphate buffer (pH 7.0), 15 Mm magnesium sulphate, 1.7 Mm reduced glutathione and 3.5 Mm methylglyoxal in a final volume of 0.8 ml. The reaction was started by the addition of MG and the formation of thioester was measured by observing the increase of absorbance at 240 nm for 1 min in a spectrophotometer. Concentration of Glyoxalase I was calculated from the standard curve and expressed in µg/ml.

Statistical analysis

All data obtained was subjected to one-way analysis of variance (ANOVA) and the significance of difference between the mean values was compared by Duncan's multiple range tests using MSTAT-C. Differences at $P \leq 0.05$ were considered significant

RNA isolation

Total RNA was isolated from leaves by LiCl precipitation method (Sambrook et al., 1989). Around 200 mg of leaf tissue was ground to a fine powder in liquid nitrogen and 2 ml of extraction buffer (0.2 M NaCl, 0.1M Tris-HCl, pH 8.5, 0.01M EDTA, pH 8.0, 1.5% SDS, 0.1% 2-mercaptoethanol and 1% insoluble PVPP) was added. Following extraction with an equal volume of extraction buffer-saturated phenol (centrifugation for 15 min at 10,000 g), the aqueous phase was transferred and re-extracted twice with equal volumes of chloroform. The RNA was precipitated overnight in 1/3 volume of 8M LiCl at -20°C. The precipitated RNA was recovered by centrifugation for 10 min at 10,000 g, washed with ice-cold 2M LiCl and dissolved in 250 µl sterile RNase free water. The RNA was then re-precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volums of ethanol at -20°C. Following centrifugation for 10 min at 10,000 g, the pellet was re-suspended in 100 µl RNase free water. Ten microgram (10 µg) of Total RNA was resolved in 1% formaldehyde agarose gel.

cDNA synthesis

First strand cDNA was synthesized from total RNA by reverse transcription with oligo (dT) primer using the kit Super Script III first-strand synthesis system for RT-PCR (Invitrogen U.S.A) according to the manufacturer's protocol. 2 µl of the first strand cDNA was used to amplify the glyoxalase I sequences.

PCR amplification of glyoxalase I

The sequences coding for glyoxalase I in *Arabidopsis thaliana*, *Cicer arietinum*, *Lycopersicon esculentum* and *Avicenia marina* were compared and consensus sequences were identified using 'megalign' programme of lasergene software (DNASTAR, USA). One set of specific primers 5'GATGAAGCAACTAAAGGTTA3' (forward) and 5'CCAATAGCCATCAGGATCTT3' (reverse) were used. The PCR reactions were carried out in 20 µl reaction volumes containing 100 µM dNTPs, 250 nM of each primer, 10X Taq assay buffer and 0.75 U Taq DNA polymerase (Sigma, USA) with 20 ng template DNA in a thermal cycler (Biorad, USA). The PCR amplification profile consisted of first a denaturation at 94°C for 4 min, followed by 35 cycles of 94°C at 1 min, 45°C for 2 min and 72°C for 1 min. Amplified DNA fragments were electrophoresed in 1.5% agarose gels stained under UV light. The PCR products were gel purified and used for cloning.

Cloning of glyoxalase I

The ligation reaction was set up with 100 ng of eluted PCR products. 1 µl (50 ng) of pGEM-T easy vector (Promega corporation, USA), 1 µl of T4 DNA ligase and 5 µl 2X rapid ligation buffer in a 10 µl total reaction volume kept at 16°C till transformation. The ligation product was transformed into competent cells of *Escherichia coli* JM109 prepared by $CaCl_2$ treatment and plated on LB/ampicillin plates with lPTG (7µl) and X-gal (40µl) and incubated overnight at 37°C. Recombinants were selected through blue-white screening on Luria agar. The presence of insert in single white colonies was confirmed by PCR with the same primer combination.

Sequence analysis

The sequencing of the PCR amplified fragment was done at the DBT facility for DNA sequencing, Indian Institute of Science, Bangalore, India. The method was done in an automated DNA sequencer using the same forward and reverse primers used for PCR amplification. Sequence analysis was done through BLAST analysis at the NCBI (National centre for Biotechnology information, USA) site and sequence alignment was done using Multlign programme (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl)

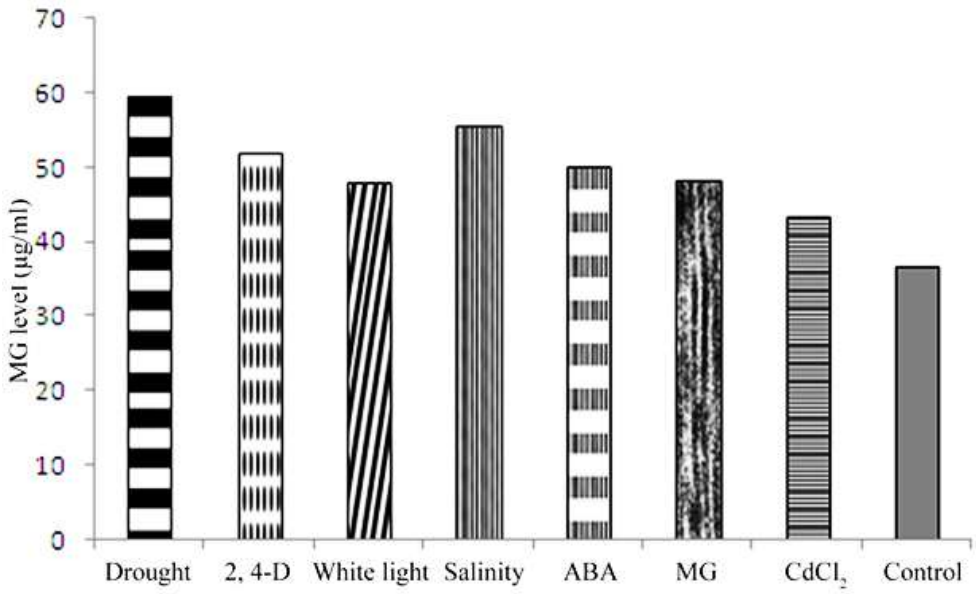

Figure 1. Effects of various stresses on MG levels in Hevea seedlings. Hevea seedlings were treated with drought, 2, 4-D (50 µM), white light, Salinity (300 mM NaCl), ABA (50 µM), MG (25 mM) and heavy metal ($CdCl_2$ (1mM)).

RESULTS AND DISCUSSION

Up-regulation of Methylglyoxal levels in *Hevea brasiliensis* under normal and stress conditions

The up- regulation of MG in rubber in response to various abiotic stresses was measured in young *Hevea brasiliensis* seedlings under control and various stresses like salt, drought, white light, MG, 2,4-D, ABA and heavy metal ($CdCl_2$) stresses. It was observed that methylglyoxal levels increased significantly under different stress treatments within 24 h and the levels ranged from 28 to 66.8 µg/ml under control and various stress conditions (Figure 1). Drought caused the highest induction (1.62 fold) of methylglyoxal followed by salinity, 2, 4-D, ABA, MG, White light and $CdCl_2$ stresses. Elevated levels of MG due to stress treatments have also been reported recently in plants (Yadav et al., 2005; Singla-Pareek et al., 2006; Hossain et al., 2009). Under stress conditions, cells become metabolically active, which is mirrored by upregulation of enzymes involved in glycolysis and TCA cycles (Umeda et al., 1994; Espartero et al., 1995). It is reported that MG levels increased under stress conditions. It may be that MG could act as a signal for plants to respond to stress (Hossain et al., 2009).

Up-regulation of glyoxalase I activity in *Hevea brasiliensis* under drought and other stress conditions

The concentrations of glyoxalase I in *Hevea brasiliensis* seedlings under normal and stress conditions were estimated. There was a significant increase in the enzyme concentration under the various stress treatments (Figure 2). Highest increase of glyoxalase I concentration was observed due to drought stress, followed by salinity, 2, 4-D, white light, ABA and MG stresses. Several research groups have reported that the activity of glyoxalase I was affected by various exogenous factors and abiotic stress treatments including salt, water, white light, ABA and heavy metal stresses (Chakravarty and Sopory, 1998; Espartero et al., 1995; Veena et al., 1999; Hossain et al., 2009). In the present study we also observed a significant increase of glyoxalase I activity due to different stress treatments especially drought and the results were in accordance with the MG levels.

Molecular characterization of glyoxalase I

In the present study we have isolated and cloned the sequence coding for glyoxalase I from *Hevea brasiliensis*. RNA in good quality and quantity without much degradation and DNA contamination was obtained by the LiCl precipitation method. After electrophoresis, the RNA was observed as a clear patch with bands of 18S and 28S rRNA. The mRNA in the isolated RNA was found to be intact as it could be successfully used for the cDNA synthesis and subsequent amplification of the gene from cDNA. The first strand cDNA from mRNA present in the total leaf RNA was synthesized through reverse transcription using the kit Super Script III first-strand

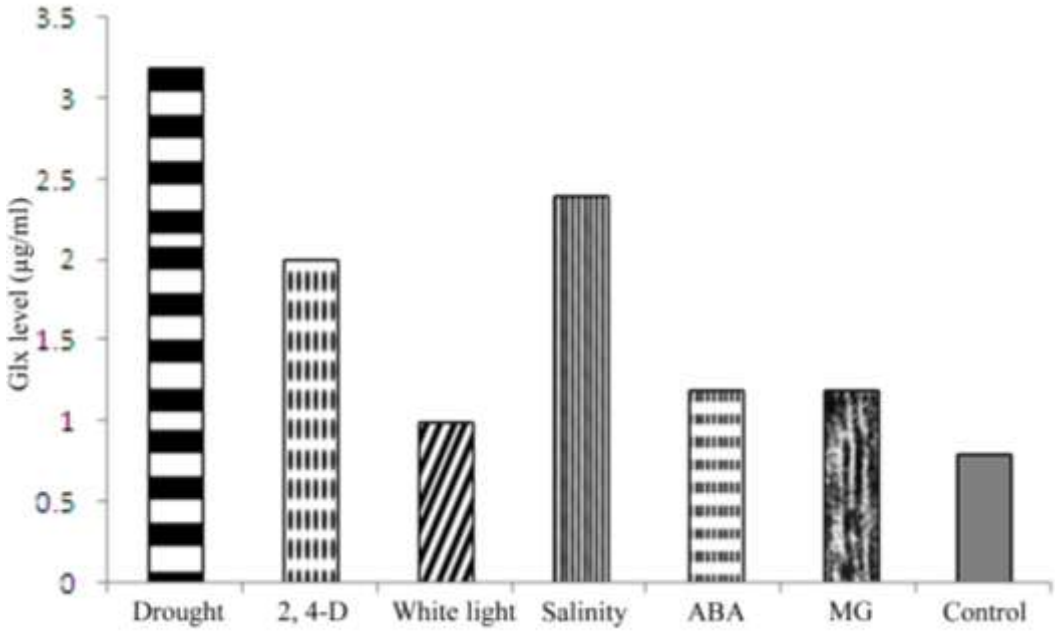

Figure 2. Effects of various stresses on glyoxalase I activities in Hevea seedlings. Hevea seedlings were treated with drought, 2, 4-D (50 µM), white light, salinity (300 mM NaCl), ABA (50 µM) and MG (25 mM).

synthesis system for RT-PCR. The glyoxalase I sequence was amplified using primers designed on the available sequence information in other species.

Under optimal PCR conditions, a prominent band of expected size (440 bp) was amplified from the cDNA (Figure 3a). The band was purified from gel for cloning and further sequence analysis. Cloning was confirmed by agarose gel electrophoresis and PCR analysis of the putative recombinant plasmids (Figure 3b and c). The PCR product, purified from the gel, was sequenced with the same primers used for amplification.

A partial cDNA sequence of glyoxalase I, which was 379 bp long, was obtained from the sequencing results excluding the primer regions (Figure 4).The sequence of *Hevea* glyoxalase (GenBank Acc. No: GU598520) had six open reading frames. The ORF finder revealed the longest ORF of 336 bp. The cDNA sequence of glyoxalase I gene from *Hevea,* obtained in the present study was subjected to online BLAST analysis to find out the similarity with the already reported sequences and multiple sequence alignment was done through Multalign programme. The present sequence showed significant similarity with several glyoxalase I genes isolated from different plant species (Figure 5). Maximum similarity for the sequenced portion of the gene was obtained with the glyoxalase I from *Ricinus communis* (90%), followed by populus EST from severe drought-stressed leaves (87%), *Glycine max,Cicer arietinum* (83%), *Cucurbita maxima* etc. It also shows similarities with glyoxalase I mRNA isolated from *Arabidopsis* (82%), *Avecenia marina* (82%), *Solanum* (81%) and *Brassica* (80%) and several other mRNA from drought-stressed leaves. Further a distant tree was constructed (figure not shown) to visualize the evolutionary relationship of glyoxalase I sequences of plants. Phylogenetic analysis indicated that *Hevea brasiliensis* and *Ricinus communis* glyoxalase I are closest in evolution. *Cucurbita* and *Allium* also showed a closer relationship with Hevea glyoxalase I.

A 126 amino acid long protein sequence was deduced from the cDNA sequence obtained. The protein sequence also showed high homology with the glyoxalases from different species. Maximum homology was obtained with the *Ricinus communis* glyoxalase protein (91%). A comparative analysis of *Hevea brasiliensis* glyoxalase I with protein sequence from *R.communis, G.max, C. arietinum and C. maxima* showed regions of high homology. Conserved domain analysis of amino acid residues detected active site, metal binding site, glutathione binding site and dimer interface.

As global demand for natural rubber increases, a major challenge for the cultivation of rubber plants is their capacity to withstand the unfavorable environmental conditions in the context of global climate change. Drought remains one of the most biologically damaging and ecologically limiting factors among all environmental constraints. Drought stress can occur at any stage of growing process, and can cause complete loss of crops or serious damage to yield. The major limiting factor that prevents its cultivation of *Hevea brasiliensis* is drought or semi-arid conditions. Therefore development of drought

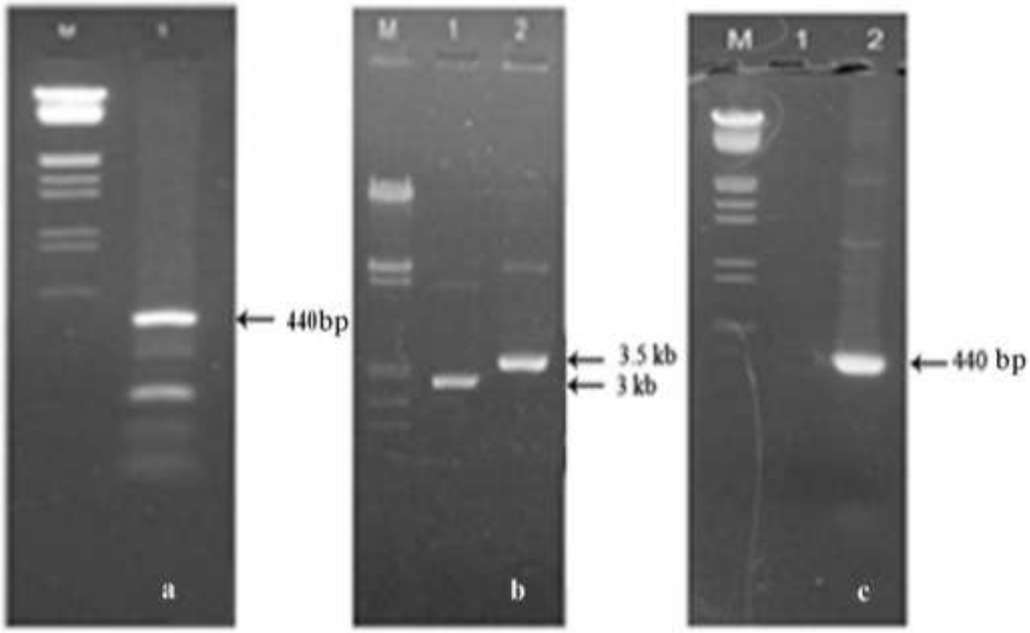

Figure 3. **(a)** The 440 bp fragment amplified with cDNA template with glyoxalase I specific primers. M=Molecular weight marker; Lane 1= 440 bp amplicon. **(b)** Recombinant plasmid isolated from E.coli. M=Molecular weight marker; lane 1=plasmid without insert (3 kb); lane 2=Plasmid with insert (3.5 kb). **(c)** 440 bp fragment amplified from recombinant plasmid with glyoxalase I specific primers. M= Molecular weight marker; lane 1= Plasmid DNA without insert; lane 2= Plasmid DNA with insert.

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1   attaaggatccaaaaataagtctcgattttattctcgcgtattgggcatgtcgttgctt  60
1   I  K  D  P  K  I  S  L  D  F  Y  S  R  V  L  G  M  S  L  L    20
61  aagaggttggatttccagacatgaagtttagcttgtactttatgggctacgaggatcca  120
21   K  R  L  D  F  P  D  M  X  F  S  L  Y  F  M  G  Y  E  D  P   40
121 gcatcagctccaagtgacccagttgaaagaactgtttggacctttggtcagaaggctaca  180
41   A  S  A  P  S  D  P  V  E  R  T  V  W  T  P  G  Q  K  A  T   60
181 attgaattaactcataattggggtactgaaagtgatcctgacttcaaaggatatcacaat  240
61   I  E  L  T  H  N  W  G  T  E  S  D  P  D  F  K  G  Y  H  N   80
241 ggaaattcagaacctcgtggctttggacatattggtatttctgtggatgatgtgtacaag  300
81   G  N  S  E  P  R  G  F  G  H  I  G  I  S  V  D  D  V  Y  X   100
301 gcatgtgagagatttgaacatctaggggtggagttcgccaaaaaacctgatgatggaaaa  360
101  A  C  E  R  F  E  H  L  G  V  E  P  A  K  K  P  D  D  G  X   120
361 atgaaaggaatagctttct                                          379
121  M  K  G  I  A  F                                             126
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Figure 4. Nucleotide sequence of Hevea glyoxalase I cDNA and deduced amino acid sequence.

important. The drought tolerant clones of rubber have been found to possess a more efficient osmoregulation mechanism.

In this study we observed increases in both glyoxalase I and methylglyoxal and enhanced accumulation of glyoxalase I protein in response to drought, salinity, tolerant genotypes of *Hevea brasiliensis* is very heavy metal, 2-4 D, ABA and white light. Our results here on the induction of glyoxalase I and methylglyoxal due to drought stress clearly suggest the role of this pathway in

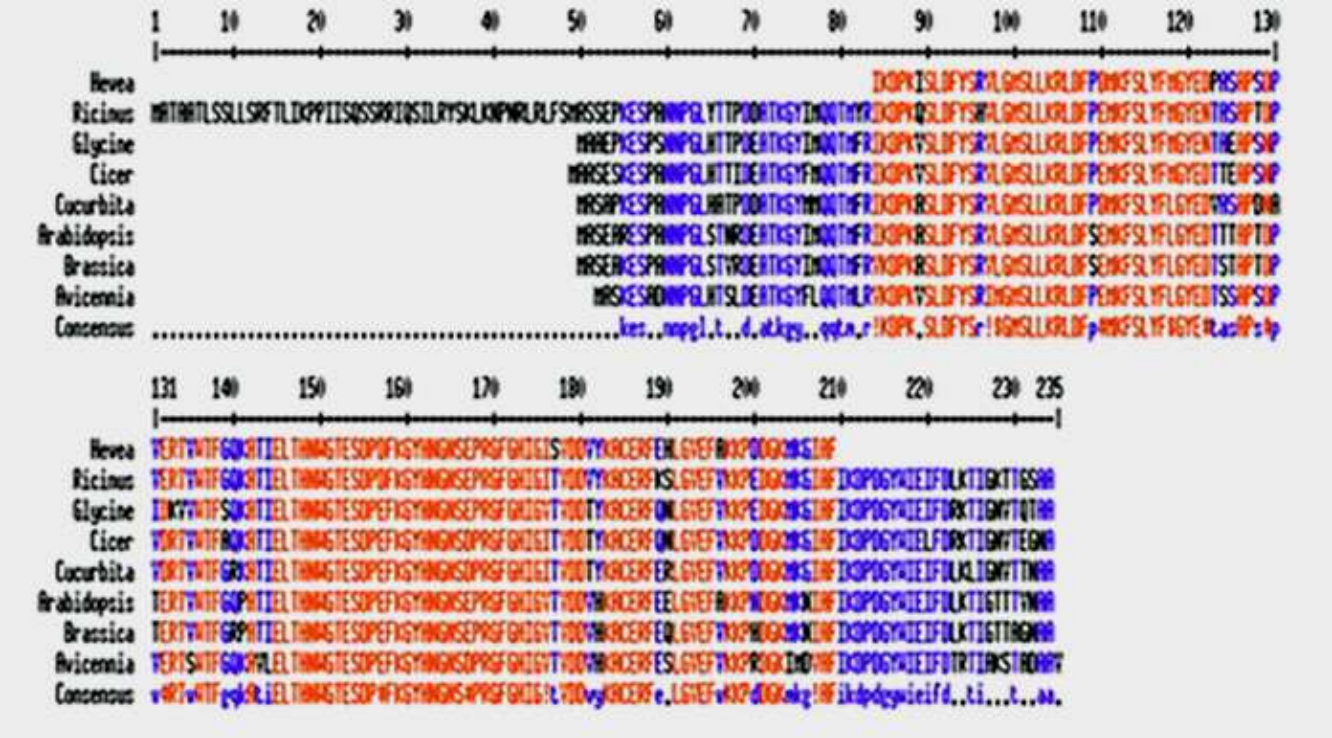

Figure 5. Multiple sequence alignment of nucleotide sequence of *Hevea* glyoxalase I and previously reported sequences in the database.

plants under drought stress. The higher glyoxalase I activity suggested that it might protect the plants against MG, which was formed under drought stress and confers tolerance by increasing the GSH-based detoxification system and decreasing lipid peroxidation. In view of the up-regulation of methylglyoxal and glyoxalase I in drought stressed seedlings of *Hevea brasiliensis* as well as from the sequence information, cloning of the glyoxalase I gene and incorporating the same through genetic transformation would be useful in improving the abiotic stress tolerance especially drought in *Hevea brasiliensis*.

Conflict of interests

The authors have not declared any conflict of interests..

ACKNOWLEDGEMENTS

The authors thank Department of Biotechnology and Microbiology, School of Life Sciences, Kannur University, Rubber Research Institute of India and Sir Syed Institute, Kannur for technical support and infrastructural facilities provided.

Abbreviations

ABA, Abscisic acid; **$CdCl_2$,** cadmium chloride; **2, 4-D,** 2, 4-dichlorophenoxyacetic acid; **Glx,** glyoxalase; **MG,** methylglyoxal; **GSH,** glutathione; **BLAST,** basic local alignment search tool; **BLASTP,** basic local alignment search tool for protein sequences.

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academicJournals

Vol. 15(11), pp. 384-391, 16 March, 2016
DOI: 10.5897/AJB2015.14418
Article Number: 14EF65457533
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nutritional food content of seed and effects of five different growing media on the seed germination and seedling growth of *Afzelia africana* SM Caesalpiniaceae

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Received 13 January, 2015; Accepted 22 April, 2015

Nutritional food content of seed and effect of five different growing media: Top forest soil (TS), sawdust (SD), 2:1 mixture of; TS+SD, TS+composted poultry manure (TS+PM) and 2:1:1 mixture of TS+SD+PM on the seed germination and seedling growth of *Afzelia Africana* SM Caesalpiniaceae were investigated. Thirteen chemical food nutrients were detected in the species seed. These occurred in varying percentages. The mixed growing media (2:1:1 mixture of TS+SD+PM, 2:1 mixture of TS+SD and TS+PM) consistently had: higher percentages of: water holding capacity and nitrogen than the single media (TS and SD). Shorter periods of initial and final seed germination were achieved when seeds were sown in the mixed growing media than in the single growing media. The highest percentage seed germination (87.4%) was achieved when seeds were sown in 2:1:1 mixture of TS+SD+PM growing medium, while the poorest percentage seed germination (30.5%) was obtained when seeds were sown in the TS growing medium, the control. Comparatively, the mixed media induced higher percentage seed germination responses than the single growing media. After four weeks growth in the nursery, values of seedling growth parameters showed that seedlings grown in the mixed growing media had better growth attributes than seedlings grown for the same period in the single media. The results also showed that the best growth attributes were developed by seedlings grown in 2:1:1 mixture of TS+SD+PM (mean: Seedling height 22.4cm., number of leaves, 22 and mean leaf area 120.6cm2).

Key words: *Afzelia Africana*, seed nutrient content, growing media, seed germination, and seedling growth attributes.

INTRODUCTION

In most developing countries, extensive research have been done on the exotic/introduced food crop species like *Manihot spp.* (cassava), *Mangifera indica* (mango), *Zea mays*, (maize), *Triticum durum* (wheat), *Musa spp.,* etc. Similar research activities on the indigenous food crops like *Afzelia Africana, Myranthus arboreus, Discorea bulbifera, Treculia africana, Brachystagia,* etc are either scare or lacking. Okafor (1983) reported that lack of

research activities on the indigenous food crop species were due to late appreciation of the values of their resources, except revenue generation for the resource poor rural dwellers, lack of knowledge of their propagation methods and lack of information on their nutritional contents. Currently, most of these valuable indigenous food crop species are endangered while some like *Discorea bulbifera* has gone extinct. Nzekwe (2006) reported that the endangered status of most indigenous tree food crop species appear related to depletion of their natural forest habitat by man's various activities like; lumbering, slash and –burn agricultural activities and fire outbreak. Above all, research activities on the indigenous food crops have not been included in most nations' research activities or in any arm of national agricultural programmes.

With the present hunger, poverty, disease and unemployment in Nigeria, there is an urgent need to reveal the nutritional food content of edible parts of the indigenous food crops and also provide information on their propagation requirements, so as to facilitate their conservation and full biotechnological exploitation of their resources. Among the popularly utilized food crop species capable of contributing to the socio-economy of the ever increasing rural population, *A. Africana SM. Caesalpiniaceae* was chosen as the study species, with the following objectives; first, assessment of the effects of five different growing media on the germination of the seeds, so as to obtain information on the medium/media that can be relied upon for obtaining high percentage seed germination, as well influence the production of high quality seedlings which when established in the field, can contribute to the conservation of the species for full biotechnological exploitation of the species resources. Second, determination of the species' seed chemical nutrient contents, in order to obtain, information on the possible contribution of the seed to human healthy growth and development when consumed as food condiment.

