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León Irapuato,
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*Molecular Mycology and Plant Pathology
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Isfahan
Iran*

Dr. Beatrice Kilel

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*Institute of Molecular Medicine
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1 University road Tainan 70101,
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Jalan Raja Muda Abdul Aziz
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Ago-Iwoye.
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*National Agricultural Biotechnology Center,
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*DuPont Industrial Biosciences
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DLF Phase III
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Dr. Sang-Han Lee

*Department of Food Science & Biotechnology,
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Full Length Research Paper

Molecular survey of the Texas Phoenix decline phytoplasma population in Florida, USA

K. Ntushelo*, N. A. Harrison and M. L. Elliott

Department of Plant Pathology, Fort Lauderdale Research and Education Center, University of Florida,
3205 College Avenue, Fort Lauderdale, FL 33314, USA.

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A nested polymerase chain reaction (PCR) assay was used to amplify 16S-23S intergenic spacer (IGS) region from DNA samples individually extracted from 25 *Sabal palmetto* (cabbage palms) showing symptoms of Texas Phoenix decline (TPD) in West Central Florida. The IGS region was also amplified from DNA from other palm species showing symptoms of TPD and lethal yellowing (LY). A subset of the aforementioned phytoplasma DNA samples (*Sabal* and other palm species) together with additional samples from various hosts collected from different geographical localities were further studied to compare the collected phytoplasma strains using sequence analysis of the glycoprotease (*gcp*) genes. Restriction fragment length polymorphisms (RFLP) analysis of the PCR-amplified 16S-23S IGS region and the *gcp* gene using a three restriction enzymes showed that the population of the phytoplasmas infecting *S. palmetto* in West Central Florida is probably homogenous. The *S. palmetto* phytoplasma also appeared similar to all the 16SrIV-D phytoplasmas infecting other palm species and different from all phytoplasmas belonging to the 16SrIV-A subgroup. We recommend more work using genes or genomic regions other than the 16S-23S IGS region and the *gcp* gene to be done.

Key words: 16S-23S intergenic spacer region, glycoprotease gene, phytoplasma, Texas Phoenix decline, lethal yellowing.

INTRODUCTION

Until 2005, the only phytoplasma disease of palms known to occur in Florida was Lethal Yellowing (LY) caused by phytoplasma subgroup 16SrIV-A. While *Cocos nucifera* L. (coconut palm) is the primary palm susceptible to this subgroup, the disease has been observed in 35 other palm species (Harrison and Jones, 2004). During the 40 plus years that LY has been active in Florida, no palm species native to Florida has been affected by this disease. Even with the detection of a decline phytoplasma disease in Florida in 2005, native palm species remained unaffected (Harrison et al., 2008). Thus, it was a surprise in 2008 to determine that the lethal decline of *Sabal palmetto* (Walter) Lodd. Ex Schult.

& Schult. f. (cabbage palm), the most common native palm throughout Florida, being observed in west central Florida was caused by a phytoplasma (Harrison et al., 2009). Initial DNA-based characterization of the phytoplasma affecting *S. palmetto* determined that this strain is identical to the subgroup previously documented as affecting *Phoenix* spp. in Texas and later on *Phoenix* spp. and *Syagrus romanzoffiana* in west central Florida, namely subgroup 16SrIV-D (Harrison et al., 2008, 2009). In all its host palms, the disease caused by phytoplasma subgroup 16SrIV-D is called Texas Phoenix decline (TPD). Because *S. palmetto* is the dominant native palm, and often the dominant tree, in natural areas

*Corresponding author. E-mail: khayantushelo@hotmail.com.

throughout Florida, association of the TPD phytoplasma with *S. palmetto* has caused great concern as its biology, its genetic characteristics and the extent of devastation to be expected are not known.