A. Africana S.M. Caesalpinaeae is a medium size woody tree of the tropical rainforest zone of Nigeria (Okafor, 1983). The tree is dichotomously branched, deciduous and flowers between August and November, while the fruits ripen between December and April. The fruit (A pod), green when Juvenile and black when ripe, is kidney shaped. The seed, capsule shaped has red/yellow "cap" at the microphylar region, partially covering the black seed (fig. 1). The seed is roasted/fried to remove the seed coat, after which the cream coloured cotyledons are powdered before use as soup condiment/seasonal/ thickener. The seed imparts sweet taste and aroma to soup, thus *Afzelia* preparations taste good (Ejiofor and Okafor, 1997). In addition to the use of the seed in preparing soup, inter-state trade on the seed generates substantial revenue for the resource poor rural dwellers and the middle men traders. *Afzelia* is nitrogen fixing species and thus could potentially play a useful role in agricultural system (Palm, 1995). The species is planted in allies where it fixes nitrogen thus returns nutrient to the soil (Palm, 1995). Locally, the species is not widely cultivated due to lack of information on its propagation requirement (Okafor, 1983).

Despite popular demand for the seeds, the species has not been purposely cultivated like other cash crops, but the trees are protected in farms where they are used for staking climbing food crops like *Telfairia spp.* There are no reports on the species propagation methods and the chemical nutrient contents, hence justifying the present study. The species is under exploited and endangered. The main source of the seeds, the most desired part of the species, is still from the few trees in the fast disappearing forests.

MATERIALS AND METHODS

The twig and mature fruits of the species were obtained from protected trees in the fruit nursery of the National Horticultural Research Institute (NIHORT), Okigwe, Imo state, in April 2012. Okigwe is located between Lat. 05°29'N and Long. 07'31E, at an altitude of 122 mm above sea level, with an annual rainfall of 2000 mm. Okigwe is in the humid zone of the tropical rainforest, with distinct dry and wet seasons. The materials were identified and confirmed at the herbarium of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, following the procedure described by Hutchinson and Dalziel (1964). Two thousand and five seeds (Figure 1) obtained from the trees were split into two parts of 1000 seeds each. The first batch (1000 seeds) was used for the analysis of the seed chemical nutrient contents, while the second batch (1000 seeds) was used to assess the effects of five different media on seed germination, seedling growth and development. The chemical food nutrient contents of the seeds were analysed in the Food Science laboratory of the Department of Crop Science, University of Nigeria, Nsukka. Prior to use, the seeds for germination study were subjected to viability test by soaking them in water for 15 to 25 min. Seeds that floated were discarded, being considered non-viable, while seeds that sank were used for the study. A total of 675 seed of uniform size were selected after soaking in water. Five media, namely, sawdust (SD), garden soil (TS), 2:1 mixture of garden soil and sawdust (TS + SD), 2:1:1 mixture of garden soil, sawdust and poultry manure (TS +SD + PM) and 2:1 mixture of garden soil and poultry manure (TS + PM), were used. Garden soil (TS) was used as the control. Topsoil was collected around the Botanic Garden of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, while sawdust was collected from Nsukka Urban timber market. After enquiry, sawdust of a softwood species, *Ceiba pentardra* was

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Abbreviations: SD, Sawdust; **TS,** garden soil; **PM,** poultry manure.

Figure 1. *Afzelia africana* seeds.

Figure 2. Germinated *Afzelia* seeds showing hook-shaped hypocotyls and stretched hypocotyls.

collected. Poultry manure was purchased from the poultry farm of the Department of Animal Science, University of Nigeria Nuskka. Prior to use, the poultry droppings were exposed for 3 weeks to compost. In formulating the mixed media, 2:1 mixture of: TS +SD and TS +PM, 100 kg of TS was thoroughly mixed with 50 kg each of SD and PM respectively. 2:1:1 mixture of TS + SD + PM medium was formulated by thoroughly mixing 150 kg of TS and 50 kg each of SD and PM. Prior to potting, samples of each medium were taken for physicochemical analysis, with emphasis on their water holding capacity and nitrogen contents. Each medium was filled in twenty five medium size black poly pots perforated at the sides for drainage of excess water, after which seeds were sown to the depth of about 2 to 2.5 cm and at the rate of three seeds per poly pot. Each treatment (medium) received 75 seeds and was replicated 5 times. Thus, 5 poly pots gathered together constituted a replication. The experiment, a completely randomized design (CRD), was carried out on an open, elevated concrete platform in the Botanic Garden, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The replications were displayed in a completely randomized manner. The seeds were considered germinated when the cotyledons become exposed or the seedlings grew to about 1 cm above the medium. Germinated seeds were randomly sampled and their morphology observed (Figure 2). Seven days after seed germination, the seedlings were thinned down to a seedling per poly-pot. The periods of first and last seed germination were recorded.

Table 1. Percentage water holding capacity and Nitrogen content of the media assessed for the seed germination of *Afzelia africana*.

Potting (% Media)	Some media physicochemical properties	
	% Water holding capacity	% Nitrogen
TS	25.2	0.002
SD	16.5	0.001
2:1 TS+SD	7.5	0.003
TS+PM	70.5	0.010
2:1:1 TS+SD+PM	84.6	0.050

Key: TS = Topsoil, SD = Sawdust, PM = Poultry manure.

Table 2. Periods of initial and final seed germination in the five different media seed germination periods (days).

Potting media	Initial	Final
TS	12-14	38-42
SD	12-14	39-40
2:1 TS+SD	7-9	28-30
2:1 TS+PM	10-12	22-24
2:1:1 TS+SD+PM	7-9	18-20

Madia interpretation in table 1.

Table 3. Effects of media on percentage germination of the seeds of *Afzelia africana*.

Potting media	% Seed germination
TS	30.5^{c}
SD	33.6^{c}
2:1 TS+SD	68.5^{ab}
2:1 TS+PM	56.3^{b}
2:1:1 TS+SD+PM	87.4^{a}
X	52.28
SE	21.44

Values with the same alphabet(s) are not significantly different from one another (P = 0.05). Media interpretation in table 1.

At the termination of the seed germination, the seedlings were allowed further four weeks growth after which, some of their growth parameters (seedling height, number of leaves and leaf area) were determined. Heights of 10 seedlings of each treatment were determined using meter rule, and the mean recorded. Similarly, mean number of leaves of 10 seedlings from each treatment was recorded after counting. Leaf area was determined by tracing out the leaf on a standard graph paper calibrated in cm^2, after which, the number of cm^2 covered by the leaf were counted. For each treatment, mean of ten seedlings was recorded. Data obtained in the seed germination and in some seedling growth parameters were subjected to the analysis of variance (Steel and Torrie, 1980) and the means were separated by Duncans Multiple Range test (DMRT).

RESULTS

Results of the physicochemical properties of the five different media (Table 1) show that the mixed media, TS + SD, TS + PM and TS + SD + PM consistently had higher percentages of water holding capacity and total nitrogen contents than the single media, TS and SD. The results further showthat the medium, SD had the least values of the two physicochemical properties. The initial seed germination (Table 2) was observed 7 to 9 days after sowing in 2:1:1 TS + SD + PM, 2:1: TS + SD media and 10 to 12 days later in TS + PM medium, while the single media; TS and SD took the longest period, 12 to 14 days to germinate. Irrespective of medium and the period of initial seed germination, germinated seeds developed hook-shaped hypocotyl that later carried the cotyledons above the medium as the hypocotyl stretched out. Final seed germination showed that seeds sown in the mixed media took shorter period than seeds sown in the single media to accomplish germination. The effects of media on percentage germination are summarized in Table 3. Generally, higher percentage germination responses were obtained when seeds were sown in the mixed media; 2:1 mixture TS +SD, TS + PM and 2:1:1 mixture of TS + SD + PM, than in the single media; TS and SD. The highest percentage seed germination (87.40 ± 21.4%) was achieved when seeds were sown in 2:1:1 mixture of TS + SD + PM medium, and the least, (30.5 ± 21.4%) when seeds were sown in TS medium, the control. Effects of media on the development of the seedling growth parameters; (Figure 3 and Table 4); seedling height, number of leaves and leaf area, showed variations. Seedlings grown in the mixed media; TS + SD + PM, TS + PM and TS + SD for four weeks produced higher values of all the growths parameters assessed than seedlings grown in single media, TS and SD. Seedlings grown in 2:1:1 mixture of TS + SD + PM consistently produced seedlings with the highest values of all the growth parameters assessed; while seedlings grown for the same period in SD medium produced the least.

Figure 3. *Afzelia* seedlings at the end of the study (4 months).

Table 4. Mean Values of Some growth Parameters of *Afzelia africana* seedling grown for 4 weeks in the nursery.

Media	Seedling weight (cm)	Number of leaves	Leaf area (cm^2)
TS	16.5^{d}	19.5^{b}	120.6^{b}
SD	14.7^{cd}	18.5^{c}	118.7^{b}
TS+SD	17.5^{bc}	20.5^{b}	120.8^{b}
TS+SD	19.5^{b}	22.0^{ab}	180.4^{a}
TS+SD+PM	22.4^{a}	24.5^{a}	196.3^{a}
X	18.12	20.6	147.36
SE	2.64	2.65	33.85

Values with the same alphabets along the vertical column are not significantly different (P = 0.05); media interpretation as in table 1.

The results of the analysis of the chemical food nutrient content of the seed of *A. africana* (Table 5) showed that the seed contained appreciable amount of 13 basic chemical food nutrients required for human healthy growth and development.

DISCUSSION

Detailed information on the requirements of most indigenous food tree crops in the tropics for seed germination, seedling growth and development are scarce (Okafor, 1983; Mbakwe and Nzekwe, 2005). Numerous earlier reports (Bruckner, 1997; Smith, 1998; Wilson et al., 2001; Baiyeri, 2003) showed that materials used in formulating potting media significantly determine the physicochemical properties of the media. Hence variations in the ratio of base materials used in formulating the five media involved in this study appear to explain the differences in their percentage water holding capacity and nitrogen contents. The medium, mixture of 2:1:1 TS + SD + PM, which had the highest proportion of poultry manure (PM), also had the highest percentage water holding capacity and nitrogen content. This was followed by the other mixed media; 2:1: TS + SD and TS + PM, while the single media, TS and SD, had the least. Smith (1998) pointed out that single media have numerous macro-pore spaces that encourage water loss

Table 5. Chemical nutrient, food contents of the seed of *Afzelia africana*.

Nutrient contents	Concentration %
Carbohydrates	38.36
Crude protein	23.29
Fat	25.95
Crude fibre	1.60
Moisture	9.10
Ash	3.30
Calcium (Ca)	0.18
Magnesium (Mg)	0.47
Sodium (Na)	0.023
Potassium (K)	0.390
Iron (Fe)	0.75 6 mg/100 g
Vitamin B	0.005 mg/100 g
Vitamin A	108.66 1 u

by drainage. The author reported that single media can be amended by the addition of organic matter which reduces the macro-pores to numerous micro-pores, known for the retention of large quantity of absorbed water and good aeration. The large quantity of water retained (as early as water was applied) by mixed media probably created water vapour saturated atmosphere around the seed, thus making water constantly available within soils inter spaces.

In this study, the short periods of initial germination, duration of seed germination and high percentage seed germination responses obtained in the mixed media; 2:1; 1 TS +SD+ PM, 2:1, TS + SD and TS + PM appear related to the existence of water vapour saturated environments of the seeds sown in them, which in turn reflected the variations in the media physicochemical properties. The long periods of initial and final seed germination, observed in the single media, TS and SD, could probably be due to low water holding capacity, following water losses by drainage, and the period expended before water vapour saturated atmosphere was created as well as poor aeration. Baiyeri (2003) pointed out that medium of low water holding capacity is prone to poor aeration. The high percentage seed germination obtained when seeds were sown in 2:1:1 mixture of TS + SD + PMI (87.4%) thus, implied that the medium satisfied the qualities of good potting media. The base materials are readily available; the technique of formulating the medium is non-technical and can be easily adopted by local nurserymen and farmers. The medium hence appears recommended for routine propagation of the species.

The results of the determination of seedling growth parameters (seedling height, number of leaves and leaf area) showed that despite variations, seedlings grown for4 months in 2:1:1 mixture of TS+SD+PM medium consistently produced seedlings with superior values of all the growth parameters, followed by seedlings grown in the other two mixed media, TS+SD and TS+PM. The results appear reflective of the physicochemical properties of the media, particularly their nitrogen contents, which is high in the mixture of 2:1:1 TS+SD+PM. Noggle and Fritz (1976) reported that high nitrogen content of growth media favours vegetative growth of plants. Chemical food nutrient contents of *A. africana* (Table 5) showed that the carbohydrate content (36.76%) was higher than the values reported by Ejikeme et al. (2010) for *A. africana* seeds from Abakiliki (30.1%), Enugu (33.2%) and Nsukka (32.2%) in the Eastern part of Nigeria. The value was however higher than those reported for oil seeds such as cotton seed (21.9%), flax seed/linseed (34.3%), peanut (12.5%), rape seed (8.3%), sesamon seed (0.9%) and sunflower seed (Food Standards Agency and Institute of Food Research, 2002). Carbohydrates provide nutritional energy value of 17 kcal/g when consumed in food as: sweetening, gel or paste-forming, thickening agents, stabilizers and precursors for aroma and colouring substances (Belitz et al., 2009). The energy value (473.75 kcal) of *A. Africana* seed was relatively low. Considering the fact that *A. africana* is used mainly for soup thickening, it is a healthier alternative for diabetics and slimmers than cocoyam flour with 72.38 to 79.48% carbohydrate content (Onyishi, 2012).

The crude protein (23.29%) and fat (25.95%) were lower than the ranges (26.29 to 26.60% and 32.06 to 34.58%, respectively) detected in *A. africana* from Enugu, Nsukka and Abakiliki by Ejikeme et al., (2010). The high protein content which surpassed that of some legumes such as African yam bean, pigeon pea and cowpea would contribute in the reduction of protein malnutrition being experienced in developing countries. The protein content fell within the range 17 to 40% reported for legumes (Bojňanská et al., 2012). It however, contrasted with the 7 to 13% of cereals (Bojňanská, 2004), and was nearly equal to the upper level of protein range of meats (18 to 25%) (Čuboň et al., 2011). The fat content was found to be lower than the values recorded for peanut (46%), sesamon seed (58%) and sun flower (47.5%) (Food Standards Agency and Institute of Food Research, 2002) used mainly for vegetable oil production. The level of fat in *A. africana* seed would reduce oil requirement in food products formulated with its addition. Onyechi et al. (2013) reported that *Afzelia* snacks are healthier snacks for diabetics than the *Detarium* snacks because of its lower fat content. However, the fat content of snacks made from different cultivars of African yam beans (1.93%) (Odenigbo, 2001) were lower, compared to *Detarium* (11.11 to 14.87%) and A*fzelia* (11.62 to 14.21%) snacks (Onyechi et al., 2013). The level of crude fibre (1.6%) detected was low compared with gourd seeds (2.8%) and soybeans (4.28%) reported by Ogungbenle (2006) and Temple et al. (1991), respectively. Fiber increases the endogenous excretion of energy-containing nutrients, the excretion of bacteria,

and affects the digestion and absorption of nutrients (Miles, 2013). Utilizing *A. Africana* seed in food formulations will definitely increase the fiber content of the diet, which has been shown by Kelsay et al. (1978) to decrease the availability (apparent digestibility) of the energy containing nutrients, and thus the energy of the diet.

The moisture content (9.1%) was found to be higher than the values (5.88%) and (7.45%) reported by Adebayo and Ojo (2013) and Ejikeme et al. (2010). According to the Australian Oilseeds Federation (2013) the most appropriate storage conditions for oilseeds are to maintain the temperature of the seed below 20°C and seed moisture less than 7%.The sample contained sufficient amount of ash (3.30%) that was within the range of ash (2.88 and 3.2%) detected by Ejikeme et al. (2010), implying that it is rich in mineral elements. The iron content of the seed is 0.756 mg/100 g, while the recommended daily intake of iron is about 15 mg. Iron deficiency has been identified as the most common nutritional deficiency disease on the planet, particularly among pregnant women than infants and young children being the two most vulnerable demographic groups (Theuer, 2008). This situation which may be combated by altering dietary practices (Ziegler and Fomon, 1996) will be alleviated by frequent consumption of iron rich foods. The sodium content (0.023 mg/100 g) was low compared to 25, 20 and 3 mg reported for cotton, sesame and sunflower seeds, respectively. The American Diabetes Association (2002) reported that the amount of sodium in the diet should be limited since sodium helps to increase blood pressure and has the tendency to retain fluids. The level of calcium detected (0.18%) in *Afzelia* was also low, but would contribute to the concentration of calcium in the diet if used as a thickener. Calcium is very important for strong bone formation, essential for maintaining total body health, needed not just to keep the bones and teeth strong but to ensure proper functioning of muscles, nerves and also needed for blood clot (Bell et al., 1996).

The value recorded for potassium (0.390%) was not up to the level (1350 mg) detected in cotton seed (US Department of Agriculture, Agricultural Research Service, 2003) and the 3.5 mg US RDA for both men and women. Potassium is the principal cation in intracellular fluid and functions in acid-base balance, regulation of osmotic pressure, conduction of nerve impulse, muscle contraction particularly the cardiac muscle, cell membrane function and Na+/K+-ATPase (Soetan et al., 2010). A high level of potassium provides a protective effect in instances of excessive sodium intakes (Dzomeku et al., 2006). The magnesium content (0.47%) was found to be lower than the values recorded for the range of magnesium (63 to 440 mg) of major oilseeds (Food Standards Agency and Institute of Food Research, 2002) and the US Recommended Daily Allowance of 350 mg for men and 280 mg for women. Magnesium is an activator of many enzyme systems and maintains the electrical potential in the nerves (Adeyeye and Agesin, 2007). It also works with calcium to assist in muscle contraction, blood clotting, and the regulation of blood pressure and lung functions (Swaminathan, 2003). The value of these minerals were however lower than those reported by Adebayo and Ojo, (2013) for fresh *A. Africana* seed flour from Ekiti State, Nigeria; K (1580 mg/100 g), Fe (2.6 mg/100 g), Ca (340 mg/100 g) and Mg (1.6 mg/100 g), which may probably be due to geographical location and varying agronomic conditions. The level of mineral elements in *Afzelia* seed in this study suggest that the species seed flour when consumed in diet (soup) can significantly complement the availability of the mineral elements from other components of the diet, such as vegetables and meat. Thus, *A. africana* can be a cheap source of obtaining mineral elements for human healthy growth and development, particularly among rural dwellers.

Vitamin contents of *A. Africana* observed in this study varied with that earlier reported. Pro-vitamin A (108.66 IU) was found to be below the recommended daily intake of pro-vitamin A (6.0 to 12.0 mg) (Belitz et al., 2009). Vitamin A is essential for growth, healthy eyes as well as structuring and functioning of the cells of the skin (Ihekoronye and Ngoddy, 1985). The vitamin B_2 content of the seed, which is also important in carbohydrate and protein metabolism, was 0.005 mg/100 g. The values were however lower than 0.3 to 1.6 mg (Belitz et al., 2009) recommended daily intake of vitamin B_2 and the values (0.023 /100 to 0.028 mg/100 g) reported by Ologunde et al. (2011) for cashew nuts from three Nigerian states. Based on the results of this study, it can be concluded that the species can be propagated by sowing the seeds in and growing the seedlings in 2:1:1 TS + SD + PM medium; that the seed of *Afzelia* when eaten can supplement for most of the basic nutrients required for healthy growth and development of man, particularly rural dwellers, whose diet is dominated by carbohydrate.

Conflict of interests

The authors have not declared any conflict of interest.

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academicJournals

Vol. 15(11), pp. 392-400, 16 March, 2016
DOI: 10.5897/AJB2015.14874
Article Number: 2ACC70257539
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of African eggplants to *Fusarium* spp. and identification of sources of resistance

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Received 20 July, 2015; Accepted 5 November, 2015

Eggplant (*Solanum* spp.) production in Arumeru district and other parts of Africa is severely affected by wilting diseases of unknown etiology. *Fusarium* spp. characterized through morphological and sequence analysis of the translation elongation factor associated with Fusarium wilt of eggplants was used to test the response of three different eggplant species. Three *Solanum* spp. accessions were tested in a screen house at the seedling stage for resistance to two isolates each of *Fusarium equiseti* (corda) Sacc, *Fusarium solani* (Mart.) Sacc and *Fusarium oxysporum* (Schlecht). The study indicated that accessions MM 1131 (*Solanum macrocapon)* and N 19 (*Solanum anguivi*) accessions are susceptible to *F. equiseti.* Accession N 19 *(S. anguivi*) was susceptible to *F. solani* while both N 19 *(S. anguivi*) *and* MM 1131 (*S. macrocarpon*) was also susceptible to *F. oxysporum f. sp. melongenae.* Ninety-three accessions of cultivated and wild eggplants were subsequently evaluated in two screen house trials for resistance to *Fusarium* wilt. A root dip technique was used to inoculate the accessions with isolate Fs 40 (*F. oxysporum* f.sp. melongenae). Seventeen of the 93 accessions were found to be resistant and they belonged to *Solanum macrocarpon and Solanum aethiopicum* species. Accessions in *S melongena* were found to be the most susceptible. Eggplant accessions that showed high levels of resistance could potentially serve as valuable sources of *Fusarium* wilt resistance in eggplant breeding programs in Tanzania and beyond.

Key words: African eggplants, *Fusarium* spp. susceptibility, resistance.

INTRODUCTION

Eggplant (*Solanum* spp.) is a multi-species, diploid and seed propagated crop that is cultivated widely in sub-saharan Africa. African eggplant (*S. aethiopicum* L.*)* and *S. macrocarpon* L. are the most popular native traditional

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Table 1. Description, source, morphology, molecular identification and pathogenicity of the Fusaria from wilting eggplants (*Solanum* spp.) that were used in this study.

Isolate code	Host	Site	Collection (2009)	Morphology identification	Differential test
FS 3	*S. macrocarpon*	WVC	July	*F. lacertarum* *	+
FS 22	*S. anguivi*	WVC	August	*F. solani*	+
FS 24	*S. melongena*	Shangarai	August	F. oxyporum*	+
FS 27	*S. aethiopicum gr.Kumba*	AVRDC	July	*F. equiseti* *	+
FS 35	*S. anguivi*	WVC	July	*F. solani**	+
FS 40	*S. melongena*	Shangalai	August	*F. oxysporum* *	+

Identification followed by an * were isolates confirmed by analysis of the ITS region and the α - elongation factor; + Isolate tested of it's pathogenicity; WVC-ARUSHA: World Vegetable Center-ARUSHA.

vegetables in West, and Central Africa. The African eggplant is widely cultivated as a major source of food and is a rich source of vitamins, fibers and minerals. It is also cultivated for medicinal purposes in some countries of Africa (Shippers, 2002) Losses in eggplant production in Africa due to wilt diseases have not been statistically evaluated. Previous research has shown that Fusarium wilt and Verticillium wilt pathogens are the major causal agents of wilting in eggplants (Kouassi et al., 2014).

The search for sources of resistance to Fusarium wilt pathogens has been done using the wild relatives of *S. melongena* and two genes carrying wilt resistance have been tagged (Mutlu et al., 2008; Toppino et al., 2008). *S. anguivi* and *S. aethiopicum* have been utilized in breeding programmes for development of disease resistant eggplant varieties (Altinok et al., 2014; Toppino et al., 2008). Several eggplant accessions have also been utilized in the development and production of disease resistant rootstocks for grafting high yielding varieties (Boyaci et al., 2011; Yoshida et al*.,* 2004).

Two eggplant accessions of *S. aethiopicum* gr. Gilo and *S. aethiopicum* gr. Aculeatum *(Solanum integrifolium*) are known to carry a gene for resistance designated as *Rfo-sa1,* to the fungal wilt disease caused by *F. oxysporum* f. sp. *melongenae* (Toppino et al., 2008). Work done by Altinok et al. (2014) and Iwamoto and Ezura (2006) showed that the high diversity of eggplant germplasm represents a valuable source of wilt resistance genes that could be introgressed into cultivated varieties. The objective of this study was to investigate the differential response of a limited number of African eggplant (*Solanum* spp.) accessions to a range of Fusarium isolates and to search for sources of resistance to the most virulent isolate.

MATERIALS AND METHODS

Differential response of African eggplants (*Solanum spp.*) to inoculation with isolates of *Fusarium* spp*.*

Six isolates of *Fusarium* spp. Coded Fs 24 (1480 JQ244840), Fs 3(1474 JQ244844), Fs 35 (1477 JQ244847), Fs 27(1481 JQ244856), Fs 40(1479 JQ244846), Fs 22 were used in this test. The numbers in parenthesis are accession numbers of the isolates nucleotide sequences deposited in the NCBI genebank. They were isolated from eggplants showing wilting symptoms collected from farmers fields in Arumeru district, AVRDC-RCA eggplant research field (Table 1). Morphological, cultural and molecular characterizations were carried out to confirm the identity of the isolates (Ghoneem et al., 2009; O'Donnell et al., 2009; Seifert, 1996).

Three accessions of *Solanum* spp., SIVONKWE (*S. aethiopicum* gr.Gilo), N 19 (*S. anguivi*), and MM 1131 (*S. macrocarpon*) were used in this study. Inoculation was done on seedlings at the six leaf stage (Altnolk, 2005). The seedlings were lifted gently from the trays and the soil washed off. The root tip of each seedling was cut to two thirds in length. The roots were immersed for 3 min in a suspension of 1×10^6 conidia per ml of *F. oxysporum* f. sp. *melongenae* harvested from 14 day old cultures grown on PDA at 25°C. The control plants were inoculated with distilled sterile water. The seedlings were planted in 15 cm diameter plastic pots containing sterile soil (forest soil mixed with sand at the ratio of 3:1) and placed in a screen house. The experiment was set up in a randomized complete block design with five replicates.

Identification of sources of resistance to eggplant wilt *(Fusarium oxysporum f.sp. melongenae)*

One isolate of *F. oxysporum* f.sp. melongenae coded Fs 40 isolated from infected plants of cultivated aubergine (*S. melongena*) was used. This isolate was used due to its high mean disease index in the differential response test and its high prevalence in the cultivated eggplants (*S. melongena*).

Preparation of inoculum

Single spore cultures were grown on PDA, for consistent sporulation and pigmentation, Petri plates were kept 40 cm below cool white fluorescent tubes and illuminated for 12 h periods at alternating 25°C day/20°C night cycles. Conidia was harvested from 14 day old cultures grown on Potato Dextrose Agar (PDA) at 25°C by adding sterile water to the plates and scraping the surface of the culture with a sterile glass slide. The resulting conidial suspension was filtered through two layers of cheesecloth to remove mycelia fragments. Spore concentrations were then determined using a hemacytometer and adjusted in distilled water to a concentration of 1×10^6 conidia per ml which was adopted in the two trials. The inoculum was used to inoculate seedlings in the susceptibility test.

Susceptibility test

Evaluation for wilt resistance was done according to Yoshiteru et al.

(1996) with modifications. Ninety three eggplant accessions (Table 2) of *S. macrocarpon, S. aethiopicum*, *S. anguivi*, *S. dasyphyllum* and *S. melongena* were inoculated at 5-week-old stage (2-3 true leaves emerged) using root dip inoculation technique. Six seedlings of each accession were planted in 15 cm diameter plastic pots containing sterile soil. The soil was drenched with 5 ml of inoculum ($1x10^6$spores/ml) of *F. oxysporum* f.sp melongenae and the seedlings grown in a screen house. The experiment was laid out in a randomized complete block design with three replicates.

Disease evaluation and statistical analysis

Differential response of the eggplants to the isolates

Plants were monitored daily for wilt development and symptomology and the extent of disease severity recorded at intervals of four weeks starting from the 4th week Scoring was done on a 1 to 5 disease scale where 1 = no symptoms; 2 = slight wilting and yellowing of the lowest leaves; 3 = half of the leaves wilted or showing yellowing; 4 = almost all the leaves wilted or showed yellowing; and 5 = all the leaves wilted, showed yellowing or plant died. The response for each accession and cultivar was determined against a mean disease index calculated according to the following formula (Matsubara et al*.,* 2004):

$$= \frac{\sum(\text{Number of plants} \times \text{degree of symptom})}{\text{Total number of plants} \times 5}$$

Susceptibility test for wilt resistance

The response for each accession and cultivar was determined against a percentage disease index calculated according to the following formula (Matsubara et al*.,* 2004).

$$\text{Disease index} = \frac{\sum(\text{number of plants} \times \text{degree of symptom})}{\text{Total number of plants} \times 5} = \times 100$$

Less than 20% = symptomless; 20 - 40% = slight wilting and yellowing of the lowest leaves; 40 - 60% = almost all the leaves wilted or showing yellowing; 60 - 80% = almost all the leaves wilted or showing yellowing and 80 - 100% all the leaves wilted, showing yellowing or plant died. R: Resistant (< 40%); PR: Partially resistant (40 - 50%); S: Susceptible (>50).

Statistical analysis

Data was analyzed for significant differences using ANOVA and comparison of means among accessions was done using Tukeys HSD. Co STAT statistical package (COHORT Software, Minneapolis, USA) was used to analyze the data. The level of probability was set at P=0.05. Means on the same column followed by a common letter are not significantly different according to the Tukeys test ($p \leq 0.05$).

RESULTS

Differential response of African eggplants (*Solanum* spp.) to inoculation with isolates of *Fusarium* spp.

Typical symptoms of eggplant Fusarium wilt were observed when seedlings of MM 1131 and N 19 (susceptible lines) were inoculated with isolates *F. oxysporum, F. solani* and *F. equiseti*. Symptoms on the inoculated seedlings included sudden drooping of leaflets starting from the apical part and progressing downward, yellowing of the leaves which began from one side of the leaf and final wilting of the whole plant. These symptom developments were also observed by Beladid et al. (2004). Complete death of the susceptible seedlings occurred during the 5th week.

The differential tests indicated that isolate Fs 27 (*F. equiseti)* and Fs 24, 40 (*F. oxysporum* f.sp melongenae) generally resulted to a higher mean disease index (>2.5) across the three accessions used compared to the other isolates which had a disease index of < 2.0 (Table 3). *Fusarium equiseti* and *F. oxysporum* were pathogenic to both MM 1131 (*S. macrocapon)* and N19 (*S. anguivi)*. *Solanum anguivi* was susceptible to all the Fusarium isolates except *F. laceratum*. *Solanum aethiopicum* gr. Gilo, (accession Sivonkwe) showed significant resistance to all the isolates except Fs27. Fs 3 was nonpathogenic to any of the *Solanum* spp (Table 3). Re isolation and culturing of the pathogen from the infected stem tissues on PDA yielded colonies of *F. oxysporum*, *F. solani* and *F. equiseti* inoculated and therefore proved positive for Koch's postulates.

Identification of sources of resistance to eggplant wilt *(Fusarium oxysporum f.sp. melongenae)*

The inoculation method adopted gave good disease incidence in all trials, and provided a useful screening system for resistance to Fusarium wilt. Symptoms started after 7 days of inoculation. There were significant differences between the two trials and this was attributed to the different prevailing weather conditions at the time the two trials were carried out. Temperatures were higher and the condition was dry in the duration the 1st trial was carried out compared to the 2nd trial which was characterized by heavy rains and temperatures as low as 13^oC during the nights. The maximum and minimum temperatures in the 1st trial ranged from 21 to 34°C while in the 2nd trial ranged from 13 to 29°C therefore the symptoms were more severe. The two trials showed that the strain used in inoculating the accessions (Fs 40) was virulent to the accessions.

12% of the accessions tested were considered as resistant while 71% were partially resistant and 10% susceptible According to Table 4, MM 1044, MM 11044 and MM 10260 were accessions found to be resistant within the *S. macrocarpon* species. There were also very susceptible accessions found in the *S. macrocarpon* species such as MM 855 and MM 283. This shows a high genetic variability within this species and an assumption on this species being totally susceptible or totally being resistant is ruled out. There were no accessions within

Table 2. List of eggplant species and cultivars tested.

Species	Cultivar	Origin
S. aethiopicum	SOS 1	AVRDC gene bank*
S. aethiopicum	UG-AE-4	Uganda
S. aethiopicum	UG-AE-10	Uganda
S. aethiopicum	UG-AE-21	Uganda
S. aethiopicum	TZSMN 2-8	Tanzania
S. aethiopicum	TZSMN 3-10	Tanzania
S. aethiopicum	TZSMN 75-7	Tanzania
S. aethiopicum	ML-AE-5	Malawi
S. aethiopicum	ML-AE-12	Malawi
S. aethiopicum	DB3	Ghana
S. aethiopicum	Manyire Green	Tanzania
Solanum spp.	Landrace	Tanzania
S. aethiopicum	OAA (089)	Cameroon
S. aethiopicum	Small oval	Tanzania
S. aethiopicum	N20	AVRDC gene bank*
S. aethiopicum	Heart shaped	Tanzania
S. aethiopicum	Sivonkwe	AVRDC gene bank*
S. aethiopicum gr. shum	MM347	Congo
S. aethiopicum	MM01150	AVRDC gene bank*
S. aethiopicum gr. Gilo	MM1371	Tanzania
S. aethiopicum	MM1106	AVRDC gene bank*
S. aethiopicum gr. Aculeatum	MM134	France
S. aethiopicum gr. Aculeatum	MM1474	INDE
S. aethiopicum gr. Aculeatum	MM1102	Burkina Faso
S. aethiopicum gr. Aculeatum	MM1483	Inconnue
S. aethiopicum gr. Gilo	MM10181	Ghana
S. aethiopicum gr. Gilo	MM803	Gabon
S. aethiopicum gr. Gilo	MM870	Madagascar
S. aethiopicum gr. Gilo	MM1641	Africa(Ouest)
S. aethiopicum gr. Gilo	MM10086	Togo
S. aethiopicum gr. Gilo	MM10245	Zambia
S. aethiopicum gr. Gilo	MM1480	Inconnue
S. aethiopicum gr. Gilo	MM1188	Inconnue
S. aethiopicum gr. Gilo	MM11010	Cote D' Voire
S. aethiopicum gr. Gilo	MM196 TER	Burkina Faso
S. aethiopicum gr. Gilo	MM868	Tchad (Bousso)
S. aethiopicum gr. Gilo	MM458	Japon
S. aethiopicum gr. Gilo	MM10079	Togo
S. aethiopicum gr. Gilo	MM10213	Ghana
S. aethiopicum gr. Gilo	MM1162	Uganda
S. aethiopicum gr. Kumba	MM574	Senegal
S. aethiopicum gr. Kumba	MM1642	Africa(Ouest)
S. aethiopicum gr. Kumba	MM1107	Burkina Faso
S. aethiopicum gr. Kumba	MM1207	Mali
S. aethiopicum gr. Kumba	MM267	Mauritania
S. aethiopicum gr. Shum	MM1121	Zambia
S. aethiopicum gr. Shum	MM1119	Togo
S. aethiopicum gr. Shum	MM1161	Bernin
S. aethiopicum	UG-AE-1	Uganda
S. aethiopicum	UG-AE-3	Uganda
S. aethiopicum	UG-AE-14	Uganda

Table 2. Contd.

S. aethiopicum	UG-AE-15	Uganda
S. aethiopicum	UG-AE-23	Uganda
S. aethiopicum	UG-AE-24	Uganda
S. melongena	Black beauty	Tanzania
S. dasyphyllum	MM1164	Togo
S. dasyphyllum	MM12126	Uganda
S. macrocarpon	MM10256	Ghana
S. macrocarpon	MM714	Zimbabwe
S. macrocarpon	MM10260	Ghana
S. macrocarpon	MM11044	Cote D' Voire
S. macrocarpon	MM132	France
S. macrocarpon	MM1131	Togo
S. macrocarpon	MM 150	Cote D' Voire
S. macrocarpon	MM283	AVRDC gene bank*
S. macrocarpon	MM12209	Zaire
S. macrocarpon	MM252	Ghana
S. macrocarpon	MM855	Togo
S. macrocarpon	UG-AE-6	Uganda
S. macrocarpon	UVPP	Tanzania
S. macrocarpon	CR001	Cameroon
S. macrocarpon	MM01139	AVRDC gene bank*
S. macrocarpon	MM01064	AVRDC gene bank*
S. melongena	S. 00677	AVRDC gene bank*
S. melongena	S. 00718	AVRDC gene bank*
S. melongena	S. 00735	AVRDC gene bank*
S. melongena	S. 00811	AVRDC gene bank*
S. melongena	S. 00017	AVRDC gene bank*
S. melongena	S. 0052	AVRDC gene bank*
S. melongena	S. 00204	AVRDC gene bank*
S. melongena	S. 00256	AVRDC gene bank*
S. melongena	S. 00736	AVRDC gene bank*
S. melongena	S. 00567	AVRDC gene bank*
S.melongena	TS00567	AVRDC gene bank*
S.melongena	TS00131	AVRDC gene bank*
Solanum anguivi	N19	AVRDC gene bank*
S. anguivi	MM1103	Burkina Faso
S. anguivi	MM905	AVRDC gene bank*
S. anguivi	MM159	AVRDC gene bank*
Solanum spp.	TZSMN 15-2	Tanzania
Solanum spp.	ML-AE-4	Malawi
Solanum spp.	ML-AE-6	Malawi
Solanum spp.	ML-AE-9	Malawi

*Germplasm without place of origin data.

the *S. melongena* species categorized as being resistant.. Accesions MM 1161, MM 1616, UG AE– 21, MM 1119 and SOS1 in the *S. aethiopicum* species were resistant to the Fusarium isolate used. This species also exhibited a high genetic variability in its resistance to *F. oxysporum* f.sp. *melongenae* (FOM). Accessions used within the *S. anguivi* and *S. dasyphyllum* species were found to range from partially resistant to susceptible

DISCUSSION

The survival and activity of *Fusarium spp.* is greatly dependant on many factors, with the most important ones being soil moisture, soil and air temperatures (Mui-Yun,

Table 3. Behavior of three African eggplants after inoculation with different isolates of *Fusarium* spp.

Accession code	Mean disease severity (1 - 5);Isolates tested for pathogenicity						
	Fs 24 F.oxy	Fs 35 F.sol	Fs 22 F.sol	Fs 27 F. equi	Fs 3 F. lac	Fs 40 F.oxy	Control
MM 1131	3^{ab}	1.2^{cd}	1.2^{cd}	3^{ab}	1.0^{d}	2.6^{abcd}	1.2^{cd}
N 19	2.8^{abc}	2.6^{abcd}	2.8^{abc}	4^{a}	1.6^{bcd}	2.8^{abc}	1.4^{bcd}
SIVONKWE	1.2^{cd}	1.8^{bcd}	1.4^{bcd}	2.4^{abcd}	1.4^{bcd}	1.4^{bcd}	1.0^{d}

*****Values on each column followed by a letter in common are not significantly different at ($P \leq 0.05$). Foliar symptom scale (1-5), higher numbers indicate severerity of disease.

Table 4. Reaction of eggplant accessions and cultivars after artificial inoculation with *Fusarium oxysporum* f.sp *melongenae* (FS 40) expressed as disease incidence (%) in the two trials.

Species	Accession code	Trial 1	Trial 2	Average	Tukeys test
S. macrocarpon	MM 10260	36.7	20	28.3^{R}	e
S. aethiopicum	MM 1119	33.3	23.3	28.3^{R}	e
Solanum spp.	UG AE 6	33.3	26.7	30^{R}	e
S. aethiopicum	SOS1	30	33.3	31.7^{R}	e
S. aethiopicum	MM 1616	43.3	20	31.7^{R}	e
Solanum spp.	ML AE 6	40	26.7	33.3^{R}	e
Solanum spp.	ML AE 4	30	40	35^{R}	de
S. aethiopicum	MM 1161	36.7	36.7	36.7^{R}	de
S. macrocarpon	MM 11044	36.7	36.7	36.7^{R}	de
S. aethiopicum	MM 10079	40	33.3	36.7^{R}	de
S. aethiopicum	MM 1207	40	33.3	36.7^{R}	de
S. aethiopicum	UG AE-21	30	43.3	36.7^{R}	de
Solanum spp.	MM 1692	40	36.7	38.3^{R}	de
S. aethiopicum	TZ SMN AE 3-10	50	30	40^{R}	de
S. aethiopicum	MM 11008	36.7	43.3	40^{R}	de
S. macrocarpon	MM 1044	46.7	33.3	40^{R}	de
Solanum spp.	LANDRACE	50	30	40^{R}	de
Solanum spp.	MM 01139	36.7	46.7	41.7^{PR}	de
S. aethiopicum	MM 1160	43.3	40	41.7^{PR}	de
S. aethiopicum	N 20	40	43.3	41.7^{PR}	de
Solanum spp.	ML AE 9 GKK 149	43.3	40	41.7^{PR}	de
Solanum spp.	MM 1498	50	33.3	41.7^{PR}	de
Solanum spp.	SITE 101	40	43.3	41.7^{PR}	de
S. aethiopicum	MM 11010	50	36.7	43.3^{PR}	de
S. aethiopicum	MM 348	50	36.7	43.3^{PR}	de
S. dasyphyllum	MM 12126	46.7	40	43.3^{PR}	de
S. melongena	S 00813	46.7	40	43.3^{PR}	de
Solanum spp.	UG AE-23	40	46.7	43.3^{PR}	de
S. aethiopicum	MM 347	43.3	43.3	43.3^{PR}	de
S. dasyphyllum	MM 1164	43.3	43.3	43.3^{PR}	de
S. macrocarpon	MM 1062	43.3	43.3	43.3^{PR}	de
S. aethiopicum	MM 10086	40	50	45^{PR}	de
S. aethiopicum	MM 1106	43.3	46.7	45^{PR}	de
S. aethiopicum	MM 1107	46.7	43.3	45^{PR}	de
S. aethiopicum	MM 1121	46.7	43.3	45^{PR}	de
S. aethiopicum	MM 1162	50	40	45^{PR}	de
S. aethiopicum	MM 1642	43.3	46.7	45^{PR}	de

Table 4. Contd.

S. aethiopicum	MM 803	46.7	43.3	45^{PR}	de
S. aethiopicum	SMALL OVAL TYPE	50	40	45^{PR}	cde
S. aethiopicum	TZ SMN-AE 2-8	56.7	33.3	45^{PR}	cde
S. aethiopicum	TZ SMN AE 52-3	50	40	45^{PR}	cde
S. aethiopicum	MM 10252	46.7	43.3	45^{PR}	cde
S. macrocarpon	MM 1048	50	40	45^{PR}	cde
S. macrocarpon	MM 12209	40	50	45^{PR}	cde
S. macrocarpon	MM 132	36.7	53.3	45^{PR}	cde
Solanum spp.	MM 1007	40	50	45^{PR}	cde
S. aethiopicum	MM 10213	46.7	46.7	46.7^{PR}	cde
Solanum spp.	ML AE 5 29-7	46.7	46.7	46.7^{PR}	cde
S. aethiopicum	MM 1102	50	43.3	46.7^{PR}	cde
S. aethiopicum	MM 870	50	43.3	46.7^{PR}	cde
S. anguivi	MM 159	50	43.3	46.7^{PR}	cde
S. melongena	S 00736	50	43.3	46.7^{PR}	cde
Solanum spp.	UG AE- 5	43.3	50	46.7^{PR}	cde
Solanum spp.	UG AE-14	43.3	50	46.7^{PR}	cde
S. aethiopicum	MM 574	50	46.7	48.3^{PR}	cde
S. melongena	OOO17	43.3	53.3	48.3^{PR}	cde
S. melongena	S 00718	43.3	53.3	48.3^{PR}	cde
S. melongena	TZ 00567	30	66.7	48.3^{PR}	cde
Solanum spp.	UG AE - 24	43.3	53.3	48.3^{PR}	cde
S. aethiopicum	MANYIRE GREEN	53.3	46.7	50^{PR}	bcde
S. aethiopicum	MM 10245	60	40	50^{PR}	bcde
S. aethiopicum	UG AE-10	60	40	50^{PR}	bcde
S. aethiopicum	MM 196	46.7	56.7	51.7^{S}	bcde
S. melongena	TS 00131	56.7	46.7	51.7^{S}	bcde
Solanum spp.	MM 138	56.7	46.7	51.7^{S}	bcde
S. aethiopicum	MM 1158	60	46.7	53.3^{S}	bcde
S. melongena	OO 204	46.7	60	53.3^{S}	bcde
S. aethiopicum	MM 868	53.3	53.3	53.3^{S}	bcde
S. aethiopicum	MM 1188	56.7	53.3	55^{S}	bcde
S. aethiopicum	MM 1483	60	50	55^{S}	bcde
S. aethiopicum	MM 1615	50	60	55^{S}	bcde
S. melongena	OO 677	50	60	55^{S}	bcde
S. aethiopicum	MM 1371	56.7	56.7	56.7^{S}	abcde
S. anguivi	MM 905	56.7	56.7	56.7^{S}	abcde
S. macrocarpon	MM 150	56.7	56.7	56.7^{S}	abcde
S. aethiopicum	MM 134	63.3	50	56.7^{S}	abcde
S. aethiopicum	MM 1480	66.7	46.7	56.7^{S}	abcde
S. aethiopicum	TZ SMN AE 75-7	63.3	50	56.7^{S}	abcde
S. melongena	OO 567	60	53.3	56.7^{S}	abcde
S. melongena	S 00735	60	53.3	56.7^{S}	abcde
S. aethiopicum	MM 1474	56.7	60	58.3^{S}	abcde
S. aethiopicum	MM 267	46.7	70	58.3^{S}	abcde
S. macrocarpon	CR 001	56.7	60	58.3^{S}	abcde
S. aethiopicum	MM 10181	63.3	56.7	60^{S}	abcde
S. aethiopicum	DB 3	63.3	56.7	60^{S}	abcde
S. anguivi	MM 1103	60	60	60^{S}	abcde
S. melongena	BLACK BEAUTY	60	60	60^{S}	abcde
S. anguivi	N 19	66.7	63.3	65^{S}	abcd
S. macrocarpon	MM 1131	63.3	66.7	65^{S}	abcd

Table 4. Contd.

S. aethiopicum	MM 1308	66.7	70	68.3^{S}	ab
S. macrocarpon	MM 283	70	76.7	73.3^{S}	ab
S. melongena	OO 256	80	73.3	76.7^{S}	a
S. macrocarpon	MM 855	80	83.3	58.7^{S}	a

Each value is the mean % disease incidence of six plants. Means followed by the same letter are not significantly different following Tukeys test, ($P \leq 0.05$).

2003). The significant differences within the two trials were as a result of the prevailing environmental conditions during the time the two trials were carried out. *F. oxysporum* is a warm weather pathogen and wilting is more prevalent when the temperatures are high (28°C) and in moisture stressed soils. Infected plants may remain symptomless in wet seasons (Lester et al., 1988).This explains the higher disease severity of the accessions in the first trial compared to the second trial. Similar reports indicate that *Fusarium spp.* requires soil and air temperatures of 25 to 28°C (Mui-yun, 2003) to effectively infect their hosts.

Among the isolates used for the pathogenicity test, *F. equiseti* caused the highest wilting in *S. macrocarpon* and *S. anguivi* accessions followed by *F. oxysporum* f.sp melongenae. Pathogenicity of *F. oxysporum* f.sp melongenae to eggplants has also been reported by several authors (Altinok, 2005; Zhuang, 2005; Cho and Shin, 2004), however accession SIVONKWE in the *S. aethiopicum* gr. Gilo proved to be resistant in this case. This study confirms previous work showing accessions of *S. aethiopicum* gr. Gilo to carry resistance to *Fusarium oxysporum* f.sp melongenae though other accessions within the species have also been found to be susceptible (Stravato and Capelli, 2000; Toppino et al., 2008). The resistant Sivonkwe accession can be used in breeding for resistance to *Fusarium* spp. and other wilt related pathogens. *Fusarium equiseti* pathogenicity in eggplants has not been reported and further studies on its economic importance on cultivated eggplants would contribute significantly to wilt control. *Fusarium equiseti* and *F. solani* have been reported to cause heavy wilting and severe seedling root rot in sunflower (Sharfun-nahar and Mushtaq, 2007). The colonization and pathogenicity of *F. equiseti* on tomatoes has also been observed by Jamiołkowska, (2009). It's pathogenicity has been linked to a pathogenic factor known as equisetin and trichothecenes (Hestbjerg et al., 2002; Wheeler et al., 1999).

The screening of the 93 accesions *to F. oxysporum* f.sp melongenae exhibited a whole range of reactions, that is, resistance, partial resistance and susceptible. Accessions within the *Solanum dasyphyllum* species (MM 1164, MM 12126) found to be partially resistant to *F. oxysporum* f.sp melongenae are wild eggplant species which have not been cultivated but these accessions would be valuable if used as rootstocks. *S. macrocarpon* is not susceptible to most diseases and is resistant to damping off caused by *Thielaviopsis basicola* (Shippers, 2002). Certain cultivars of *S. macrocarpon* have been reported to be resistant to *Fusarium* wilt (Grubben and Denton, 2004). Interspecific hybridization between *S. macrocarpon* and *S. melongena* is known to produce fertile hybrids and therefore can be used in transfer of the resistant traits to cultivated eggplants. *Solanum macrocarpon* and *S. dasyphyllum* can also be crossed easily and therefore produce fully fertile hybrids (Shippers, 2002). *Solanum aethiopicum* gr. Gilo accessions exhibited reactions ranging from resistance, partial resistance to susceptibile. Similar results were reported by Stravato and Cappelli (2000). This may be explained by the existence of genetic variability within the *S. aethiopicum* groups Gilo and Shum as reported by Sekara et al. (2007).*Solanum aethiopicum* groups, Shum and Kumba were found to carry a higher resistance than Group Gilo. *Solanum aethiopicum* (MM 1161, MM 1616, UG AE– 21, UG AE-6, SOS1, MM 10079, MM 1207 and MM 11008 and TZSM NAE-3-10) are valuable for breeding for resistance. Further evaluation for resistance to Verticillium and bacterial wilts would be important for eggplant improvement. Accession SOS1 (*S. aethiopicum* gr. *Gilo* X *S. aethiopicum* gr. *Aculeatum)* which was found to be resistant by Toppino et al. (2008) was also resistant in this study. Previous work has reported eggplant (*S. melongena*) to be susceptible to *F. solani* resulting to crown rot, vascular discoloration and wilt (Nabi et al., 2013; Romberg and Davis, 2007; Chakraborty et al., 2008). Consideration of the *Fusarium* spp. causing wilt in *S. anguivi* and *S. macrocarpon* is important when breeding for resistance to wilt for accessions within this species. More screening should also be done to test the resistance of the *S. aethiopicum* species groups to *F. equiseti* and *F. solani.*

Categorizing host reactions (resistant, partially resistant, susceptible) can be useful in indicating an accessions response to disease in disease favourable environments. The present study clearly shows that resistance to fusarium wilt exist in non-commercial eggplant germplasm which can be exploited to reduce losses.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Egerton University for hosting the student for the Msc programme and the World vegetable Center-Arusha (formerly AVRDC) who funded this project for study. They also appreciate the director of world vegetable centre for giving the student the opportunity to use the facilities and resources in the organization.

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academicJournals

Vol. 15(11), pp. 401-407, 16 March, 2016
DOI: 10.5897/AJB2015.14883
Article Number: F200ADD57542
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A commercial micropropagation protocol for virupakshi (AAB) banana via apical meristem

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Received 24 July, 2015; Accepted 19 January, 2016

***In vitro* micropropagation of banana (*Musa* spp.) cv.virupakshi (Hillbanana) was studied. Suckers were collected from the germ plasm block of Jain R&D (originally established from the suckers from Palani Hills, Tamil Nadu) during summer. The sucker surface sterilized with 1% NaOCl for 30 min gave 100% survival without any contamination. Apical meristems that were isolated and cultured on MS based media supplemented with benzylaminopurine (BAP) 10.0 mg/l and IAA1.0 mg/l gave higher number of shoots (134.3 shoots/explant) within168 days (24 weeks). Kinetin 2.0 mg/l and NAA0.5 mg/l gave early rooting in just five days with 6.6 roots per plant. Observations were recorded after every four weeks up to six sub-culturing. Acclimatization was done in poly house, followed by shade house under 50% light conditions. The hardened plants when shifted to the field showed luxurious growth. The regenerated micro propagated banana plants were tested for genetic uniformity through 13 inter simple sequence repeat (ISSR) markers recommended by NCS-TCP, DBT. Profiles obtained by all the three ISSR primers namely, 834, 840 and 850, respectively exhibited similar banding patterns, which revealed the existence of genetic uniformity in micro- propagated plants.**

Key words: Micropropagation, Virupakshi, hill banana, banana bunchy top virus.

INTRODUCTION

Banana is one of the most economically important fruit crops that can be produced in almost all parts of India as a vital source of energy. Plantains and bananas (*Musa* spp.) constitute staple food for rural and urban consumers in the humid tropics and are the fourth most important global food commodity after rice, wheat and milk. Annual production of *Musa* spp. in the world is about 101.99 million tons (FAO, 2012).

India is the largest producer and consumer of banana with many varieties under cultivation. Among the cultivars that are grown and consumed in specific areas and purposes, hill banana is one amongst them. Hill bananas (AAB genome) have two eco types namely Virupakshi and Sirumalai, known for their special flavor, long shelf life and are unique to some pockets of Tamil Nadu, India. This variety is grown in limited area with traditional planting material and cultivation methods without much scientific intervention. This has resulted in infection and

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accumulation of several diseases in plantations, the most important among them being banana bunchy top virus (BBTV). This has reduced the productivity, quality and profitability for the farmers.

BBTV has been the sole cause for reduction in hill banana cultivation from 18, 000 ha in 1970s to about 2,000 ha at present (Elayabalan, 2010). BBTV is transmitted by the aphid *Pentalonia nigronervosa* (Hu et al., 1996). Availability of virus free planting material may help in restoring the area, productivity and income. Micro propagation is an established method to multiply virus free planting material in banana. *In vitro* micro propagation has been widely adopted as an alternative means for production of disease free banana planting material. There are many reports on micro propagation of other banana varieties (Sadik et al., 2012) using shoot tip, but none or very limited on hill banana.

Recently, *in vitro* micro propagation technique for 'Virupakshi' through embryonic cell suspension culture has been reported. However, the method needs expertise for developing suspension cultures. Keeping these facts in view, in the present research investigation, the study has successfully developed a commercially viable micro propagation protocol for hill banana.

MATERIALS AND METHODS

Micro propagation

The research investigation was conducted at Tissue Culture Laboratory of Jain R&D, Jain Irrigation Systems Limited, Jalgaon (Maharashtra) India. Sword suckers were collected from virus free and true to type Hill banana plants cv. Virupakshi from the germ plasm block of Jain R&D farm. Before sucker collection, the mother plants were indexed for viruses (BBTV and CMV) to ensure that they are free from viruses. The suckers were brought to laboratory and cleaned under running tap water for 30 min. Following this, two to four leaf sheaths were carefully removed, and explants were trimmed into 50 x 30 mm by cutting the top and base. The explants were surface sterilized using 1% Ridomil and two drops of Tween-20 for 30 min.

Further, explants were surface sterilized using 1% NaOCl for 30 min followed by four to five washings with sterile reverse osmosis water four times each for 5 min. Excised shoot tips (10 X 5 mm) were inoculated vertically in 50 ml of modified Murashige and Skoog medium (MS) media (Murashige and Skoog, 1962) (basic salts plus thiamine HCl 2.0 mg/l, nicotinic acid 0.5 mg/l, pyrodoxine HCl 0.5 mg/l, meso-inositol 10 mg/l, L-tyrosine10 mg/l) supplemented with IAA (0.5 to 1.0 mg/l), benzylaminopurine (BAP) (0.0 to 20 mg/l), agar 0.5% (w/v) and sucrose 3% (w/v). The pH of medium was adjusted at 5.8±0.02 before auto claving at 15 psi for 15 min. All cultures were incubated at 25±2°C and were exposed to a photoperiod of 16/8 h light and dark cycling under 3000 lux density provided by white cool fluorescent tubes (40 W, Philips, India) with 70±5% relative humidity maintained in the culture room. Ten (10) explants were taken for each treatment and replicated thrice.

After initiation, explants were transferred into multiplication medium (MS+BAP+IAA, Table 3). Sub-culturing was carried out after every four weeks. In the first sub culturing, shoot tip was cut into two equal halves and transferred into multiplication medium. In further sub culturing, explants were cut into two equal halves and inoculated into multiplication medium, this process was repeated up to six sub-culturing.

After last sub culturing, 3 to 4 cm long shoots were detached from clumps and transferred to the rooting medium supplemented with various concentrations of kinetin (0.0 to 2.0 mg/l) and NAA (0.0 to 1.0 mg/l). Observations were recorded on time taken for root initiation, number of roots, root length and root weight after 4 weeks. Rooted plantlets were removed from glass bottles and washed under running tap water to remove culture media from plantlets because sucrose in agar encourages growth of microorganisms. After washing the plantlets, roots were dipped in fungicide solution (0.1% Ridomil) and transferred to 20 cc plant tray containing peat moss medium for primary hardening. Initially, plant trays were transferred into poly tunnel for 18 days (24 to 26°C, 80 to 90% RH and 7000 lux natural light). After that, plants were transferred to green house for 10 days (26±2°C, 70 to 80% RH and 10000 to 15000 lux natural light). Primary hardened plants were finally transferred in 1.2 L bags having press mud cake, silt, coconut husk and farm yard manure (1:1:1:1) under 50% shade net house for 45 days at 40% RH. Secondary hardened plants were ready for field plantation.

Table 1. Primers used for testing of genetic fidelity of micro propagated plants.

Primer	Sequence
UBC 807	GA GAG AGA GAG AGA GT
UBC 808	AGA GAG AGA GAG AGA GC
UBC 811	GAG AGA GAG AGA GAG AC
UBC 812	GAG AGA GAG AGA GAG AA
UBC 818	CAC ACA CAC ACA CAC AG
UBC 830	TGT GTG TGT GTG TGT GG
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 836	AGA GAG AGA GAG AGA GYA
UBC 840	GAG AGA GAG AGA GAG AYT
UBC 841	GAG AGA GAG AGA GAG AYC
UBC 842	GAG AGA GAG AGA GAG AYG
UBC 850	GTG TGT GTG TGT GTG TYC
UBC 868	GAA GAAGAAGAAGAAGAA

Genetic fidelity testing

Total genomic DNA of micropropagated plants was isolated using two gram fresh leaf from 13 micro propagated plants and mother plant using CTAB method (Dellaporta et al. 1983). The DNA pellet was dissolved in Tris EDTA (TE) buffer (200 µl) and quantified on nano drop spectrophotometer. DNA was stored at -20°C. Working stock of genomic DNA was diluted to 50 ng/µl concentration. Polymerase chain reaction (PCR) was performed using different inter simple sequence repeat (ISSR) primers (Table 1) suggested for banana by NCS-TCP, DBT (Anonymous, 2008). A total of 25 µl PCR reaction was performed using 10 mM Tris-HCl , pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 1.0 µM primer, 100 ng genomic DNA and 1 unit of *Taq* DNA polymerase. Amplification was performed infollowing steps: 5 min at 94°C, [45 s at 94°C, 45 s annealing temperatures specified for each primer and 2 min extension at 72°C] x 35 cycles with final extension for 7 min at 72°C. The gel electrophoresis was carried out using a submarine horizontal gel assembly in 1.5% agarose gel with ethidium bromide. The PCR product was visualized in a gel documentation system (Alpha Innotech Corporation, USA).

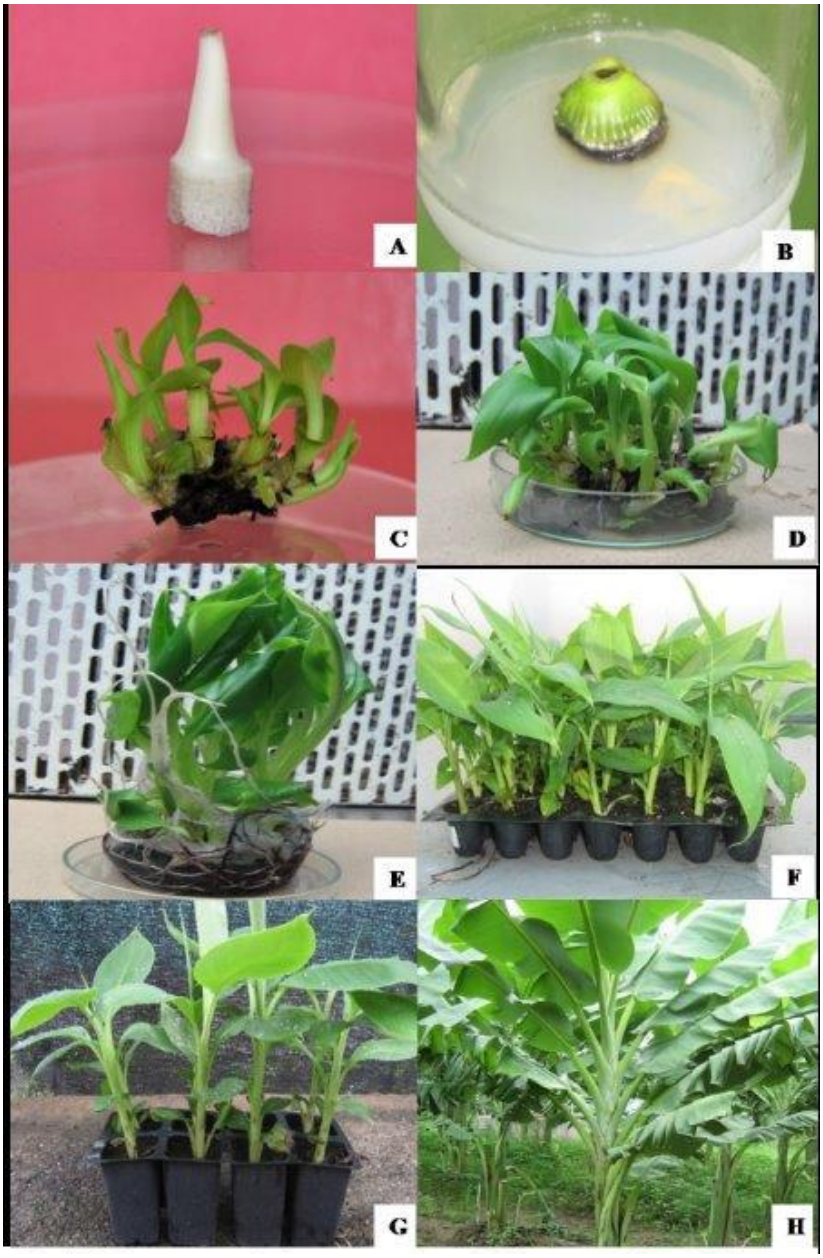

Figure 1. Various stages of micro propagation of hill banana cv. Virupakshi. (A) Trimmed sucker (*Ex* plant), **(B)** initial apical meristem, **(C)** shoot multiplication, **(D)** elongation, **(E)** rooting, **(F)** primary hardened plants, **(G)** secondary hardened plants, and **(H)** micro propagation plants in field.

RESULTS AND DISCUSSION

In vitro culture initiation and multiplication

Among the different concentrations of BAP and IAA, modified MS+BAP10.0 mg/l+IAA1.0 mg/l was found to be the best for initiation and multiplication (Figure 1A to C, Table 2). This combination produced 134.33 shoots per sucker and 2 cm long plantlets followed by modified MS+BAP10.0 mg/l+IAA0.5 mg/l, which produced127.66 plantlets/sucker with average plant height (1.8 cm) after 6th sub culturing. It was observed that when the concentration of BAP was further increased up to 20 mg/l along with IAA0.5 mg/l, the treatment caused an adverse effect on proliferation and the number of plantlets reduced to 57.33% (Table 2). It appears that high concentration of cytokine is detrimental to shoot proliferation in banana plants. Similar observations were

Table 2. Effect of phytohormones on shoot multiplication.

Treatment (mg/l)	Average no. of explants survived after sub culturing				Average plant height (cm)
	3rd	4th	5th	6th	
Control	8.0	10.33	13.66	17.66	3.33
BAP5.0+IAA0.5	9.0	18.33	33.33	60.33	2.67
BAP10.0+IAA0.5	10.33	23.33	54.33	127.66	1.8
BAP15.0+IAA0.5	9.66	21.66	46.00	87.99	1.73
BAP20.0+IAA0.5	7.33	14.33	28.66	57.33	1.67
BAP5.0+IAA1.0	9.66	19.66	38.33	86.66	2.33
BAP10.0+IAA1.0	10.33	23.33	56.00	134.33	2.00
BAP15.0+IAA1.0	8.66	18.99	40.00	100.00	1.83
BAP20.0+IAA1.0	8.33	17.66	33.33	70.33	1.67
CD	1.450	2.051	2.703	5.724	0.788
SEM±	0.484	0.685	0.903	1.912	0.263

Table 3. Effect of phytohormones on rooting.

Treatment (mg/l)	Days taken for root induction	No. of roots	Root length (cm)	Root weight (g)
Control	10.0	3.0	5.4	0.0374
Kinetin 0.5+NAA0.5	7.3	4.0	6.0	0.0391
Kinetin 1.0+NAA0.5	6.3	4.3	6.9	0.0418
Kinetin 1.5+NAA0.5	5.6	5.3	7.0	0.0425
Kinetin 2.0+NAA0.5	5.0	5.6	12.6	0.0734
Kinetin 0.5+NAA1.0	7.3	5.0	5.76	0.0332
Kinetin 1.0+NAA1.0	7.0	5.3	6.4	0.0399
Kinetin 1.5+NAA1.0	5.6	5.6	9.4	0.0556
Kinetin 2.0+NAA1.0	5.0	6.6	15.3	0.0908
CD	0.744	0.742	1.459	0.002
SEM±	0.248	0.248	0.487	0.001

also reported by Vuylsteke (1989) and Arinaitwe et al. (2000) during multiplication of 'Nzizi', 'Kibuzi' and 'Ndiziwemiti', respectively.

Rahman et al. *(*2004) recorded highest number of leaves per shoot at 30 days interval with 5 mg/lit BAP which was at par with BAP4 mg/l +NAA 1.5 mg/l. Higher concentrations of BAP and kinetin beyond optimum levels were also reported to cause necrosis and reduction in shoot formation during *in vitro* multiplication of Nendran (Rabbani et al., 1996). Other experiments observed highest number of shoots per explants at 28 days (3.11) with 5.0 mg/l of BAP and kinetin (Damasco and Barba, 1984). BAP alone 10.0 mg/l gives 10.10 shoots per ex plant (Gupta, 1986) with BAP and higher proliferation rate with increase in number of cycles (first cycle 11.32 and 4th cycle 17.78 number of shoots) was also observed.

In vitro rooting

The data presented in Table 3 clearly revealed kinetin 2.0 mg/l+NAA1. 0 mg/l gave early rooting (Figure 1E) in just five days, with higher number of roots (6.6 roots/plantlet) and root length (15.3 cm) in four weeks. The next best combination was kinetin 2.0 mg/l+NAA0.5 mg/l which induced lower number of roots (5.6) and root length (12.6 cm) in four weeks; while, lowest number of root (3.0) and root length (5.4 cm) was observed in control. It was observed that when cultures were shifted to lower concentrations of kinetin (1-2 mg/l) and auxins (NAA 0.5 to 1.0 mg/l) the number of roots and root length were increased while the time taken for root initiation had decreased. Low cytokinin of concentrations with lower cytokine into auxin ratio is useful for root initiation (Wong, 1986).

Naphthalene acetic acid (NAA) was found to be effective at very low concentrations for root initiation of banana. According to Cronauer and Krikorian (Cronauer and Krikorian, 1984). NAA 1.0 mg/l is suitable for root initiation in Musa *sp*. Arinaitwe et al. (2000) observed rooting on MS medium containing NAA 1.2 µM during the study of multiplication rate effects of cytokinins on Kibuzi, Bwara and Ndizwemit banana cultivars. Pruski et al. (2005) also reported best rooting response in a

Table 4. Comparative growth of plantlets during primary and secondary hardening.

S/N	Primary hardening				Secondary hardening			
	Plant height (cm)	No. of leaves	No. of roots	Root weight (g)	Plant height (cm)	No.of leaves	No. of roots	Root weight (g)
1	8.80	5.66	4.33	1.23	13.73	5.00	6.33	7.09
2	9.50	5.66	5.83	1.34	14.73	5.33	7.33	7.10
3	8.8	4.66	7.66	1.32	15.46	5.66	6.00	7.04
4	8.73	7.16	6.66	1.63	16.13	6.00	6.33	8.68
5	9.20	5.66	7.16	1.40	17.03	6.33	7.33	7.10
6	9.30	6.66	6.33	1.32	15.00	6.66	6.66	8.04
7	8.70	7.33	8.00	1.51	16.26	7.33	7.66	8.76
8	9.70	7.16	7.33	1.43	15.76	6.66	7.66	7.37
9	8.70	6.33	5.66	1.13	16.50	7.00	6.33	8.09
10	9.50	6.00	6.66	1.52	17.00	5.33	6.66	7.98
CD	0.722	1.581	1.715	0.247	1.957	0.990	1.085	0.766
SEM±	0.243	0.532	0.577	0.083	0.659	0.333	0.365	0.258

combination of IBA and NAA. However, De Langhe (1985) and Novak et al. (1990) used half strength MS+1.0 mg/l IBA, whereas Cronauer and Krikorian (1984) used auxin-free MS for rooting of banana microshoots. On the other hand, Banerjee and De Lahange (1986) and Azad and Amin (2001) obtained rooted banana shoots in half strength MS medium supplemented with 0.2 mg/l IBA. Akbar and Roy (2006) reported 1.0 mg/l IBA for best rooting response of *in vitro* cultured plants.

Hardening of *in vitro* raised plant

It is generally seen that maximum mortality of micropropagated plants occurs during acclimatization phase because plantlets undergo rapid and extreme changes in physiological functioning, histological and biochemical changes (Pati et al., 2013). To avoid this problem, it is necessary to harden the plantlets in two stages *viz.* primary and secondary hardening. During primary hardening (Figure 1F), plants showed hundred per cent survival and produced more number of leaves (6.3 leaves/plant), root (7.6 roots/plant) and root weight (1.63 g roots/plant) in the peat moss (Table 4). Problems of growth phase, primary hardening and secondary hardening need to be overcome for arriving at a commercially successful protocol of hardening (Anonymous, 2002). Vasane and Kothari (2006) reported that press mud cake mixed with soil was used as the optimal medium for producing sturdy plants during the secondary hardening (Figure 1G) process of banana cv. grand nain plantlets. Plants were showing luxurious growth after transfer into field (Figure 1H).

Genetic fidelity testing in regenerated plants

A protocol to be commercial must produce plants of true to type showing no genetic variation between the mother plant and the micro propagated plants. The Department of Biotechnology (DBT) has recommended a list of primer pairs that should yield identical banding patterns of the PCR products from mother plant and the micro propagated plants. Using 13 primers as recommended by DBT, the banding patterns of the mother plants and the micro propagated plants obtained from them, respectively were compared. Regenerated plants of hill banana cv. Virupakshi did not show any genetic variation between mother and micro propagated plants. Profiles obtained by three ISSR primers viz. 834, 840 and 850 as representing the patterns obtained are shown in Figure 2. As seen in these primers, all the 13 primers exhibited similar banding patterns for the mother plants and their micropropagated plants. These patterns reveal the existence of genetic uniformity in micro propagated plants.

Conclusion

Therefore, from the current research investigations, it is concluded that the study micro propagation protocol is successful for mass scale production of hill banana cv. virupakshi. This protocol can further be employed for the germplasm storage and production of disease free planting material.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

BAP, Benzylaminopurine; **ISSR,** inter simple sequence

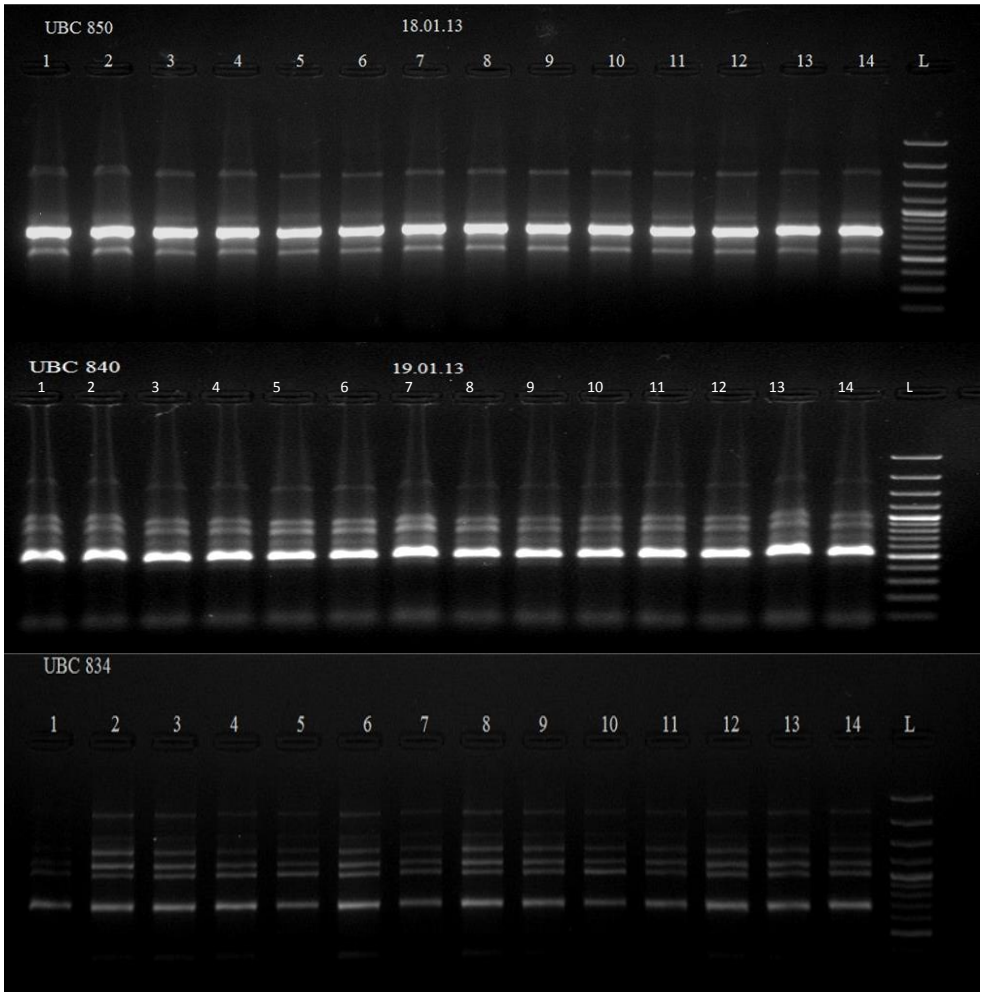

Figure 2. DNA finger printing pattern of mother plant (lane 1) and micro propagated plant (lanes 2 to 14) obtained by ISSR marker 834, 840 and 850. Lane L is DNA ladder.

repeat.

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Vol. 15(11), pp. 408-416, 16 March, 2016
DOI: 10.5897/AJB2015.14991
Article Number: 20C623757545
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Detection of extracellular enzymatic activity in microorganisms isolated from waste vegetable oil contaminated soil using plate methodologies

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Received 18 September, 2015; Accepted 1 February, 2016

In the present study, from a total of 100 strains isolated from waste vegetable oil contaminated soil, 38 bacterial and 14 fungi strains that presented positive lipolytic activity were obtained by detection through Rhodamine B Agar 0.02% w/v as a screening method. Additionally, two other enzymatic activities were determined. Positive proteolytic activity was evaluated in Casein Hydrolysis Agar and chitinolytic activity was identified by change in coloration in Bromocresol Purple Agar. Using these methodologies, we were able to report 18 microorganisms with two enzymatic activities and 6 microorganisms with all three enzymatic activities, thereby establishing these techniques as suitable and fast approaches for detection and semi-quantification of extracellular enzymatic activity.

Key words: Enzyme, lipases, proteases, chitinases, rhodamine B, soil.

INTRODUCTION

The enzymatic activity of a microorganism is in many cases influenced by the environment. The evaluation at the simplest level with the diversity analysis of present enzymes and ratios between and within major elements like C, N and P provides an insight into the microbial community response to changing nutrient resources (Caldwell, 2005).

The isolation from an oil contaminated soil could give an indication of the metabolic capacity of the microorganism's adaptation to these conditions. Among the enzymes commonly identified, three enzymes, lipases, proteases and chitinases are of high importance. Lipases, belonging to the family of hydrolases, are capable of catalyzing diverse reactions like, alcoholysis, hydrolysis, esterification and transesterification (Hasan et al., 2010). Lipases have been isolated and purified from fungus, yeasts, bacteria, plants and animals (Fuji et al., 1986; Pahoja and Sethar, 2012) and for their characteristics, they are used in food and textile industry, for biodegradable polymers synthesis and biodiesel production, among others (Fuji et al., 1986; Falch, 1991; Snellman et al., 2002; Noureddini et al., 2005; Hasan et al., 2010; Sangeetha et al., 2011).

As well, proteases are thoroughly distributed in nature

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and have microbes as their main source. As one of the main industrial enzymes, they are primarily used in detergents for their remotion capacity of protein based textile stains, with additional applications in leather, food, pharmaceutical industry and bioremediation processes (Anwar and Sleemuddin, 1997; Vijayalakshmi et al., 2011; Banerjee et al., 1999; Vishwanatha et al., 2010).

Finally, chitinolytic activity was determined considering that chitinases are found in bacteria, fungus, insects, plants and animals (Chernin et al., 1997; Gooday, 1990) and have a wide range of biotechnological applications, especially in chitin oligosaccharides and N-acetyl-D-glucosamine production (Pichyangkura et al., 2002) as well as bioconversion of chitin wastes to unicellular proteins (Vyas and Deshpande, 1991).

The aim of this study was to determine enzymatic activities of three of the most important enzymes found on bacterial and fungal strains using fast and reliable plate methodologies that allowed to process, identify and provide semi-quantification of extracellular enzymatic activity.

MATERIALS AND METHODS

Sampling

The samples were taken from waste cooking oil contaminated soil using a clean spatula at 5 cm of depth following a 10 km straight line with sampling every 2 km. The samples were placed in plastic bags, then transported to the laboratory and kept at room temperature.

Isolation and conservation

The samples were processed through serial dilution in 0.85% sterile saline solution, taking 1 g of the collected soil from each location. Nutritive Agar (NA) was used for bacterial isolation, sterilized and poured in Petri dishes to solidify. Single streak inoculation was performed with incubation at 37°C for 120 h. Potato dextrose agar (PDA) was used for fungi isolation and prepared by dissolving 39 g in 1000 ml of distilled water. Additionally, PDA with chlorampenicol at 1% (PDAC) was prepared and poured in Petri dishes to solidify. The incubation was performed for 21 days at 25°C. In addition submerged fermentations were prepared with nutritive broth supplemented at 1% with vegetable oils, including olive (OLI) (Cárdenas et al., 2001), canola (CAN) and waste vegetable oil (WVO), respectively. These remained in agitation for 96 h at 25°C and 150 rpm for latter inoculation through serial dilution in PDA, NA and PDAC. All media were sterilized in autoclave at 121°C for 15 min. Once morphological characteristics were determined, the pure strains were conserved in 20% glycerol at -20°C.

Sample and assay preparation

After a pre-culture of 24 h, all the bacterial suspensions (0.85%) saline were adjusted to 1 in the McFarland scale and centrifuged at 10,000 rpm, 4°C for 20 min and the supernatant was recovered (Rajeswari et al., 2011; Vijayalakshmi et al., 2011). Primary lipolytic activity selection of the bacterial strains was made by fluorescence intensity through simple streak in plates with Rhodamine B Agar. Semiquantification of lipases, protease and chitinase potential was determined using bacterial solutions adjusted to 1 on the McFarland scale, inoculating 10 µl of supernatant in 3 mm holes in each of the selective mediums and incubated for 48 h. For fungal strains, 1 cm sterile paper circles were placed on the center of Petri dishes adding 2 µl of fungal solution adjusted to 1 x 10^{-6} conidias/ml and incubated at room temperature for 144 h.

Lipolytic activity

Due to the fact that the soil from where the samples were taken was contaminated with waste vegetable oil, the first screening applied to the microorganisms isolated was the determination of lipolytic activity. Base media contained 1.25 g of yeast extract, 4.5 g of nutritive broth and 10 g of bacteriological agar in 450 ml of distilled water. The lipoidal emulsion was prepared with 200 ul of Tween 80, 30 ml of olive oil and 50 ml of water adjusted to pH 7. Both were sterilized at 121°C for 15 min separately. The dye was prepared aseptically adding 50 mg of Rhodamine B to 50 ml of sterile water and adding 20 ml of dye solution to lipoidal emulsion and mixing vigorously. For a final volume of 500 ml of Rhodamine B Agar at 0.02% w/v, 50 ml of dye and lipoidal emulsion were added to base media (Alken-Murray). All samples were analyzed under UV light at 350 nm. Together with the presence of fluorescence for identification of positive lipolytic activity, a selection criteria of fluorescence intensity for bacterial strains was followed (Rabbani et al., 2013; Carissimi et al., 2007) with 3 categories (1, 2 or 3), selecting those that coincide with category 3 from a mean of 6 repetitions.

Proteolytic activity

Casein hydrolysis agar containing 1 g of KH_2PO_4, 0.5 g of KCl, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2.2H_2O$, 25 ml with 15% of powdered skim milk, 10 g of glucose and 12 g of agar in 1 L of distilled H_2O was prepared. The milk powdered preparation was done by adding 3.75 g of powdered skim milk to 25 ml of distilled water mixed to homogenize in a creamy texture before adding it to the media; once added, pH was adjusted to 5.4 and sterilized at 121°C for 10 min (Mata Villegas, 2008).

Chitinolytic activity

Bromocresol purple agar was prepared containing 4.5 g of colloidal chitin, 0.3 g of $MgSO_4 \cdot 7H_2O$, 3 g of $(NH_4)SO_4$, 2 g of KH_2PO_4, 1 g of monohydrate citric acid, 15 g of agar, 0.15 g of Bromocresol Purple and 200 ul of Tween 80, pH was adjusted to 4.7 and sterilized at standard conditions (Lunge and Patil, 2012).

Enzymatic rate determination

For proteases and chitinases found in fungi, an enzymatic rate determination was used, with the formula:

$$\text{Enzymatic Activity index} = \frac{\text{Colony diameter + halo diameter}}{\text{Colony diameter}}$$

Statistical analysis

The obtained results were analyzed using analysis of variance (One-way ANOVA) with statistical significance of $p<0.05$, followed by a post hoc Tukey test.

Table 1A. Effect of the diverse inductors on the isolation of positive bacterial strains for lipolytic activity.

Source	Bacterial strains	Lipases	Positive strains (%)
Direct Isolation	26	12	46.2
Induction with OLI	21	12	57.1
Induction with WVO	16	10	62.5
Induction with CAN	11	4	36.4
Total	74	38	51.4

Table 1B. Additional enzymatic activities determined for bacterial strains.

Source	Chitinases	Positive strains[1] (%)	Proteases	Positive strains[1] (%)
Direct isolation	0	0.0	2	7.7
Induction with OLI	2	9.5	3	14.3
Induction with WVO	3	18.8	0	0.0
Induction with CAN	0	0.0	2	18.2
Total	5	6.8	7	9.5

[1]= Percentage calculated from the total of isolated strains.

RESULTS AND DISCUSSION

Initially, 74 Gram negative bacterial strains were obtained from which, 38 were positive for lipase production by fluorescence observation under UV light at 350 nm (Table 1A) and it was observed that the best inductor for lipases was WVO with 62.5% of positives strains.

To this primary screening, chitinases and proteases identification was added (Table 1B), obtaining 5 positive strains by observation of a coloration change from yellow to purple and 7 positive strains through hydrolysis halo observation, respectively (Figure 1).

For fungi, 26 strains were obtained from which, 14 presented lipolytic activity and where induction with olive oil (Table 2A) was the most succesfull with 100% of the strains positive. Furthermore, 15 strains presented chitinolytic activity (Table 2B) shown by coloration change and 11 strains presented proteolytic activity shown by hydrolysis halo (Figure 2).

Lipases

Different techniques have been used for lipase detection, including those with Tween 80 and tributyrin as substrate in solid media (Cárdenas et al., 2001; Sierra, 1957); these involve the development of clear areas around the colonies as an enzymatic activity indicator. However, tributyrin shows activity for esterases as well as lipases (Kim et al., 2001), which makes it a non-specific method (Kumar et al., 2012).

Another technique, using a chromogenic substrate Rhodamine B, has an action mechanism where the union with fatty acids and mono- and diglycerids has proved to be a fast and specific method for analysis which is insensitive to pH changes (Kouker and Jaeger, 1987; Hou and Johnston, 1992; Willerding et al., 2011; Rabbani et al., 2013), reason why it was chosen for the lipolytic activity detection of the worked samples. Following the procedure of fluorescence intensity, only the strains with category 3 were selected to proceed to inoculation of the bacterial supernatant in Rhodamine B agar Petri dishes in order to semi-quantify extracellular lipase activity.

The results were reviewed after 48 h of incubation, time reported to allow better contact between substrate and strains (Feng et al., 2010; Boonmahome, 2013) and shown in Table 3, in which strain 12 presented the highest enzymatic activity with a halo diameter of 10.7 mm followed by strain 38 with 10.6 mm, both considered as good producers according to the criteria established by Hou and Johnston (1992). From the results of the statistical analysis ($p<0.05$), strains 12, 38, 35, 30 and 31 were selected for further study and given that from this analysis the selected strains were part of the same group, from spectrophotometric quantification, we were able to discern and stablish significant difference between them (data not shown).

For the determination of fungal strains with lipolytic activity (Figure 3) fluorescence intensity was not included because based on the visual analysis performed, differences within strains were not detected. We focused on the ability of the fungi to grow in a solid media that contained olive oil (Savitha et al., 2007). They were observed at 144 h of incubation, obtaining the results shown in Table 3. This technique was considered useful as reference, emphasizing that the growth of the fungi in

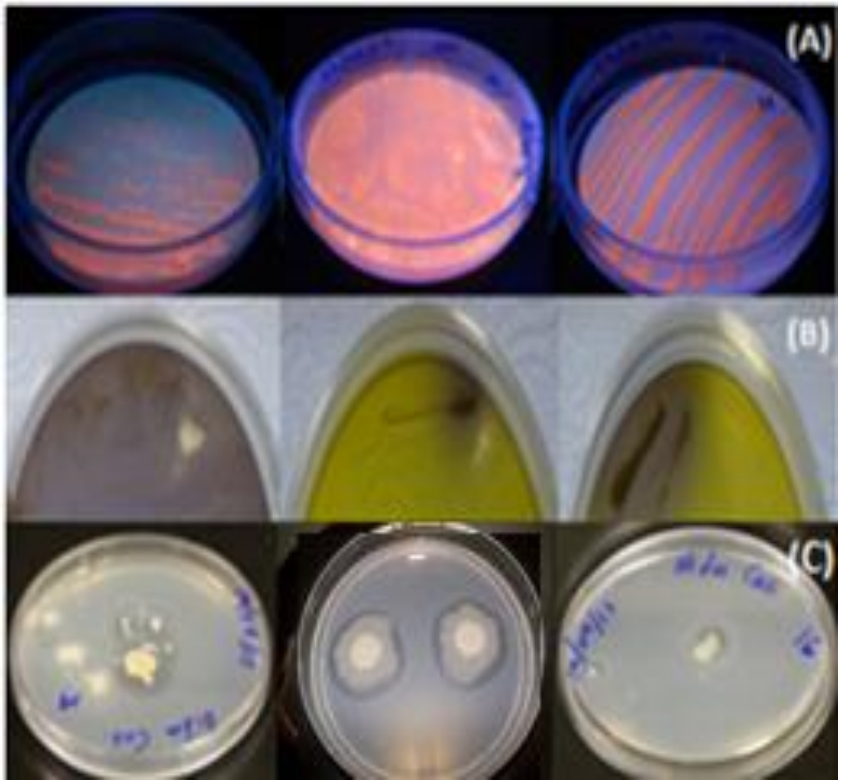

Figure 1. A) Positive bacterial strains for lipolytic activity observed under UV light at 350 nm. B) Positive bacterial strains with chitinolytic activity in bromocresol purple agar. C) Positive bacterial strains with proteolytic activity in casein hydrolysis agar.

Table 2A. Effect of the diverse inductors on the isolation of positive fungal strains for lipolytic activity.

Source	Fungal strains	Lipases	Positive strains (%)
Direct isolation	17	6	35.3
Induction with OLI	3	3	100.0
Induction with WVO	0	0	0.0
Induction with CAN	6	5	83.3
Total	26	14	53.8

Table 2B. Additional enzymatic activities determined for fungal strains.

Source	Chitinases	Positive strains[1] (%)	Proteases	Positive strains[1] (%)
Direct Isolation	7	35.5	7	41.2
Induction with OLI	3	100.0	1	33.3
Induction with WVO	0	0.0	0	0.0
Induction with CAN	5	83.3	3	50.0
Total	15	57.7	11	42.3

[1]= Percentage calculated from the total of isolated strains

Rhodamine B helps to determine which strains had a higher development using olive oil as a substrate (Rifaat et al., 2010; Rajeswari et al., 2011) and to categorize in a semiquantitative way, the strains that presented a higher

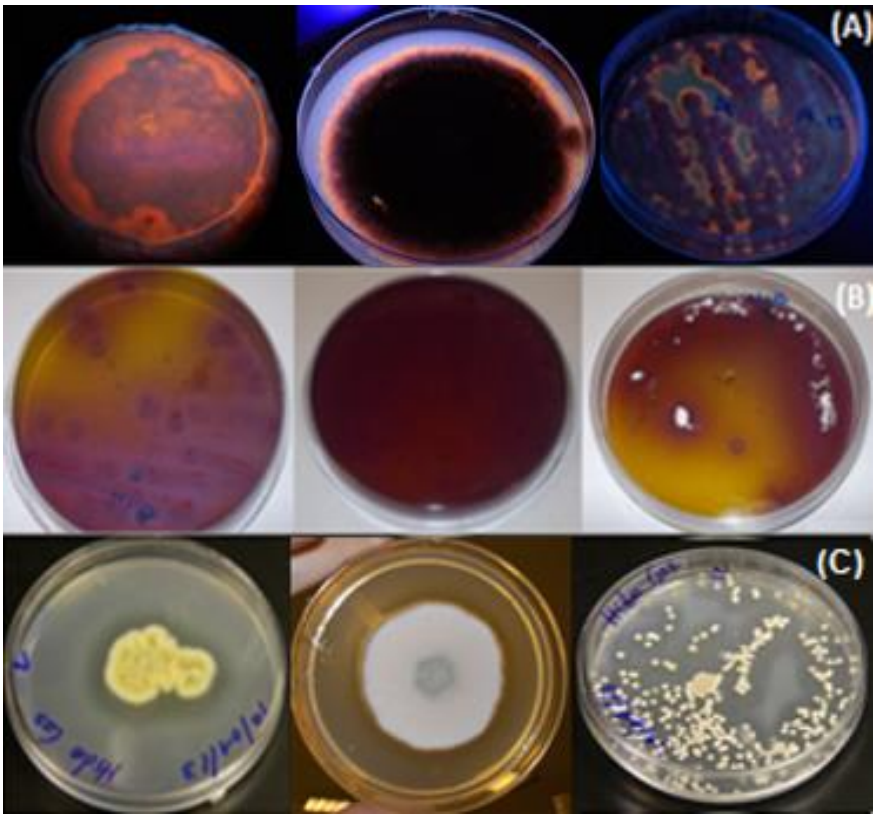

Figure 2. Positive fungal strains for: A) lipolytic activity; B) chitinolytic activity; C) proteolytic activity.

Table 3. Halo size for lipolytic activity.

A. Halo size for lipolytic activity of bacterial strains									
Strain	2^{ab}	5^{bc}	11^{a}	12^{c}	30^{abc}	31^{abc}	35^{abc}	36^{ab}	38^{c}
Halo (mm)	3.8	8.8	2.0	10.7	7.5	6.8	4.6	4.0	10.6

B. Halo size for lipolytic activity of fungal strains											
Strain	1^{ab}	2^{ab}	3^{ab}	4^{ab}	11^{ab}	18^{ab}	19^{ab}	22^{a}	23^{ab}	24^{b}	26^{a}
Halo (mm)	30.7	28.7	24.3	25.0	39.3	40.5	30.2	32.0	39.4	38.7	31.0

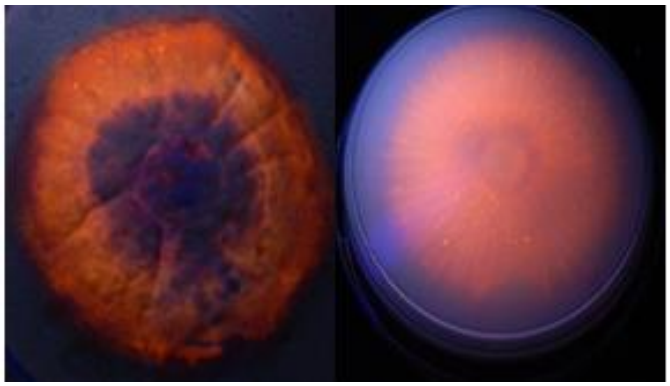

Figure 3. Fungal strains with lipolytic activity.

lipolytic potential in the incubation time stablished. Based on this, strain 18 had the highest growth with a 40.5 mm of maximum halo.

Proteases

The results obtained for bacterial proteases (Table 4) showed strain 18 as the main producer with a maximum hydrolysis halo of 25.0 mm after 48 h of incubation. This showed a similar activity when compared with the highest reported by Sánchez et al. (2004), differing in time of incubation, an important role as some strains require more time (up to 96 h) to be able to show their highest

Table 4. Halo size for proteolytic activity.

Strain	3[c]	8[a]	14[c]	15c	18[d]	22b[c]	24[ab]
Halo (mm)	17.5	8.3	19.0	17.8	25.0	15.0	6.7

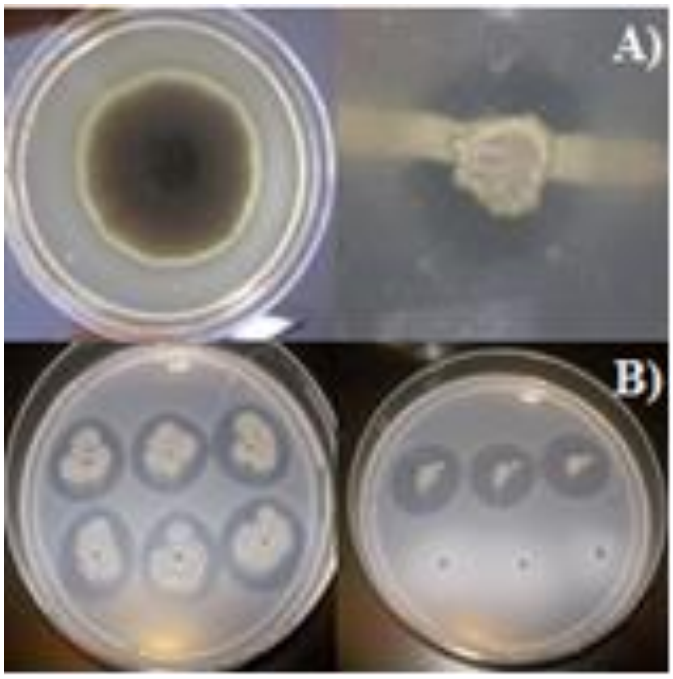

Figure 4. A) Strains with hydrolysis halos for fungal proteolytic activity. B) Strains with hydrolysis halo for bacterial proteolytic activity.

Table 5. Halo size for proteolytic activity of fungal strains proteolytic and proteolytic enzymatic rate.

Strain	1[ab]	2[a]	3[a]	4[a]	5[c]	6[ab]	10[bc]	18[a]	24[a]	25[ab]	26[d]
Halo (mm)	2.0	1.2	1.0	1.0	6.7	3.0	5.2	1.5	1.0	2.3	13.3
P.E.R.[1]	2.06	2.04	2.03	2.03	2.31	2.14	2.18	2.03	2.03	2.06	2.75

[1] P.E.R. = Protease enzymatic rate.

potential (Sánchez et al., 2004; Rodas Junco et al., 2009). Proteases, a group of enzymes whose function is peptide chain hydrolysis of proteins to polypeptide or free amino acids (Alnahdi, 2012), were tested in accordance with their function in skimmed milk agar which contains casein, (Santhi, 2014). This approach allowed us to observe the breaking of links resulting from the enzimatic activity as a halo around the colony (Figure 4).

Within fungal strains that showed protease activity (Table 5), it can be observed that strain 26 presented the highest activity with an enzymatic rate of 2.75 equivalent to 13.3 mm. Comparing the activity obtained from actinomycetes and microorganisms isolated from tropical soil, a superior behaviour of our isolated strains can be observed (Rodas Junco et al., 2009), which indicates a wide variety of protease producing microorganisms. When contrasting our results with those obtained by several authors, it can be observed that the difference in locations from which the samples were gotten is very wide and therefore the microorganisms present diverse adaptations to these particular environments giving an insight into the plasticity of this enzymes. It is also noticiable that considering the differences in the percentage of skim milk used in this assays and therefore

Table 6. Halo size for bacterial chitinolytic activity.

Strain	20[ab]	22[a]	30[a]	31[bc]	34[c]
Halo (mm)	19.0	15.0	12.0	50.0	50.0

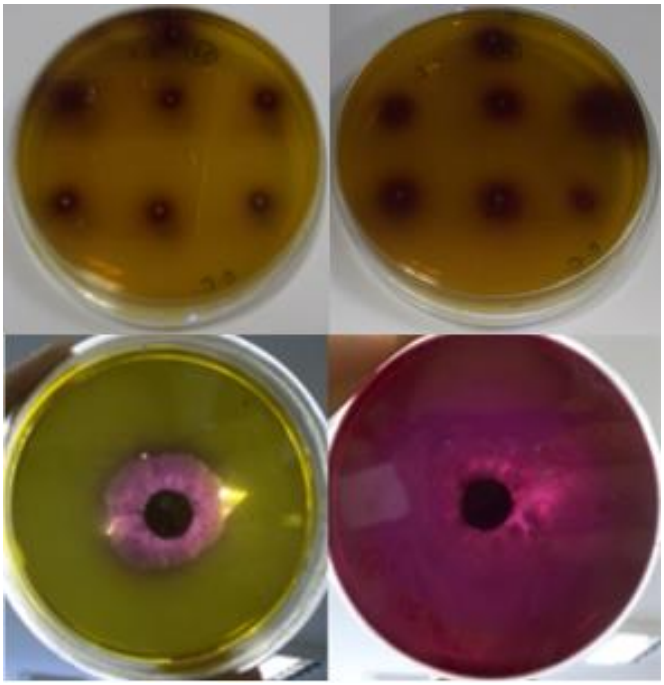

Figure 5. A) Bacterial strains with chitinolytic activity. B) Fungal strains with chitinolytic activity.

the amount of casein added to the media, ranging from 1 to 15%, it can be observed that even at 1%, this is a suitable and sensible assay that requires a minimmun amount of substrate added to the media (Kamat et al., 2008; Ahmad et al., 2014).

Chitinases

For bacterial chitinases (Table 6), strain 31 and strain 34 showed chitinolytic potential according to the criteria of El-Tarabily et al. (2000) and Rodas Junco et al. (2009) with halos up to 50 mm in 48 h of incubation (Figure 5) which also correspondent to reports made by Kuddus and Ahmad (2013).

Chitinases are found in an extensive variety of organisms in which fullfill different functions, specifically in fungi where they have autolytic, nutritional and morphogenetic roles (Patil et al., 2000). To determine the activity of chitinolytic enzymes, one of the most common procedures consist of visualization of the breake of chitine to N-acetiglucosamine. In this study, bromocresol purple in a media supplied with colloidal chitine was used

Table 7. Halo size for chitinolytic activity of fungal strains and chitinolytic enzymatic rate.

Strain	Halo (mm)	C.E.R.
1[ab]	8.7	2.39
2[a]	7.3	2.30
3[ab]	7.0	2.50
5[a]	6.7	2.22
6[a]	4.0	2.19
10[ab]	9.2	2.49
11[a]	3.8	2.24
18[a]	6.8	2.21
19[a]	1.5	2.14
22[b]	15.3	3.65
23[a]	5.0	2.17
24[a]	5.0	2.16
25[a]	4.7	2.16
26[a]	5.6	2.29

[1]C.E.R= Chitinolytic enzymatic rate.

through which the change in pH of acid to basic facilitates the detection with coloration turning from yellow to purple (Agrawal and Kotasthane, 2012; Lunge and Patil, 2012). Given that chitinases have different roles in fungi and bacteria, they serve nutrition and diverse functions such as parasitism (Patil et al., 2000; Ahmad et al., 2014); it was expected, for the nature of the sample, to find this enzymes.

The results obtained for fungal chitinases showed strain 22 with high production based on enzymatic rate and halo size (Table 7) according to the results obtained by Agrawal and Kotasthane (2012) that classified different groups (1 = no chitinase activity; 2 = low chitinase activity; 3 = medium chitinase activity and 4 = high chitinase activity).

Conclusion

From a total of 100 strains isolated from waste vegetable oil contaminated soil, 52 positive isolates for lipolytic, proteolytic or chitinolytic activity were found which were categorized based on their capacity to grow in solid media supplmented with sutiable substrates. With this results, these useful and time-saving techniques are highlighted for detection and categorization of enzymatic activities, reporting 18 microorganisms that present at least two enzymatic activities and 6 with all (three) enzymatic activities.

Conflict of interests

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to thank the biotechnology institute of the Autonomous University of Nuevo León for the support shown in the realization of this work. This work was supported by CONACYT with scholarship number 392281.

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academicJournals

Vol. 15(11), pp. 417-422, 16 March, 2016
DOI: 10.5897/AJB2015.15120
Article Number: 1D8870457565
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Histological and biochemical evaluations of the liver and kidney of Wistar rats fed with fish meal of *Sarotherodon melanotheron* captured with *Tephrosia vogelii's* powder

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Received 23 November, 2015; Accepted 19 January, 2016

To evaluate the toxicity of fish captured with *Tephrosia Vogelii* (TV), an ichtyotoxic plant, the Wistar albino rats were fed with the flour of tilapias *Sarotherodon melanotheron* poisoned with TV leaves powder. This study aimed to evaluate the poisonous effects of TV on various organs of rats, especially the liver and kidney. Three groups of Wistar rats were constituted and fed for 28 days. Histological sections were done on the liver and kidneys. Plasmatic levels of alanine aminotransaminase (ALAT) and aspartate aminotransaminase (ASAT) were measured. The histological sections carried out on their organs presented no lesions. However, the biochemical parameters, ALAT and ASAT showed a slight change. This study shows that the ingestion of fish poisoned with *T. vogelii* does not provoke any digestive lesion in Wistar rats, but the slight changes in biochemical parameters makes it foreseeable to prohibit fishing with *T. vogelii* and to prevent their consumption by humans.

Key words: *Tephrosia vogelii*, ichtyotoxic, Wistar rat.

INTRODUCTION

Fishing provides 44% of halieutics products. There are 90,000 tons of fish used for this purpose in Benin per year (DP, 2011). Fishing contributes to 3% of the National Gross Domestic product (GDP) (Food and Agricultural Organization (FAO), 2008). Moreover, because of increasing demography and fish consumption, which is 9.2 kg per year per capita (ImorouToko, 2011), the demand for halieutics products remains unsatisfied. The use of many prohibited ways for fishing like the powder of *Tephrosia vogelii* in rivers is a secular practice in Africa and American- Southern areas (Kerharo et al., 1974). It is true that this practice allows for the collection of a large

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amounts of fish that are sold in local markets for human consumption. However, the toxicity of *T. vogelii* is not very selective and has been observed in both macrofauna (fish, batrachians and reptiles) and microfauna. *T. vogelii is* very toxic, even with very weak dilution and by simple contact with cold-blooded animals (Elouard et al., 1982). The rotenone, principal component of *T. vogelii,* is carcinogenic, according to Bourgois (1989). Unfortunately in Benin, information about its negative effects on consumers and fishers of fish captured with *T. vogelii is* not evaluated, which is the importance of this study. There are scanty reports in this field. Thus, the aim of our present study was to evaluate: 1) the effect of the powder of *T. vogelii* on the liver and kidney of Wistar rats, 2) the effects of the fish meal poisoned with the powder of *T. vogelii* on the plasmatic level of ALAT of the Wistar rats, and 3) the effects of the fish meal poisoned with the powder of *T. vogelii* on the plasmatic level of ASAT of the Wistar rats.

MATERIALS AND METHODS

Animals and experimental model

Thirty (30) male Wistar rats were used in this study. Their weights were between 130 and 150 g. Before assays, they were acclimatized to the conditions of breeding of the Institute of Applied Biomedical Sciences (IABS) at the fairground of Cotonou. They received water and a standard food distributed *ad libitum*. They were divided into three groups of ten (10) rats: group 0 (control): 10 rats fed with a ration without fish meal poisoned during 28 days; group 1: 10 rats fed with a ration of fish meal poisoned at a concentration of 400 powder mg/L of *T. vogelii* during 28 days; and group 2: 10 rats fed with a ration of fish meal poisoned at a concentration of 800 powder mg/L of *T. vogelii* during 28 days.

The temperature of experimental location was maintained at 22°C, the ambient humidity was 60% and a photoperiod of 12 h/24 followed. The fish were captured in the Lac Nokoue; their average weight and length were respectively 140 ± 2 g and 13 ± 0.5 cm.

Plants

The leaves of *T. vogelii* were collected in the Commune of Bohicon, Benin. The leaves were dried during two weeks at 22°C in the Laboratory of Histology of IABS. They were then reduced to fine powder and conserved in sterile plastic bags. The powder was served to poison fish.

Laboratory materials

The proportioning of the enzymes was carried out using a spectrophotometer of mark "Thermo Electron"; ALAT and ASAT kits were from ‘‘Etablissement Français du Sang Alpes-Méditerranée 149, Bd Baille 13392 Marseille Cedex 5’’.

Obtaining the fish meal of *Sarotherodon melanotheron* poisoned with the powder of *Tephrosia vogelii*

S. melanotheron fish were fished in Nokoue Lake in the acadjas. They were maintained alive in a container filled with water. Then, fish were poisoned with the powder of *T. vogelii of* four hundreds (400) and eight hundred (800) milligrams per liter of water. They were then cured and reduced to flour for the food of the Wistar rats.

Blood sampling and proportioning of ALAT and ASAT

From the caudal vein, 2 ml of blood was taken in vacuum tubes from each rat. Blood sampling was done at days 0, 14 and 28. The proportioning of ALAT and ASAT in sampled bloods was done using commercial kits.

Autopsy of the rats and removal of their bodies

Following the blood sampling, an autopsy was carried out on 20% of each group. The liver and the kidney of the rats were collected and then fixed in 10% formol for histological examination after coloring with hematoxylin-eosin. Observations were done with a light microscope.

Statistical analyses

After the treatment, data on the ALAT and the ASAT were analyzed by analysis of variance (ANOVA) (type one) with the software Statistica 6.0 (1998). To know the differences, the test of Newman-Keuls was used with an error risk p: $p > 0.05$ (the difference is not significant) and $p < 0.1\%$ (highly significant difference).

RESULTS

Behavioral observation of the rats after intoxication

The poisoned Wistar rats did not present any sign of disease (weakening, diarrhea, or vomiting). No mortality was recorded.

Histological characteristics of the liver and the kidney of the rat fed with fish poisoned with powder of *T. vogelii*

Among the various organs of the rats fed with the fish meal containing *T. vogelii, there was* slight structural modification on the liver and kidney. The histological sections of the liver and kidney of groups 0, 1 and 2 are presented in Figures 1, 2, 3 and 4.

Biochemical parameters

Plasmatic levels of ALAT

The plasmatic levels of the ALAT (UI/L) in the control rats were 69.3 ± 0.8, 70.3 ± 0.8 and 61.6 ± 4.2, respectively at days 0, 14 and 28. Regarding the rats of group 1, the values of the ALAT were 72.3 ± 0.5 at day 0, 74.4 ± 0.6 at day 14, and 76.2 ± 0.3 at day 28. In group 2, the values of the ALAT were 73.1 ± 0.1 at day 0, 75.2 ± 0.5 at day 14, and then 77.3 ± 0.2 at day 28. At day 0, there was no significant difference at 5% threshold between the

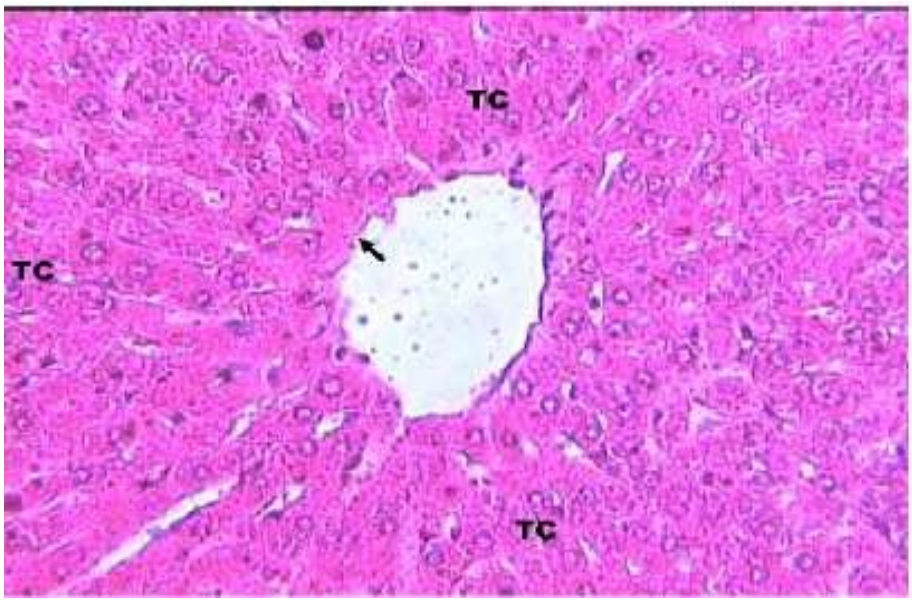

Figure 1. Normal liver of Wistar rat in group 0: ×20 (HE). Liver lobule showing a central vein (arrow) around which the liver cells are organized in cell spans (TC) arranged in radial way.

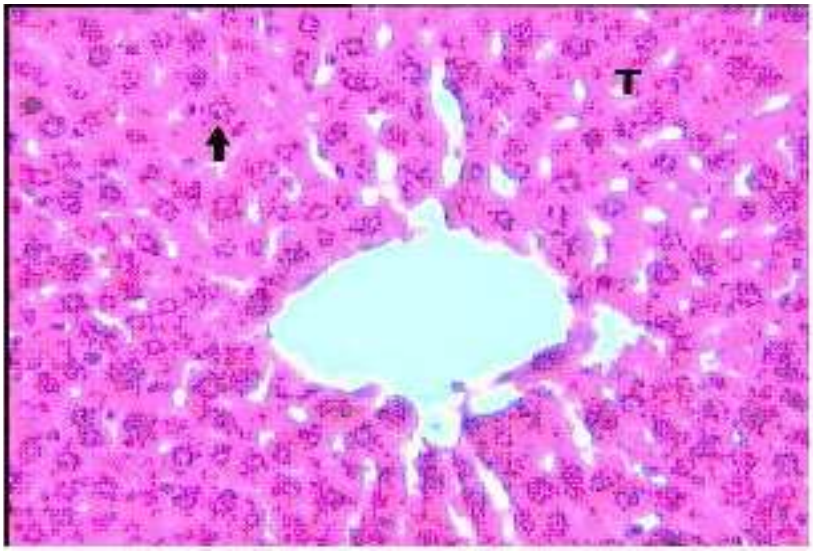

Figure 2. Wistar rat liver, groups 1 and 2: × 20 (HE). Slight necrosis of hepatocyte marqued by a homogenization of the structure of cytoplasm that becomes eosinophilic with disappearance of limit cells.

various groups with regard to the plasmatic level of the ALAT. The plasmatic levels of the ALAT did not vary either significantly at days 14 and 28 for group 0; but, in groups 1 and 2, values increased very significantly with the experimental duration. This is more remarkable in the group 2 rats fed with fish poisoned with *T. vogelii* at a rate of 800 mg/L, which have a significant difference at the threshold of 0.1% (Table 1).

Plasmatic levels of ASAT in the Wistar rats

The plasmatic levels of the ASAT (UI/L) in the control rats were 41.0 ± 3.1, 45.6 ± 2.3 and 47.3 ± 6.8, respectively at days 0, 14 and 28. In group 1, the level of the ASAT was 47.3 ± 4.3 at day 0, 50.3 ± 1.2 at day 14, and 54.6 ± 2.1 at day 28. In group 2, the values of the ASAT were 47.6 ± 3.3 at day 0, 5.2 ± 1.1 at the day 14, and 53.4 ± 0.3 at

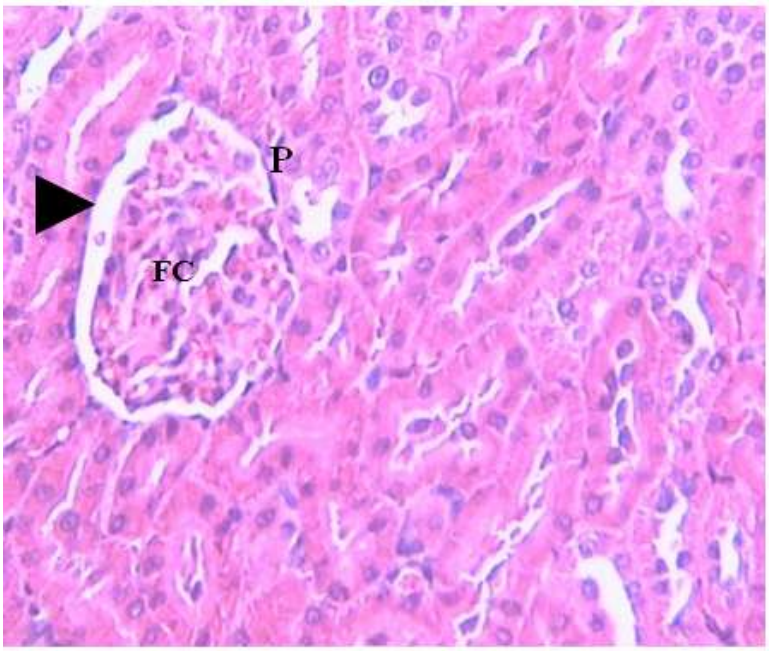

Figure 3. Normal Wistar rat kidney, group 0: × 20 (HE). Renal cortex showing a glomerulus with the capillary flocculates (CF), glomerular room (arrowhead) and the vascular pole (P) and the excretory ducts.

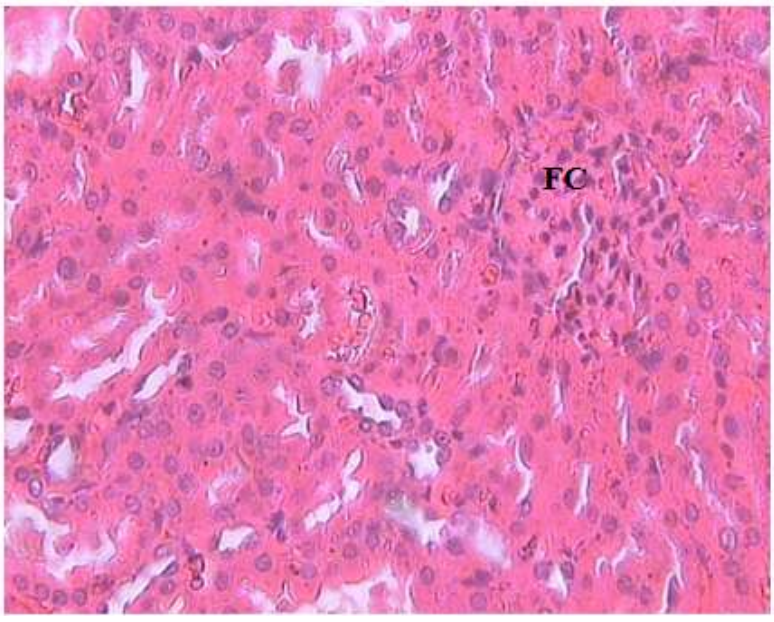

Figure 4. Wistar rat kidney, groups 1 and 2: × 100 (HE). Structural changing marqued by the thickening of the interstitium with compresses of the flocculus capillary and disappearance of glomerular room (FC).

day 28. At the beginning of the experimentation, there was no significant difference at a threshold of 5% between the various groups with regard to the value of the ASAT. It is the same with the experimental duration on the plasmatic levels for the ASAT in control group. However, the values of this hepatic enzyme evolved very significantly in groups 1 and 2 as the experimental duration was prolonged (Table 2).

Table 1. Plasmatic levels of ALAT (UI/L).

Group	Day		
	0	14	28
Group 0	69.3 ± 0.8[a]	70.3 ± 0.8[a]	61.6 ± 4.2[a]
Group 1	72.3 ± 0.5[a]	74.4 ± 0.6[b]	76.2 ± 0.3[c]
Group 2	73.1 ± 0.1[a]	75.2 ± 0.5[b]	77.3 ± 0.2[c]
Probability	0.75[NS]	<0.001**	<0.001**

Averages followed by the different letters are highly significant: P <0.001: highly significant; NS= not significant.

Table 2. Plasmatic level of ASAT (UI/L).

Group	Day		
	0	14	28
Group 0	41.0 ± 3.1[a]	45.6 ± 2.3[a]	47.3 ± 6.8[a]
Group 1	47.3 ± 43[a]	50.3 ± 12[b]	54.6 ± 2.1[c]
Group 2	47.6 ± 3.3[a]	52.1 ± 1.1[b]	53.4 ± 0.3[c]
Probability	0.75[ns]	<0.001**	<0.001**

Averages followed by the different letters are highly significant: P <0.001: highly significant; NS= not significant.

DISCUSSION

Effect of the powder of *Tephrosia vogelii* on the liver and kidney of Wistar rats

During our experimentation, no clinical sign of intoxication such as vomiting or hyper-salivation was observed in the Wistar rats. This is justified by the quantity of powder of *T. vogelii* involved which could not have been sufficient to cause the symptoms of a possible intoxication. Indeed, according to Morris and Powell (2000), the absorption of the rotenone, active ingredient of *T. vogelii* was relatively slow and incomplete in the stomach and intestine. Moreover, the liver metabolizes quickly the components of the rotenone (Ling, 2003). In addition, the poisoned fish were eviscerated before being reduced to powder for the food of the rats. This method was adopted in order to imitate the art of fish cooking before their consumption by humans.

The fish meal eaten by Wistar rats was obtained after curing of poisoned fish. According to Ross Robertson and Smith-Vaniz (2008), the rotenone, one of the toxic substances of *T. vogelii*, was thermolabile. The rotenone was then destroyed through the curing of fish used to intoxicate the rats; but, among the various organs of the rats fed with the fish meal poisoned with *T. vogelii* , some lesions were found on the cells and structures of the liver and Kidney. Therefore, as little as *T. vogelii* powder in the body can induce several disturbances on the organs. These results are contrary to those of Ross Robertson and Smith-Vaniz (2008) who assert that the poisoned fish kept in their flesh negligible quantity of the toxic substance which accumulates mostly in the internal organs (internal and gills). These internal organs were not consumed by the rats in the present study.

Indeed, Dzenda et al. (2008) affirmed that the lesions of the liver and kidneys appeared after a chronic intoxication. In the same way, according to Morris and Powell (2000), rats having consumed more than 2.5 mg/kg of rotenone during two years did not develop any pathological signs, which could be due to rotenone. Therefore, from this experimentation, it is noticed that rotenone is not the only substance which could cause lesions on Wistar rats' liver and kidney because the amount of deguelin seems higher compared to rotenone (Kalume et al., 2012). For Dzenda et al. (2008), *T. vogelii* contains, in addition to rotenone, deguelin; while the former is thermolabile, the latter is not destroyed by heat. The biochemical disturbances could be related to the activity of this heat-resisting compound.

Effects of the fish meal poisoned with the powder of *Tephrosia vogelii* on the plasmatic level of ALAT of the Wistar rats

The normal values of the ALAT in the rats fed with fish not poisoned with *T. vogelii* are in accordance with those defined by Kamdem et al. (1981); they are an average of 74.7 ± 3.8. In addition, the muscular exercise can modify the plasmatic level of the ALAT as remarked by Lecoanet (1981). The levels of the ALAT approximately increased in the rats of groups 1 and 2 fed with fish poisoned with 400 and 800 mg/L powder of *T. vogelii*. These results confirm the remark through histological section because, significantly elevated levels of ALAT suggest the existence of medical problems such as viral hepatitis, diabetes, congestive heart failure, liver damage, bile duct problems, infectious mononucleosis, or myopathy. So ALAT is commonly used as a way of screening for liver problems. The increase in its values after the feeding of the rats with fish poisoned with *T. vogelii* indicates a beginning of functional disturbances in these organs. For example, according to Scheurer et al. (2002), in the viral hepatitis, values from 500 to 1500 UI/L were reported in humans; better, values higher than 3000 UI were met in the presence of acute toxic necrosis or serious hypoxia during a hepatic ischemia.

Effects of the fish meal poisoned with the powder of *Tephrosia vogelii* on the plasmatic level of ASAT of the Wistar rats

The normal values of the ASAT obtained in the rats of group 0 in the present study differ from those reported by Kamdem et al. (1981) which were, respectively 178 ± 20.99 and 200 ± 26 UI/L. These results, on the other hand, harmonize with the rate of 42.9 ± 10.1 UI/L established by Kaneko (1989). The factor 'age' is

responsible for these cases. The consumption of fish meal poisoned by *T. vogelii* increased the rate of the ASAT in the rats of groups 1 and 2. This enzymatic increase, although light, means surely a beginning of functional deteriorations of the liver of these rats. This enzyme is found in many tissues: liver, heart, kidney, muscles, intestines and the ASAT and ALAT ratios (ASAT/ALAT ratio) are commonly measured clinically as biomarkers for liver health. Nevertheless, its activity is more significant in liver, heart and the muscles as remarked by Schuck and Alain (1997). The increase in the rate of ASAT is constant during acute hepatitis (Banting et al., 1975). In the present study, the results of the histological section examination revealed hepatic lesions; however, the significant increase in the biochemical parameters could certify the structural modification on liver. This could be confirmed with structural modifications like necrosis tissue hepatic observed in rats poisoned directly by the powder of *T. vogelii,* according to results of Morris and Powell (2000).

Conclusion

This study aimed to evaluate toxicity of *T. vogelii*. The livers and kidneys of Wistar rats revealed histological lesions. However, the plasmatic levels of alanine-amino-transaminase and aspartate-amino-transaminase were slightly increased in the rats fed with fish meal of *Sarotherodon melanotheron* captured by *T. vogelii*. Based on the toxicity of *T. vogelii* employed for the fishing, it is urgent and necessary that further studies continue on longer duration, and with higher amounts of intoxication to encircle closely the mode of action of degueline and rotenone which are active substances of *T. vogelii*. Total and fast evisceration of fresh fish and the prohibition of the ichtyotoxic plants as resource of fishing are actually the main ways to protect fish consumers in Benin.

Conflict of interests

The authors have not declared any conflict of interest

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academicJournals

Vol. 15(11), pp. 423-430, 16 March, 2016
DOI: 10.5897/AJB2015.15007
Article Number: 3742E1D57561
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production of thermophilic and acidophilic endoglucanases by mutant *Trichoderma atroviride* 102C1 using agro-industrial by-products

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Received 23 September, 2015; Accepted 19 January, 2016

Many traditional mutagenic strategies have been used to improve cellulase production by micro-organisms, especially fungi species. *Trichoderma* species are among cellulolytic fungi, those that have been most extensively studied, due to their efficient production of these enzymes. In the present study, N-methyl-N´-nitro-N-nitrosoguanidine (NTG) was used as mutagenic agent to obtain cellulolytic mutant from wild strain *T. atroviride* 676. After mutagenic procedures, two strains (102C1 and 104C2) were selected as promising cellulase-producing mutant. The effect of the carbon (sugarcane bagasse: SCB) and nitrogen (corn steep liquor: CSL) sources on endoglucanase production by the mutants 102C1 and 104C2 was studied using submerged cultivations at 28°C. Different concentrations of SCB and CSL were used and nine different media were generated. Mutant 102C1 showed the best results when using 2.5% SCB and 0.7% CSL. A central composite rotational design (CCRD) was performed to estimate optimal conditions of pH and temperature for endoglucanase activity of strain 102C1, which were pH 3.6 and temperature 66°C. The characterization of this acidophilic and thermophilic endoglucanase activity produced by the mutant strain 102C1 allows its use in biotechnological applications, particularly in the hydrolysis of agro industrial residues, such as SCB, for bioethanol production.

Key words: *Trichoderma atroviride*, CMCase, nitrosoguanidine, sugarcane bagasse, corn steep liquor.

INTRODUCTION

Cellulose, a glucose polymer linked by β-1,4 bonds, is considered to be the primary product of photosynthesis and carbon renewable resources that is highly abundant in nature (Gottschalk et al., 2010; Deswal et al., 2014). Lignocellulosic material can be used to produce ethanol after cellulose hydrolysis with cellulases. The production

cost of cellulase is still the major obstacle associated to hydrolysis of cellulosic materials into fermentable sugars. Therefore, improvements towards a cost effective cellulase production is essential to make this process economically viable (Adsul et al., 2007; Soccol et al., 2010; Borges et al., 2014). The cellulase complex secreted by filamentous fungi consists of three main components, endo-1,4-β-glucanase (EC 3.2.1.4), β-1,4-glucan cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). These three enzymes act in synergy during the conversion of cellulose to glucose (Wilson, 2011).

Significant research efforts have been invested in evaluating and understanding the enzymatic hydrolysis of lignocellulosic substrates by cellulases produced by *Trichoderma* species (van Wyk and Mohulatsi, 2003; Palonen et al., 2004), which are several hundred times more active than those produced by bacteria (Adsul et al., 2007; Martins et al., 2008). Various *Trichoderma* strains have been extensively evaluated and implemented in processes for bioethanol production (Rosgaard et al., 2006), including studies on low cost production of cellulases using lignocellulosic residues (Kovàcz et al., 2008; Grigorevski-Lima et al., 2013).

Trichoderma reesei is the most studied species of *Trichoderma* concerning cellulase production. Many traditional mutagenic strategies have been used to improve this characteristic, nevertheless, these attempts have not been totally successful yet (Chand et al., 2005; Kovàcz et al., 2008; Kovàcz et al., 2009; Jiang et al., 2011), since current enzyme production involves high costs and the production process is not yet fully defined (Adsul et al., 2007).

The identification of new cellulase high producing mutants will contribute to lower ethanol production costs especially when using sugarcane bagasse and corn steep liquor as the sole organic substrates. The use of these residues proves an efficient utilization of crops, where not only primary products, but also their by-products are use, which turns the process economically sound.

In the present study we used a mutagenic strategy to obtain a mutant library from *T. atroviride* 676, previously described as a good cellulase producer (Grigorevski-Lima et al., 2013). This library was screened to identify the most promising cellulase producer mutant and best levels of sugarcane bagasse, as carbon source, and corn steep liquor, as nitrogen source, in the production of CMCase by the selected mutant. Finally, a central composite rotational design (CCRD) experiment was performed to estimate the optimal conditions of pH and temperature for best endoglucanase activity of the selected strain.

MATERIALS AND METHODS

Microorganisms

T. atroviride 676 was isolated from the Amazon rainforest soil, and was obtained from the culture collection of Centro de Pesquisa Leonidas e Maria Deane, FIOCRUZ, Manaus, Brazil. Earlier, this strain proved promising for cellulase production (Grigorevski-Lima et al., 2013). During the present research mutants were obtained from this wild strain, using two subsequent mutations, and these used for cellulase production. Spore suspensions of the fungi were prepared according to Hopwood et al. (1985) after cultivation (28°C/15 days) in yeast extract-malt extract-agar medium (Shirling and Gottlieb, 1966) and maintained as stock cultures in 20% (v/v) glycerol at -20°C. Spore concentration was determined using Neubauer counting chamber.

Mutants strains

These were obtained by using nitrosoguanidine (NTG) and were based on Kovàcz et al. (2008). In a first experiment, the system was prepared using 100 µl of a spore suspension (10^7 spores ml^{-1}) of *T. atroviride* 676 and 2.0 ml of a sterile solution of 1.0% NTG and incubated for 8 min at room temperature. The suspension thus obtained was submitted to decimal dilutions and 0.1 ml of each one spread-plate inoculated in carboxymethylcellulose (CMC) medium based on Kovàcz et al. (2008), however a Congo red solution was added (Montenecourt and Eveleigh, 1977) and also, yeast extract was replaced by corn steep liquor (CSL) (SIGMA®, presented as corn steep solids, a spray-dried corn soluble). After 7 days incubation at 28°C, the grown colonies were isolated as pure cultures. Each strain was point inoculated into CMC-Congo red medium in Petri dishes, and after incubation for 7 days at 28°C, the strain presenting the wider halo (NTG21) was selected. Another mutation with NTG 1.0% was performed, as described earlier, using two strains for the experiments, strain 676 and strain NTG21, however incubation time was for 12 and 15 min, respectively. Cellulase production of the pure cultures thus obtained was confirmed by using cellulose-Congo red medium (César and Mrsa, 1996) and then cellulose-azure medium (Plant et al., 1988). As a preliminary fermentation study, the positive ones were then cultivated in 250 ml Erlemeyer flasks with 1/5 of its volume filled with a liquid medium (Mandels and Weber, 1966) containing sugarcane bagasse (SCB) (3.0%) and CSL (SIGMA®, as above) (0.3%) as C and N sources, respectively, at pH 4.8 and inoculated with 3.0 ml of a dense spore suspension. After 3-days of incubation at 28°C under agitation (200 rpm), supernatants were filtrated on fiber glass filter and used to measure the endoglucanase (CMCase) activities. The mutants showing a higher CMCase activity, at least two times the one observed by the original strain, were selected for further experiments.

Endoglucanase production

The enzyme production was performed in submerged fermentation using two selected mutants, in 250 ml Erlenmeyer flasks filled 1/5 of its volume with a culture medium based on the salt solution plus urea described by Mandels and Weber (1966) and added with different concentrations of sugarcane bagasse *in natura* (SCB – main carbon source) and corn steep liquor (CSL – main nitrogen

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source). A combination of different concentrations of carbon and nitrogen sources was performed in order to determine the good conditions for endoglucanase production by *Trichoderma atroviride* 102C1. Five concentration values were tested for SCB and CSL which were 1.1, 1.5, 2.5, 3.5 and 3.9% for SCB and 0.15, 0.3, 0.7, 1.1 and 1.25% for CSL, generating, in total, nine different media (Table 2). The initial pH of all media was adjusted to 5.0. Each set of flasks was inoculated with 25 µl of a spore suspension (10^8 ml^{-1}) of each studied strain and incubation was performed at 28°C in orbital shaking at 200 rev min^{-1} for 3 days. The supernatants, which corresponded to crude enzyme extracts, were used to determine endoglucanase activities.

Enzyme assays

Endoglucanase activity (CMCase) was estimated by reaction mixture containing 500 µl of a solution of 2.0% (w/v) carboxymethylcellulose low viscosity (CMC, SIGMA®) in 50 mM sodium citrate buffer (pH 4.8) plus 500 µl of the supernatant (Ghose 1987). This system was incubated for 6 min at 50°C. The reducing sugars concentration in the reaction mixture was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). All assays were performed in duplicates, and results were expressed as average values. Variations in the multiple assays were < 10%.

Crude enzyme partial characterization

A culture supernatant of 3-days fermentation [SCB 2.5% (*w/v*) and CSL 0.7% (*w/v*)] from *T. atroviride* 102C1 was used to investigate the temperature and pH effect on CMCase activity. The enzyme characterization was carried out by employing a response surface methodology having CMCase activity (U ml^{-1}) as the independent variable and pH (between 3.0 and 7.0) and temperature (range of 40 to 70°C) as the dependent variables. A 2^2 full factorial central composite rotational design (CCRD) was used in order to generate 11 run combinations as described in Table 3. This design is represented by a second-order polynomial regression model (as Equation 1, where Y is the predicted response CMCase activity; and X_1 and X_2 the coded forms of the input variables, pH and temperature, respectively) and the test factors coded according to Equation 1. Buffer solutions at 50 mM, (sodium citrate buffer for pH 3.0, 3.6 and 5.0, and phosphate buffer for pH 6.4 and 7.0) and was used at the optimal temperature previously determined. Data analysis was performed using the Statistica 7.0.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (1)$$

$$x_i = (X_i - X_0) / \Delta X_i \quad (2)$$

Zymogram

The culture supernatant from cells grown on the best conditions was analyzed by electrophoresis on denaturing 10% sodium dodecyl sulfate-polyacrylamide gel, copolymerized with 0.1% (w/v) CMC (SIGMA®) as substrate. Electrophoresis was performed at constant voltage (90 V) for 3 h at 4°C. After electrophoresis, gel was incubated with Triton X-100 sodium acetate (1.0 %) for 60 min in ice bath for SDS removal and then incubated with sodium citrate buffer at optimum pH and temperature for 6 minutes. For detection of the enzyme activity, the gel was submerged in 0.1 % Congo red solution for 10 min and then washed with NaCl 1 M until visualization of enzyme bands (César and Mrsa, 1996). Molecular masses were calculated from mobility of standards ranging from 14 and 225 kDa (Amersham).

RESULTS

The *T. atroviride* 676 wild strain was previously identified as promising producer of enzymes of the lignocellulolytic complex (Grigorevski-Lima et al., 2013). In the present study, mutants obtained using NTG as mutagenic agent, were screened to identify those displaying increased production of endoglucanases. In a first mutation using strain 676, 15 strains were obtained and NTG21 was selected based on a qualitative test in CMC-Congo red solid medium. In a second mutation, strain 676 and strain NTG21 were used, and then 27 strains were obtained, 24 from strain NTG21 and 3 from strain 676. When these strains were tested for cellulase production in CMC-Congo red and cellulose-azure media, they were all positive. In a subsequent preliminary test, CMCase activity was measured for each strain after three days of cultivation in medium containing SCB (3.0%) and CSL (0.3%). Out of the 27 mutants tested, 14 (52%) showed CMCase activities greater than the wild type and, among these, two (102C1, mutant of NTG21 and 104C2, mutant of 676) were especially interesting, presenting values more than 2.2 times higher than the original strain (Table 1).

The promising selected strains, 102C1 and 104C2, were cultivated for 3 days in different concentrations of SCB and CSL under submerged fermentation conditions. Maximal values of CMCase obtained from submerged fermentation are presented in Table 2. CMCase activity produced by strain 102C1 ranged from 1.01 to 2.93 U ml^{-1}, whereas values obtained for strain 104C2 where lower (from 0.29 to 1.77 U ml^{-1}). The highest CMCase production (2.93 U ml^{-1}) was detected in medium 9, when SCB 2.5% (w/v) and CSL 0.7% (w/v) were used, being 84.2% higher than strain *T. atroviride* NTG21. The pH and temperature profiles for CMCase activity produced by strain 102C1 were determined using crude extract of the strain grown in SCB and CSL at 2.5 and 0.7% concentrations, respectively, corresponding to the optimal conditions identified earlier. The maximum enzyme activity was 3.37 U ml^{-1} which was observed at 66°C and pH 3.6 (Figure 1).

The regression equation obtained after analysis of variance (ANOVA) (Table 4) showed CMCase production in coded values of sugarcane bagasse and corn steep liquor. The F-value of 111.22 and P < 0.1 value indicate the importance and relevance of the model. The obtained coefficient of regression (R^2=0.9911) indicated that 99.1% of the variability shown in the responses might be explained by the model. The equation that represents the model for the production of this enzyme (Y) under these conditions is given as follows:

EG= 2.77 - 0.76*SCB - 0.55*SCB^2 + 0.22*CSL - 0.25*CSL^2 - 0.39*SCB*SCL + 0.00262

Crude enzyme extract obtained on the 3rd day of

Table 1. CMCase activity of mutant strains obtained after treatment of *T. atroviride* NTG21 and *T. atroviride* 676 with NTG 1.0% for 15 and 12 min respectively. Values of CMCase were obtained after 3-days cultivation in a liquid medium containing SCB (3.0%) and CSL (0.3%). CMCase values obtained for the wild strains are presented for comparison.

Strains	Treatment with NTG 1.0% (Contact time, min)	CMCase (U.ml^{-1})
***T. atroviride* NTG21**	**-**	**1.59**
102 A3		3.08
102 B1		0.54
102 C1		**3.62**
102 C2		3.22
102 C3		0.37
102 C4		2.97
103 A2		3.41
104 A1		3.09
104 A2		1.93
104 A3		2.95
104 A4		1.65
104 A5	15	1.8
103 A1		0.94
103 B1		1.05
104 A7		1.29
104 C1		0.16
104 C2		0.57
101 A2		1.35
102 C5		0.42
104 A6		1.57
104 B1		1.19
102 A2		1.59
102 C6		1.73
101 B4		1.48
***T. atroviride* 676**	**-**	**1.37**
104 A1		1.69
104 C1	12	1.85
104 C2		**3.5**

Table 2. Media composition used in the different submerged fermentation conditions for CMCase production by *Trichoderma atroviride* 102C1 and 104C2.

Medium	Raw-material source		CMCase activity (U ml^{-1})	
	Sugarcane bagasse % (*w/v*)	Corn steep liquor % (*w/v*)	Strain 102C1	Strain 104C2
1	1.50	0.30	1.22	0.29
2	3.50	0.30	1.23	0.46
3	1.50	1.10	1.01	0.37
4	3.50	1.10	1.17	0.90
5	1.10	0.70	1.80	0.35
6	3.90	0.70	2.21	**1.77**
7	2.50	0.15	2.04	0.34
8	2.50	1.25	2.54	0.50
9	2.50	0.70	**2.93**	1.06

All media were supplemented with a salt mineral solution (see Material and Methods).

Table 3. Values of independent variables (pH and temperature), used in CCRD, showing the values observed and predicted by the mathematical model for CMCase activity characterization for strain 102C1 .

Run	Coded setting levels		Actual levels		CMCase activity ($U.ml^{-1}$)	
	pH	Temperature	pH	Temperature	O	P
1	– 1	– 1	3.6	44	2.27	2.12
2	+ 1	– 1	6.4	44	1.48	1.38
3	– 1	+ 1	3.6	66	**3.37**	3.36
4	+ 1	+ 1	6.4	66	1.02	1.05
5	– 1.41	0	3.0	55	2.68	2.76
6	+ 1.41	0	7.0	55	0.58	0.61
7	0	– 1.41	5.0	40	1.81	1.95
8	0	+ 1.41	5.0	70	2.62	2.59
9	0	0	5.0	55	2.72	2.78
10	0	0	5.0	55	2.78	2.78
11	0	0	5.0	55	2.83	2.78

Results are the mean of two experiments; *O* observed, *P* predict.

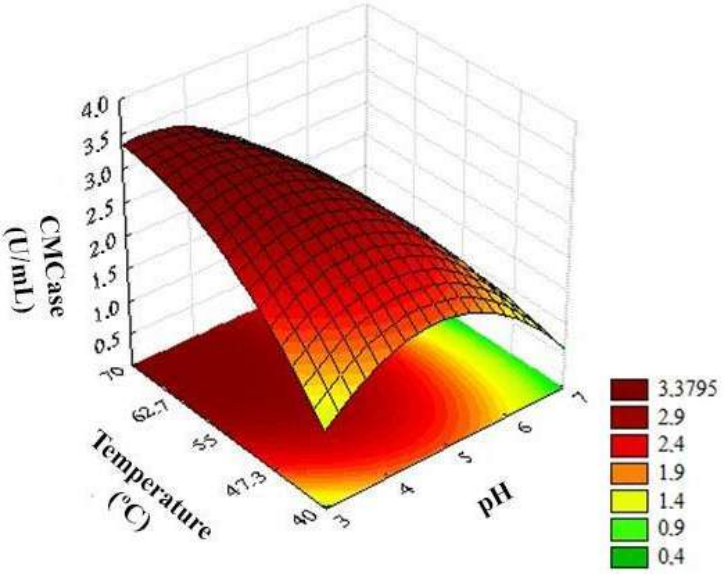

Figure 1. Response surface on partial CMCase characterization from *T. atroviride* 102C1 using pH and temperature as independent variables. The full factorial central composite design (2^2) used response surface methodology to predict the best point for CMCase activity. The values are shown in Table 2.

Table 4. Statistical ANOVA for the model of CMCase activity at different levels of pH and temperature.

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (prob>F)[a]
Model	7.40	5	1.48	111.22	<0.0001
Residual	0.067	5	0.013	-	-
Lack of fit	0.061	3	0.020	7.81	0.1156
Pure error	0.005234	2	0.002617	-	-
Total	7.47	10	-	-	-

[a]Statistically significant at 90% of confidence level; R^2= 0.9911.

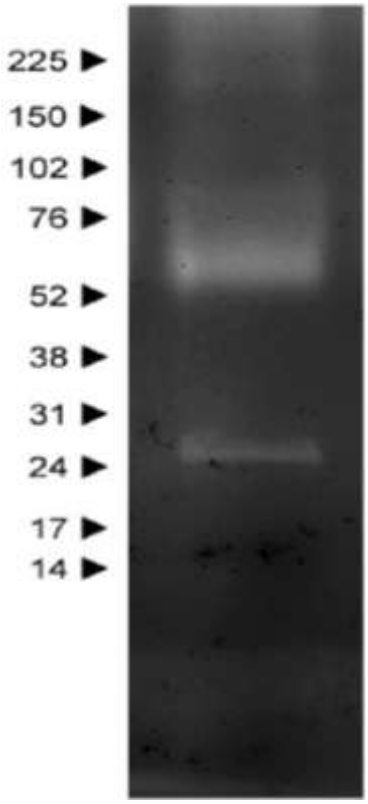

Figure 2. Zymogram analysis of CMCase activity in the supernatant of *T. atroviride* 102C1 cultures grown on best condition. The amounts loaded in the gel contained 500 mU of CMCase activity. The gel containing the MW markers was stained for proteins using the silver staining method and values for the MW markers are shown on the left side of the Figure. On the right side are shown the apparent molecular mass of CMCase bands.

fermentation in culture medium with 2.5% of sugarcane bagasse and 0.7% of corn steep liquor, which were the optimal conditions for the production of CMCase, was used to perform the zymogram experiments. Two bands with approximate molecular weights of 60.6 and 24.8 kDa were observed for CMCase activity (Figure 2).

DISCUSSION

In the present study, two mutants were selected, *T. atroviride* 102C1 and *T. atroviride* 104C2, obtained from the mutant strain *T. atroviride* NTG-21 and the wild type *T. atroviride* 676, respectively. These promising strains were tested over 3-days fermentation for endoglucanase (CMCase) production. Different combinations of SCB and CSL were tested, generating nine different mediums. In this case, the highest production was 2.93 U ml^{-1}, observed, for strain 102C1 when the concentration of C and N sources, were 2.5% for SCB, and 0.7% for CSL, respectively. When an experimental dosing was used to determine best conditions of temperature and pH for detection of enzymatic activity produced by strain 102C1, it was shown that the CMCase activity increased to 3.37 U ml^{-1} when temperature of detection was 66°C and pH 3.6.

T. atroviride 676 wild type has shown, earlier, ability to produce CMCase in lower amounts (1.37 U ml^{-1}) using the same substrates but with different concentrations of SCB (3.0%) and CSL (0.3%) as C and N sources, respectively, and also different conditions of temperature and pH for enzyme detection (Grigorevski-Lima et al., 2013). Our results using mutant *T. atroviride* 102C1 showed a 113.8% increase in enzyme activity compared to the results reported then. It is interesting to note that in our experiments, and also Grigorevski-Lima et al. (2013) research, the sugarcane bagasse used was not submitted to any treatment, as usually occurs in several studies, since, as it is well known, this would be more efficient. So, in our study, good results were also obtained using sugarcane bagasse *in natura* (untreated), which represents an economical cost-wise advantage, considering the elimination of time and efforts necessary for material processing.

The endoglucanase, referred as CMCase, is the enzyme most commonly found in cellulolytic microorganisms. Several studies have reported the production of CMCase using low cost materials as C and N sources, and mutant or wild type strains. Li et al. (2010) increased the production of CMCase up to 7% using *T. viride* mutants compared to the wild type strain. Chandra et al. (2009) observed around 3.0 U ml^{-1} in CMCase activity in *Trichoderma citrinoviride* mutant strains, which was three times higher than the wild type strain. Jiang et al. (2011) observed CMCase activity in *T. viride* mutants reaching 18 U ml^{-1}, which was also three times higher than in the wild type strain. Chand et al. (2005) measured about 0.415 and 0.60 U ml^{-1} in endoglucanase activity in *Aspergillus* mutants compared to 0.280 U ml^{-1} in the wild type strain. Another study tested *Penicillium echinulatum* using various cellulosic substrates and detected the maximum CMCase activity as 1.53 U ml^{-1} (Martins et al., 2008). Kovàcs et al. (2008) obtained 10 best *T. atroviride* cellulolytic mutants from wild strain TUB F-1505 using UV irradiation and NTG (0.1% *w/v*). The best endoglucanase activity observed in mutant strain (TUB F-1724) achieved 143.6 to 160.6 U ml^{-1}, while in the wild strain was 103 to 106 U ml^{-1}, corresponding to an increase of 50%, approximately. Hence, the 102C1 mutant strain is a good candidate for the industrial production of CMCase from untreated sugarcane bagasse and corn steep liquor since it is able to produce up to 2.93 U ml^{-1}, which is a high activity value when compared with some of those previously known.

The temperature and pH are important variables which affects the initial fermentation stage and hydrolysis rate. These profiles for optimal CMCase activity in 102C1 mutant strain supernatant were achieved at pH 3.6 and at 66°C. In a study with *Aspergillus aculeatus*, the optimal temperature for endoglucanase activity was 40°C (Naika et al., 2007). Kaur et al. (2007) observed two endoglucanases produced by thermophilic *Melanocarpus* sp. MTCC 3922 presented optimal enzyme activity at 50 and 70°C, respectively. Studies involving endoglucanases from *Trichoderma* strains described optimal activity at pH and temperature between 3.0 to 5.5 and 50 to 65°C (Gashe, 1992; Sul et al., 2004; Andrade et al., 2011). Our results show that the optimal CMCase activity occurred in the 102C1 mutant strain at more acidic pH (3.6), and at a high temperature (66°C).

The zymogram detected two intense CMCase bands with apparent molecular mass of 60.6 and 24.8 kDa (Figure 2). Javed et al. (2009) also detected an endoglucanase band produced by *Aspergillus oryzae* CMC-1 with apparent molecular mass of 25 kDa. Other studies have shown CMCase bands with different molecular masses, 51 kDa for *Trichoderma* sp. C-4 (Sul et al., 2004) and of 45 kDa for *A. aculeatus* (Naika et al., 2007), for instance. Two endoglucanase bands have also been identified in *T. atroviride* 676, the wild type of strain 102C1 (Grigorevski-Lima et al., 2013), but the reported molecular masses were 200 and 104 kDa, which are considered higher than fungal CMCase values commonly described in the literature. However, it is possible that their results represent enzyme complexes or aggregates of enzymes, which could explain the difference in values between those and the present study (Grigorevski-Lima et al., 2013).

Residue waste with biomass high-energy value is constantly generated by a variety of activities such as processing of agricultural products and by the paper and timber Industries. However, many of these residues are difficult to be degraded and become an environmental problem. Hydrolysis capabilities of cellulosic biomass play an important role enhancing the utilization of such residues. Thus, the selection of new fungal strains producing high levels of cellulases might contribute in advancing the use of cellulosic residues towards a variety of goals. Our study used untreated sugarcane bagasse as the carbon source to cultivate a fungus mutant strain producing high cellulase activity, which is an abundant material with low commercial value.

The conversion of biomass to biofuels has been the subject of intense research efforts and gained significant scientific and political force due to concerns about the shortage of fossil fuels and emission of greenhouse gases (Antoni et al., 2007; Service, 2007; Omer, 2014). The need for global energy is projected to double in the next two decades and thus, production of biofuels could become a source of carbon sustainable energy that is compatible with current and future engine technologies (Chu and Majumdar, 2012). Lignocellulosic biomass is, by far, the most abundant source of renewable sugars that can be fermented into biofuels such as ethanol. While the fermentation of corn starch or sugarcane juice by *S. cerevisiae* is a well-established technology, the hydrolysis of lignocellulosic residues is still challenging (Menon and Rao, 2012). Therefore, the development of new organisms with lignocellulolytic capacities is crucial to make this process economically viable. Recently, Oliveira et al. (2014) has characterized this same 102C1 mutant strain as an excellent xylanase producer in comparison to wild strain and others *Trichoderma* species. The authors are convinced that our fermentation results prove that our mutant might be suitable strains for practical applications, and the selection of a new mutant-type *T. atroviride* 102C1 as a good cellulase producer, allows its use in biotechnological applications, particularly in the hydrolysis of agro-industrial by-products, such as sugarcane bagasse and straw, for bioethanol production.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors thanks Marta de Souza Ferreira for technical support. This work was financially supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES).

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Vol. 15(11), pp. 431-441, 16 March, 2016
DOI: 10.5897/AJB2015.14891
Article Number: DE83FE857563
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity and structure of goats within an early livestock dispersal area in Eastern North Africa

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Received 30 July, 2015; Accepted 7 December, 2015

In this study we genotyped 14 microsatellites to assess genetic diversity, population stratification and demographic dynamics using Egyptian local goats (Zaraibi, Baladi, Saidi and Barki) and the Shami (Damascus) goat from the Middle East and West Asia close to the geographic center of goat domestication. We observed high levels of allelic and genetic diversity that was partitioned into six gene pools. Cluster analyses separated Zaraibi and Shami, which were identified with independent gene pools of potential ancestral backgrounds. The analyses provided further evidence for extensive sharing of genetic variation, revealing, varying levels of admixture among the study populations. This finding was supported by AMOVA analysis, which indicated that the proportion of genetic variation due to differences among populations was 7.06%. Our results most likely indicate that multiple waves of introduction of diverse gene pools and recent flock intermixing has created and maintained a unique set of caprine biodiversity in Eastern North Africa emphasizing the importance of the region as one of the hotbeds of African animal biodiversity.

Key words: Admixture, Bayesian clustering, bottleneck, expansion, Egypt.

INTRODUCTION

The goat (*Capra hircus*) was the first livestock species to be domesticated for purposes of human consumption. Archaeological evidence points to two possible centers of goat domestication; one in the Euphrates valley in southeastern Anatolia dating around 10,500 years before present (YBP) (Peters et al., 2005) and the other in the Zagros mountains dating to 9,900 to 9,500 YBP (Zeder and Hesse, 2000; Zeder et al., 2006). The occurrence of this phenomenon, close to the geographic center(s) of the first known ancient civilizations (Mesopotamia, Egypt, Indus valley etc) ensured a direct and tight connection between goats and most aspects of human socio-cultural

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and economic life (Boyazoglu et al., 2005). Today, there are more than 300 breeds of goats found in diverse agro-ecologies (Luikart et al., 2006) where they are a critical component of the agro-biodiversity.

Eastern North Africa (Egypt and Sudan) and the Maghreb (Algeria, Morocco, Tunisia and Libya) are important regions in the history of African goats. Radiocarbon dates suggest a rapid dispersal of goats to Africa from Southwest Asia via two routes; one along the North African Mediterranean Sea coast, and the other via the Red Sea Hills region of the Egyptian Red Sea coast (Zeder, 2008). These routes have been corroborated by mtDNA data which also support multiple waves of introduction (Naderi et al., 2008). Wetterstrom (1993) suggested a third and more recent terrestrial route via the Sinai Peninsula and Nile Delta into the Nile Valley. The genetics of local goats in Eastern North Africa and the Maghreb may have been influenced by multiple waves of introduction of goats from different genetic backgrounds and therefore are important in understanding the genetic foundation, demographic dynamics and evolutionary history of African goats.

Goats have contributed significantly to Egypt's gross national livestock product since approximately 5000 BC (Galal et al., 2005). The local goats are raised as multipurpose animals, have remained nondescript since ancient times and, a systematic assessment of their inherent genetic variation remains to be done. Nevertheless, six breeds are currently recognized, three main (Baladi, Barki, Zaraibi) and three minor (Wahati, Saidi, Black Sinai) ones, respectively (Galal et al., 2005). Some local goat breeds have been of particular interest due to their recognized features and characteristics. The Barki goat derives its name from Barka region in Libya where it is common and possibly derived from. It occurs in the Northwestern coastal desert where it is known as "Saharawi" or "of the desert"; due to its excellent adaptation to hot arid desert conditions (Galal et al., 2005). The Baladi (synonym to local or indigenous) is the native goat of Egypt. It shows high phenotypic variation among subpopulations from different agro-ecologies. It is widespread in the Nile Delta and along the Nile Valley where they are called "Local" or "Sharkawi" while in southern Egypt, they are called "Saidi". The Zaraibi is regarded as the most promising goat breed in Egypt. It occurs within a restricted geographic area in the fringes of the Northeastern Nile Delta. It is also called "Nubian" or "Nubi", after the Nuba area of Southern Egypt, where it is presumed to originate from. It is also presumed to be the progenitor of the Anglo-Nubian breed. However, these presumptions were disputed in interviews with flock owners during sampling. The Saidi is found in Southern Egypt. Phenotypically it resembles the Baladi, except that it has a larger head and body. It has a better tolerance to high temperatures due to the introgression of Sudanese goats from the South of Egypt. This introgression however, remains a matter of speculation.

Although the intensification of livestock production in the second half of the 20th century saw the widespread introduction of exotic breeds in the tropics and subtropics, the inhospitable desert conditions in Egypt, precluded the introduction of such breeds and favored the proliferation of better adapted local populations. The gene pool of local goats has therefore remained in its "pure unadulterated form" and local goats are so far the only ones that are found, and, have been described in Egypt (Galal et al., 2005). However, breeds/populations are not static entities and in the absence of stringent artificial selection, populations evolve and diverge over time to fit the diversity of local environments. Traditional management systems, (transhumance, nomadic pastoralism) as well as commercial and socio-cultural exchanges provide opportunities for intermixing of flocks from different regions and genetic backgrounds.

This study was undertaken to obtain an understanding of the degree and pattern of genetic variability among local goats from Egypt as a window to understanding the diversity of African goats. For this purpose, 14 microsatellites were genotyped in 221 individuals to: (1) Assess the within- and among population genetic diversity and (2) Investigate population structure and extent of admixture between Egyptian local goats and between them and the Shami (Damascus) goat, found across the Middle East and West Asia close to the geographic centre(s) of goat domestication.

MATERIALS AND METHODS

Sample collection and DNA extraction

We sampled 163 animals from four Egyptian goat populations (Table 1). We also sampled 58 individuals of Shami goat, which is native to the Middle East (Syria, Turkey, Lebanon, Jordan, Israel, and the Palestinian territories). The five study populations belong to the Lop-eared goat group (Porter, 2002). Similar types also occur in North Africa, the Western Mediterranean region, the Middle East, the Arabian Peninsula and the Indian subcontinent (Porter, 2002). Sampling of Egyptian goats was approval by, and analysis was done within the research premises of the Animal Production Research Institute (APRI) as the National Focal Point of animal genetic resources of Egypt.

The Shami individuals were sampled in Syria; 29 each from Hmemeh Shami Goat Research Station in Aleppo and Karahta Shami Goat Research Station in Damascus. Nine individuals of Zaraibi were sampled from a research station of the Animal Production Research Institute where selection for milk production and fecundity traits is done. The rest of the Zaraibi (31) together with the other Egyptian goat populations were sampled from farmers' flocks. In these flocks, veterinary health care and animal nutrition management are rarely practiced, mating is uncontrolled and performance recording and artificial selection are rare. Two mature animals were sampled per flock; and two flocks were sampled per village to avoid closely related individuals. All samples were collected in form of total blood with EDTA as the anticoagulant. Genomic DNA was extracted from whole blood using DNeasy® Blood and Tissue Kit (Qiagen GmbH, Germany). DNA concentration and purity were assessed using the BioPhotometer Plus (Eppendorf, GmbH, Germany).

Table 1. Indicators of allelic and genetic diversity in Egyptian and Shami goat populations analyzed using 14 microsatellite markers.

Population	N	TNA	Allelic diversity				Genetic diversity			Number of loci deviating from HWE
			AR (SD)	MNA (SD)	ENA	P_A	He (SD)	Ho (SD)	F_{IS}	
Zaraibi	40	137	7.89 (1.837)	9.79 (2.83)	4.82 (2.00)	9	0.76 (0.035)	0.64 (0.021)	0.16***	4
Baladi	28	127	8.27 (2.276)	9.07 (2.84)	5.37 (1.97)	3	0.81 (0.021)	0.65 (0.027)	0.20***	2
Saidi	47	150	8.55 (2.271)	10.71 (3.47)	5.99 (2.60)	5	0.81 (0.026)	0.67 (0.019)	0.17***	4
Barki	48	146	8.28 (2.235)	10.43 (3.39)	5.32 (2.29)	7	0.78 (0.031)	0.67 (0.019)	0.14***	1
Egyptian goats	163	190	9.26 (2.171)	13.57 (4.48)	6.70 (2.64)	24	0.82 (0.022)	0.66 (0.010)	0.20***	
Shami	58	143	8.04 (2.065)	10.21 (3.53)	5.56 (2.16)	11	0.79 (0.029)	0.71 (0.017)	0.11***	6
Overall	221	201	9.53 (2.232)	14.36 (4.58)	7.22 (2.76)	35	0.84 (0.021)	0.67 (0.009)	0.19***	

N = Sample size; TNA = total number of alleles; AR = allelic richness; SD = standard deviation; MNA = mean number of alleles; ENA = effective number of alleles; Pa = private alleles; He = expected heterozygosity; Ho = observed heterozygosity; F_{IS} = inbreeding coefficient. Significant difference was at P<0.001.

DNA amplification and genotyping

We genotyped 14 autosomal microsatellites out of the 30 recommended by the ISAG/FAO Panel on Domestic Animal Genetic Diversity (Table S1). The microsatellites were amplified in two multiplex PCR reactions each containing 100 to 150 ng DNA, 1X Platinum® Multiplex PCR Master Mix (Lifetechnologies, USA) and 10 pM of each primer in 25 ul reaction volumes. The thermal profile was as recommended by the ISAG/FAO Panel and was run on a C1000 Thermal Cycler (Biorad, USA). Genotyping was performed with the ABI3500 Genetic Analyzer (Lifetechnologies, USA) using GeneScan™ 600 LIZ® (Applied Biosystems) internal lane size standard. Allele size calling and binning were carried out with GeneMapper v3.5 (Applied Biosystems).

Statistical analyses

Total number of alleles (TNA), mean number of alleles (MNA), allelic richness (Ar) standardized for a minimum of 16 diploid individuals per population, effective number of alleles (ENA), number of private alleles (Pa) and expected (He) and observed (Ho) heterozygosity, as well as, nuclear pairwise F_{ST} values corrected for multiple testing, were calculated from allele frequencies with FSTAT 2.9.3.2 (Goudet, 2001), MICROSATELLITE TOOLKIT (Park, 2001) and POPGENE 1.31 (Yeh et al., 1997). Genetic inbreeding coefficients F_{IS} (Weir and Cockerham, 1984) were inferred in FSTAT 2.9.3.2. A nuclear AMOVA implemented in ARLEQUIN v3.11 (Excoffier and Lischer, 2010) was used to estimate and partition genetic variation within and among breeds.

The Bayesian clustering algorithm implemented in STRUCTURE v2.3.3 (Pritchard et al., 2000) was used to assess the genotypic composition of the genetic backgrounds of the populations analyzed and proportion of mixed ancestry. We performed 140,000 iterations following a burn-in of 70,000 Markov Chain Monte Carlo replications with an admixture model that allowed for correlation among allele frequencies. Ten independent simulations for each K (1 - 10) were performed to identify the most probable clustering solution by examining the modal distribution of Delta K (Evanno et al., 2005). Graphical representations of these statistics were obtained with STRUCTURE HARVESTER v0.68 (Earl and von Holdt, 2012). The outputs from multiple runs for each K were concatenated with CLUMPP (Jacobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) was used to display the assignment probabilities. To further investigate individual clustering profiles, we carried out the multivariate based Discriminant Analysis of Principal Components (DAPC) with ADEGENET v1.3-9.2 (Jombart, 2008) in R 2.15.3 (R Development Core Team, 2006). DAPC is a non model-based method provides an efficient description of genetic clusters using the discriminant functions. This multivariate analysis seeks linear combinations of the original alleles, which show linear combinations of the original alleles, maximizing differences between pre-defined clusters and minimizing variation within clusters. Based on the retained discriminant functions, the analysis derives probabilities for each individual of membership in each of the cluster. This coefficient can be interpreted as “genetic proximity” of individuals to the different clusters. These coefficients provide an “assignment measure” of individuals to predefined clusters, comparable with ancestry value derived by the structure analysis. For this analysis, we ran *K* between 1 and 40 and inferred its most optimal value using the Bayesian Information Criterion (BIC) statistic generated with DAPC in ADEGENET. We also constructed a neighbor joining (NJ) tree of phylogenetic relationships of individuals with POPULATIONS 1.2.32 (Langella, 2002) using the allele sharing distance (DAS) with 1000 bootstrap replications over loci.

We inferred excess/deficiency of nuclear heterozygosity to search for signals of population decline with BOTTLENECK 1.2.0.2 (Cornuet and Luikart, 1997) applying 1000 replications. We performed the evaluation using the stepwise mutation (SMM) and two-phase (TPM) models of microsatellite evolution. We set the proportion of SMM and its variance to 85 and 12% respectively. The significance of the tests was assessed using Wilcoxon sign-rank test (Piry et al., 1999). The mode-shift indicator test, although not a statistical test *per se*, was also performed because stable populations are expected to show larger proportions of alleles at low frequency (Cornuet and Luikart, 1997). We used the intra-locus kurtosis test (*k*-test) and the inter-locus variance test (*g*-test) (Reich and Goldstein, 1998; Reich et al., 1999) to

search for signatures of population expansions. Both tests (*k* and *g*) were performed using the macro program 'KGTESTS' (Bilgin, 2007) implemented in Microsoft Excel®.

RESULTS AND DISCUSSION

Allelic and genetic diversity

Measures of allelic and genetic diversity computed across 14 loci for each population are shown in Table 1. MNA per population had an average value of 14.36 ± 4.58, ranging from a minimum of 9.07 ± 2.84 (Baladi) to a maximum of 10.71 ± 3.47 (Saidi). The effective number of alleles was very similar in the populations studied with means ranging from 5.32 ± 2.29 (Barki) to 5.99 ± 2.60 (Saidi), except for the Zaraibi, which had a mean of 4.28 ± 2.00. The ratio between the effective and mean number of alleles per population ranged from 0.492 (Zaraibi) to 0.592 (Baladi) indicating that the distribution of allele frequencies had a minimal difference in the populations studied. The number of loci with exclusive alleles was highest in Shami (11) and lowest in Baladi (3) while the proportion of loci not in HWE was highest in Shami (6) and lowest in Barki (1). H_e had a mean value across populations of 0.84 ± 0.021 with the lowest mean in Zaraibi (0.76 ± 0.035) and the highest in Baladi and Saidi (0.81). H_o ranged between 0.64 ± 0.021 (Zaraibi) and 0.71 ± 0.019 (Shami) with a global mean of 0.67 ± 0.009, and the mean allelic richness was 9.53 ± 2.23. The mean expected heterozygosity that we observe is higher than that reported for different goat breeds and populations. They ranged from an average of 0.52 in Southeast Asian populations (Barker et al., 2001) and 0.59 in Swiss breeds (Saitbekova et al., 1999) to 0.82 in Chinese breeds (Qi et al., 2009). It is higher than the mean value of 0.69 reported for a diverse group of goats from Europe and the Middle East (Canon et al., 2006). In particular, the average allelic and genetic diversity found in our work exceeds that reported for a caprine gene pool from the geographic center of goat domestication in Iran and Pakistan (Di et al., 2011; Vahidi et al., 2014). This result was unexpected because genetic diversity, for most livestock species, tends to be negatively correlated with geographic distance from the center of domestication (Groeneveld et al., 2010; Wiener and Wilkinson, 2011). Pereira et al. (2009) reported high maternal (mtDNA) and paternal (Y-chromosome) genetic diversity among goat populations from Northern Africa. Together with our findings, these results suggest that northern Africa most likely witnessed the introduction of a diverse gene pool of goats from Southwest Asia which created a large caprine biodiversity in the region which still exists today.

A significant ($P \leq 0.001$) of F_{IS} was observed in all the populations studied ranging from 0.11 (Shami) to 0.20 (Baladi) with a mean value across populations of 0.19. Overall, these results indicate that even though the within-population H_e and H_o were not widely different, the deficit found in within-population heterozygosity (F_{IS}) was different among the populations. Inbreeding detected in the study populations is very likely to be relevant to population management and conservation. In spite of attempts to avoid sampling closely related individuals, high significant positive F_{IS} values (range = 14 to 20%) was detected indicating heterozygote deficiency confirming that the populations are not entirely panmictic. This high level of inbreeding is not unique to Egyptian goats. The values fall within the range of 4.5 and 29.3% observed in several goat populations from Europe and the Middle East (Canon et al., 2006). With several flocks sampled per population for the 4 Egyptian goats breeds, this could have resulted in fine-scale genetic substructure (Wahlund effect) reflected in positive F_{IS} values. In addition, retaining breeding animals from within-the-flock individuals is a common practice of the Egyptian shepherds which, is very likely, resulted in heterozygosity reduction, in a process called "in-favour-homozygotes selection" (Maudet et al., 2002). In the absence of written records, flock owners most likely are unable to recall accurately the long-term pedigree of their animals. Kugonza et al. (2012) observed that Ankole cattle keepers could correctly assign first-degree relatives more easily than they did for second- and third-degree relatives. Furthermore, herd sizes constrained the number of kinship assignments that could be remembered accurately based on memory recalls. For the Shami goats, as they were sampled from 2 closed governmental farms, in which, breeding of ancestrally related animals is highly expected to occur. Both Wahlunds effect and in-favour-homozygotes selection for long time periods may be the likely cause of the positive F_{IS} values.

Population structure and differentiation

The proportion of shared alleles between individuals was used to construct a NJ dendrogram (Figure 1a). The dendrogram shows that only Zaraibi and Shami are each defined by two clades. Although Baladi also separates into two clades, some of its individuals segregate into other clades as well. Individuals of Barki and Saidi do not separate into clear identifiable clades.

The possible ancestral gene pools underlying the observed genetic diversity were assessed with STRUCTURE and DAPC. As inferred by the method of Evanno et al. (2005), within the range of the number of clusters tested, $K = 1 - 10$, the most likely number of gene pools that contribute to the observed genetic variability in the five populations studied is $K = 6$ (Figure S1a). The contributions of the detected gene pools to the five study populations are graphically presented in Figure 1b. At $K = 6$, Shami and Zaraibi are each identified with two different gene pools. Respectively, the contributions of the two gene pools observed in Shami are 48.43 and 39.44%,

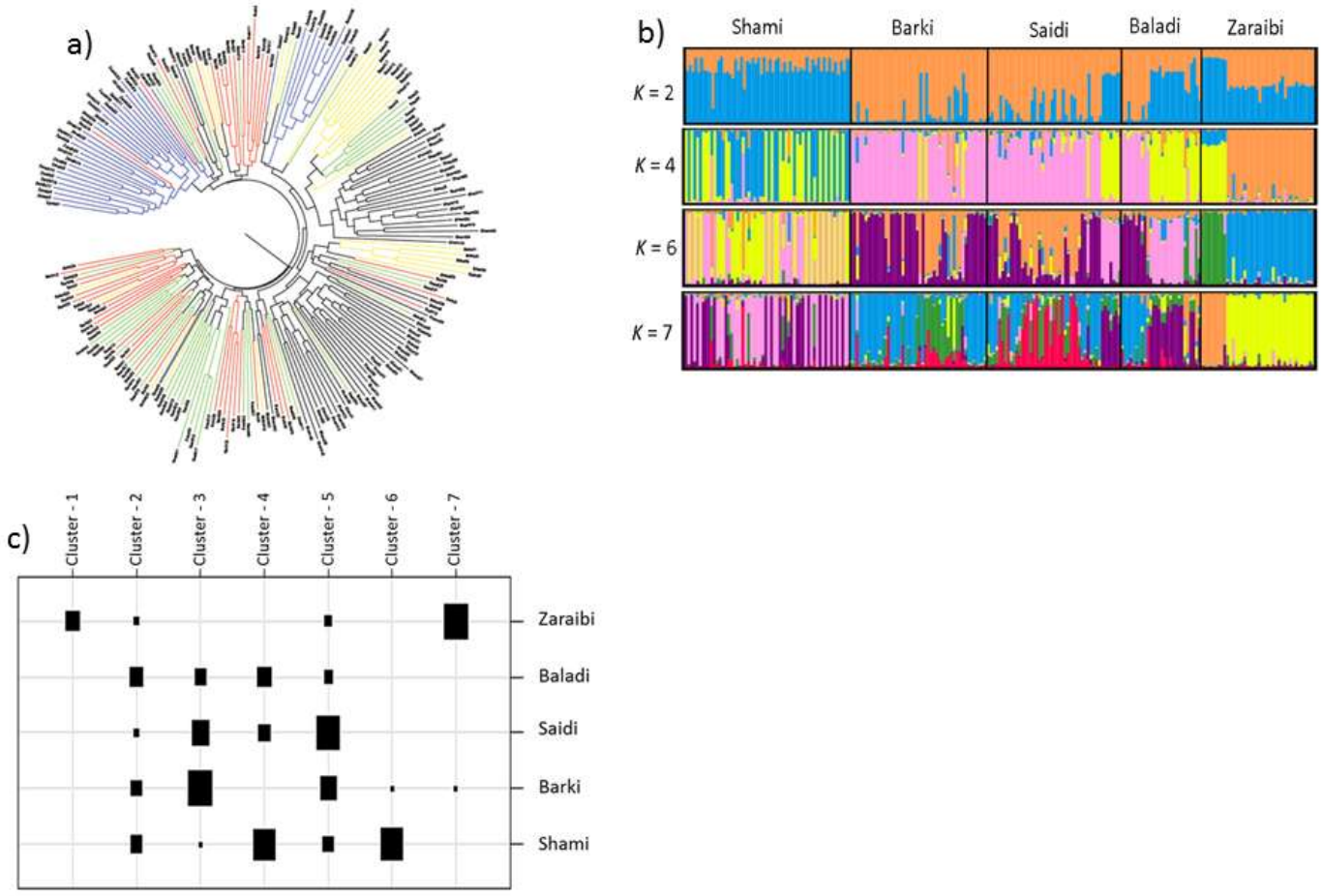

Figure 1. Individual cluster analysis. **(a)** NJ tree cluster of individuals (colour code: Blue = Zaraibi, Yellow = Baladi, Green = Saidi, Red = Barki, Black = Shami). **(b)** individual assignment probabilities generated from STRUCTURE. **(c)** Assignment of individuals to seven clusters based DAPC analyses.

while the contributions of the two gene pools observed in Zaraibi to its genetic makeup are 70.71 and 21.59% respectively. Variable frequencies of four ancestral gene pools define the genetic composition of Barki, Saidi and Baladi. No clear distinctions can be established between these three populations based on the proportions of the four gene pools. One of the gene pool observed in Shami occurs in Saidi and Baladi with a frequency of 14.30 and 41.91% respectively. The Baladi also shares one of the gene pool that is common to Barki and Saidi. This gene pool contributes 29.94, 64.16 and 37.24% of the genetic composition of Baladi, Barki and Saidi respectively. One last gene pool is observed in Barki and Saidi only, at a frequency of 24.77 and 38.88% respectively.

The BIC statistic generated by DAPC indicates that the optimal number of clusters in the data set is $K = 7$ (Figure S1b and c), showing one extra cluster generated by DAPC more than those created by STRUCTURE. Both analyses confirm that the Egyptian goats are admixed. For comparison between the analysis of the two approaches, results correspond to those of STRUCTURE at $K = 7$ are presented (Figure 1b). These results (STRUCTURE, DAPC and NJ) show extensive sharing of genetic variation among Egyptian local goats and between them with Shami. The exception is Zaraibi, which is the most genetically distinct. We therefore took $K = 6$ to represent the optimal number of gene pools that define the genetic backgrounds of the five populations; green and blue (Zaraibi), orange, pink and purple (Baladi, Barki and Saidi), and pink and yellow (Shami).

The results generated by STRUCTURE, DAPC and NJ tree were consistent with those of AMOVA which show 7.10% of the neutral autosomal genetic variation is explained by genetic differentiation between populations and 79.19% is explained by differences within individuals. Excluding Shami from the analysis reduced the variation between Egyptian populations to 6.22%. Excluding Zaraibi, which has two different gene pools, but retaining Shami, reduced the variation between populations to 6.04%. However, excluding both Shami and Zaraibi, based

on the results of STRUCTURE/DAPC/ NJ, reduced the variation between populations to 4.27%. These results indicate that Shami and Zaraibi contribute significantly to the variation present between populations.

F_{ST} values were significantly different from zero in all pairwise comparisons (Table S2). They ranged from 0.032 between Saidi and Barki (which had two common gene pools; Figure 1b and c) to 0.101 between Zaraibi and Shami (which had the most distinct gene pools; Figure 1b and c).

The estimated global F_{ST} which corresponds to the proportion of genetic variability accounted for by differences among populations was 0.071 ± 0.016 indicating that genetic diversity quantified by the neutral autosomal microsatellite markers show little differentiation among the populations analyzed. This value falls within the range of values that have been observed by most studies on goats where F_{ST} ranged between 0.04 (Sechi et al., 2005) and 0.11 (Glowatzki-Mullis et al., 2008). Vahidi et al. (2014) reported an average value of 0.062 ± 0.016 for goats from Iran and Pakistan where goat domestication took place. This comparison however depends on the level of genetic divergence of the populations analyzed. For instance, among well-defined and distinct Swiss goat breeds, the global F_{ST} value was 0.17 (Saitbekova et al., 1999) while in the study by Canon et al. (2006) it was 0.069. The latter is a lower level of genetic differentiation considering that the study analyzed 45 breeds and populations from across Europe and the Middle East. The low level of genetic differentiation in our study populations was confirmed by the poor clustering of individuals on the NJ tree (Figure 1a) generated with allele sharing distance where Zaraibi and Shami grouped in separate clusters. This low level of population differentiation amongst the Egyptian goats can be a result of either common origin as suggested by Naderi et al. (2007) or past admixture among different ancestral genetic stocks. It could also be due to extensive translocation of goats in the recent past following human movements and migrations or as commercial trading items. This has facilitated gene flow among populations and homogenized the caprine gene pool (Luikart et al., 2006; Naderi et al., 2007).

The analysis with STRUCTURE and DAPC confirmed Zaraibi and Shami, which were identified with independent clusters of potential ancestral gene pools, as genetically distinct. For the other three populations, there was clear evidence of genetic admixture, which is the result of variable contributions from four different ancestral gene pools. This result is compatible with the low genetic differentiation between Egyptian populations. It has been suggested that the Zaraibi originates from the Nuba area of southern Egypt and, based on its phenotypic characteristics, has been proposed to be the progenitor of the Anglo-Nubian breed (Galal et al., 2005).

The alternative suggestion, from flock owners, is that Zaraibi was introduced to its current location from around the Mediterranean region and has been maintained ever since as a small flock. We observe no common gene pool between Zaraibi and the other three Egyptian breeds, especially Saidi from southern Egypt, which would otherwise support an origin from southern Egypt. Our results therefore, do not support southern Egypt as a source of Zaraibi. Dispersal from areas around the Mediterranean Sea remains a possibility. Analysis of goats from the Mediterranean countries would be necessary to test this suggestion in view of findings from the analysis of various markers (mtDNA, Y-chromosome and microsatellites) which indicate that bidirectional movement of goats, sheep (Pereira et al., 2005; Canon et al., 2006) and cattle (Cymbron et al., 2005; Anderung et al., 2005) were common between northern Africa and Iberia in medieval times. The sub-clusters observed in Zaraibi and Shami (Figure 1a and b) most likely reveal the effects of reproductive isolation and breeding strategies. Thirty-one samples of Zaraibi, which form the main cluster, came from farmers' flocks while nine, which formed the minor cluster, came from a research station. This also reveals variation in breeding goals between Egyptian Zaraibi holders in East-Northern Delta and research farm. Research farms follow breeding program for genetic improvement of milk production and twinning trait in Zaraibi goats. Meanwhile, Zaraibi goats are found as household goats in the East-Northern delta, where they are kept as a hobby rather than production animal (DAGRIS, http://dagris.ilri.cgiar.org/display.asp?ID=876). Shami were sampled from two separate research stations in Syria and the clustering pattern clearly mimics these two flocks. The reproductive isolation of these two closed flocks, their different breeding management strategies, and the very likely utilization of ancestrally related animals for breeding may explain the clear genetic substructure we observe in the two breeds.

We detected signatures of introgression of Shami in Saidi and Baladi in the form of a shared gene pool (Figure 1b and c). There is no report of crossbreeding Shami with farmers' flocks other than for breeding trials in research stations (Abdelsalam et al., 2000). If the introgression was the result of crossbreeding, we expected to observe the gene pool in Barki as well. This result either reveals the introgression of Shami into Saidi and Baladi prior to their arrival in Eastern North Africa or, a more likely, a common ancestral background between the three populations. This background is gradually being lost in Egyptian populations due possibly to purifying selection and/or genetic drift. It however continues to persist in the Shami, a breed that is found in the Middle East within the proximity of the geographic center(s) of goat domestication. We speculate that the gene pool may still be maintained in the Middle East because it confers a selective advantage in the region.

One gene pool was detected in Barki, Saidi and Baladi while another was observed only in Barki and Saidi. The proportion of these gene pools in the three populations

was variable revealing an admixture trend among Egyptian goats. This may explain their high level of diversity and low differentiation due to either common origin or past admixture among populations of different genetic backgrounds. The common origin could be the hypothesis of choice considering the results of Vermeersch et al. (1994), reporting evidence that domesticated goats appeared in Southern Egypt and Northern Sudan earlier than in Northern Egypt. Therefore, it is likely that Saidi (Southern-Egypt breed) is the common origin of the other Egyptian populations. In addition to high genetic diversity, local goats in Africa and Asia are characterized by extensive phenotypic variation. The traditional management systems still in use today are defined by the absence of stringent artificial selection and by uncontrolled breeding management. These practices may have contributed to the high genetic variation we observe within these populations (Hassanane et al., 2010).

Population dynamics and demographic inferences

We performed four tests to investigate demographic dynamics and trends. We detected significant deficit in heterozygotes ($P \leq 0.05$) under the SMM model but neither a significant deficit nor excess in heterozygotes ($P \geq 0.05$) was detected under the TPM model implemented in BOTTLENECK. The graphical representations of allele frequencies revealed a normal L-shaped distribution. The *k*-test revealed several loci with non-significant ($P > 0.05$) negative *k* values; the *g*-test values were also not significant ($P > 0.29$) (Table S3). These results are characteristic of stable populations under mutation-drift equilibrium. Therefore, *KG*-tests results did not reveal significant patterns of heterozygote excess, and the mode-shift test displayed an allele frequency distribution characteristic of non-declining populations. These results do not support evidence of a recent genetic bottleneck. The *kg*-tests show all the populations to be at demographic equilibrium; none has experienced any expansions in effective population sizes in the recent past. One migrant per generation can counteract the effects of isolation and genetic drift (Mills and Allendorf, 1996). Considering the history of goats and the results of STRUCTURE, we cannot describe the Egyptian goats as being reproductively isolated from each other but, rather, would expect continuous gene flow among them. For the Zaraibi and Shami, in spite of being reproductively isolated from each other and from the other Egyptian populations, from a genetics point of view, they have not reached a critical threshold for them to be considered threatened.

Conclusion

Overall, our study indicates that within Egyptian goats, genetic diversity is high but genetic differentiation among populations is low. Although inbreeding is high, the populations analyzed are at genetic equilibrium. The observation of extensive admixture reinforces the importance of Eastern North Africa as bedrock of African caprine biodiversity while the observed enrichment of the goat gene pool in the area emphasizes the importance of the region in the history of African livestock. Knowledge on population stratification and the distribution of genetic variability within and among breeds, populations and strains are important in formulating strategies for maintaining genetic diversity, in understanding the evolutionary history of breeds and populations and, in generating insights into the history of human populations. Results suggest that northern Africa most likely witnessed the introduction of a diverse gene pool of goats from Southwest Asia that created a large caprine biodiversity in the region, which still exists today. Results clearly indicated that the indigenous goat populations before Barki, Baladi and Saidi) have been admixed and Saidi goat (Southern-Egypt) could be the common origin of other indigenous populations. Zaraibi goats are very likely to be Mediterranean-originated rather than Southern-Egyptian, as it lacks common genetic backgrounds with any of the studied indigenous goat breeds. For the animal genetic resource community in Egypt, it recommended to consider variation between Zaraibi research farm under genetic improvement practices and smallholdings in East-Northern Delta region. Baladi seems to be the most admixed population in Egypt, which also needs to be considered in any plan for genetic conservation and utilization. In spite of the different phenotypes and geo-ecological distribution of Barki and Saidi breeds, they have common genetic backgrounds. The two breeds therefore, need further investigation, probably using high-density genotyping approach.

Conflict of Interests

The authors have not declared any conflict of interests.

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Table S1. Data on microsatellite primers used in the study; microsatellite, minimum and maximum allele size, and total number of alleles detected, and multiplex in which the primer was used for full-size amplification.

Microsatellite	Allele size range		Total number of alleles detected	PCR multiplex
	Minimum	Maximum		
HTC	264	300	18	
ILSTS19	138	154	9	
INRA05	114	126	7	First
SRCRAP05	153	187	17	multiplex
SRCRSP08	202	247	20	
SRCRSP24	133	167	18	
CSRD247	217	245	15	
ILSTS87	133	151	10	
INRA023	191	215	13	
INRA063	163	183	11	Second
MAF065	113	161	22	multiplex
MCM527	148	166	10	
OarFcB20	87	111	13	
SRCRSP23	75	119	18	

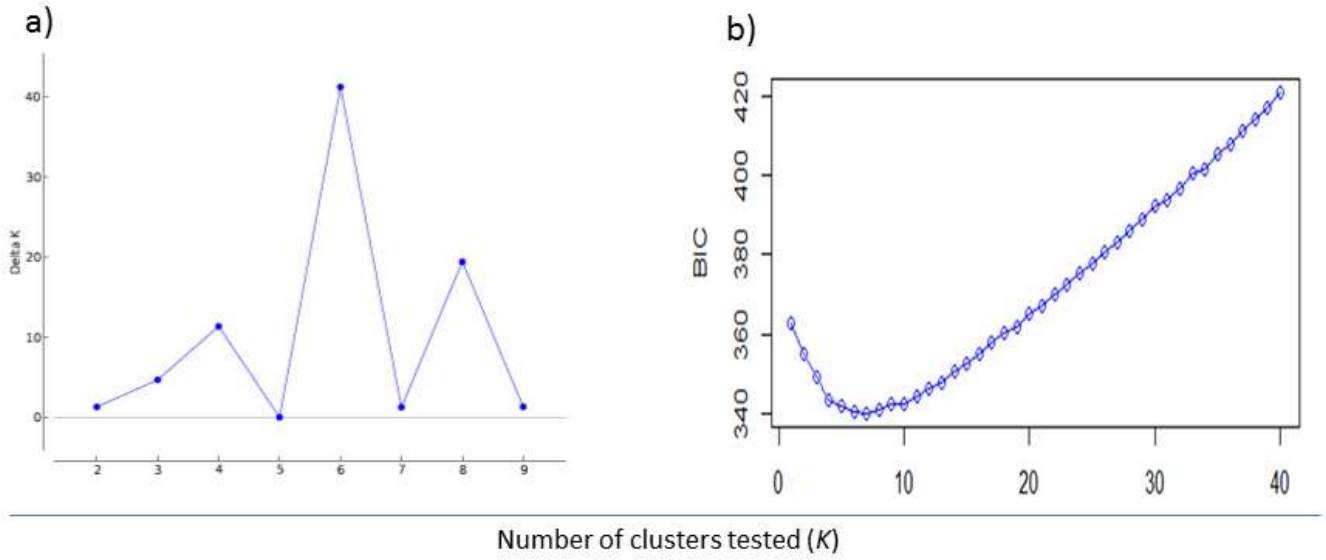

Figure S1. Distribution of (a) Delta *K* and (b) BIC values for Egyptian goats.

Table S2. Estimated pairwise F_{ST} as a measure of genetic differentiation among Egyptian and Shami goats.

Population	Zaraibi	Baladi	Saidi	Barki	Shami
Zaraibi	-	0.092*	0.068*	0.089*	0.101*
Baladi		-	0.049*	0.059*	0.077*
Saidi			-	0.032*	0.062*
Barki				-	0.076*
Shami					-

Significant difference was at $P < 0.05$.

Table S3. KG-tests results for the five populations analyzed in the current study.

Population	Number of loci with negative K value	K-test (P-value) (intra-locus)	g-test value (inter-loci)
Zaraibi	8	0.352012	0.818576
Baladi	5	0.890482	0.390459
Saidi	7	0.560168	0.329173
Barki	9	0.180484	0.32936
Shami	5	0.890482	0.412952
Overall	8	0.352012	0.332722

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