The purpose of this work was to survey the composition of the TPD phytoplasma population in west central Florida and compare with strains outside of Florida and with subgroup 16SrIV-A strains. The 16S-23S IGS region should offer more variation because of less evolutionary constraints on this region than on the 16S rRNA gene. Phytoplasma identification is based on a classification scheme that uses the highly conserved 16S rRNA gene which is useful as the basis of the classification scheme for identifying the major groups of phytoplasmas (Gundersen et al., 1994), but less conserved regions of the genome are necessary for comparing strains within a subgroup. An additional comparison was made among these phytoplasma strains (16SrIV-A and 16SrIV-D) collected from different palm hosts in Florida, Texas, Jamaica, Honduras and Mexico using the *gcp* gene. The protein encoded by the *gcp* gene, α -galactosidase endopeptidase, is possibly a host adaptation and virulence factor and is a member of the M22 peptidase family (Rawlings and Barrett, 1995).

MATERIALS AND METHODS

Plant materials and DNA extraction

The first set of samples, consisting of interior stem tissue shavings, was collected from the lower stems of *S. palmetto* with foliar symptoms indicative of decline. These symptomatic palms were located in the adjacent counties of Hillsborough and Manatee in west central Florida, USA; where diseased *S. palmetto* were most numerous. Twenty-four (24) samples were obtained (: EGS1-11, Sab1-7, and SP1, 2, 4, 6, 7 and 9) (Table 1). A second set of stem samples was obtained from TPD symptomatic *Phoenix* palms, including *P. canariensis* Chab. (Canary Island date palm) (PC1, PC2, SEG, PCT3 and SA1), *P. dactylifera* L. (edible date palm) (RPA) and *P. sylvestris* (L.) Roxb. (silver date palm) (S5-PS); and one symptomatic *S. romanzoffiana* (Cham.) Glassman (queen palm) (S1-QP) (Table 1). This second set of samples also formed part of a previous study by Harrison et al. (2008). A third set of samples were apical meristem (bud) tissues from a TPD symptomatic cabbage palm in west central Florida (Sabal1), a LY symptomatic coconut palm from Broward county in southeastern Florida (LYFL), a LY symptomatic coconut palm in Jamaica (LYJAM) and two LY symptomatic coconut palms in Mexico (LYMEX3 and LYMEX5) (Table 1). The fourth set of samples (CID3, CLDO, COYOL, JLL and PCT4), which were either stem or apical bud samples, was obtained from existing phytoplasma collections (Table 1). The areas from which the samples were collected are shown in Figure 1A and B.

For all the stem samples, tissue was removed from palms by drilling into the stem using a portable electric drill fitted with a wood boring bit as previously described by Harrison et al. (2002). The stem shavings were collected into clean sealable plastics bags. For the bud samples, tissues were collected by felling the palm and excising immature leaf bases of the stem apex. Total nucleic acids were extracted from 3 g of stem tissue or from 100 g of bud tissues.

DNA from bud tissues was extracted following the phytoplasma enrichment method of Harrison et al. (1994). From stem tissue, DNA was extracted using CTAB extraction buffer according to the procedure of Doyle and Doyle (1990). For both extraction techniques, nucleic acid was precipitated with 95% ethanol and pellets were recovered by centrifugation at 12000 x g for 15 min. The pellets were resuspended in 200 μ L TE buffer [10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8)]. Presence of DNA in the pellets was confirmed by agarose gel electrophoresis.

Polymerase chain reaction

DNA preparations from the symptomatic plants were evaluated by PCR assay, together with a negative control, which consisted of DNA from a healthy plant, and a water control (no DNA template). The PCR reaction was conducted using primer pair 16S1064F 5'-TTGGAGGAAGGTGGGGATTAC-3'/23SRev 5'-TTCGCCTTTCCCTCACGGTACT-3' (which is also a phytoplasma diagnostic primer pair) for the first reaction and primer pair TPD-16-23SF 5'-AGCTTAAACGCGAGTTTTTGGCAA-3'/TPD-16-23SR5'-GTTTCGCTCGTCTACTACCAGA-3' for the nested round. These primers were designed specifically for this study to amplify the 16S-23S intergenic spacer region. For the *gcp* gene nested PCR reaction, GCPF3 5'-GATAGGCCAGGTTCTTA3' and GCP2 5'-TCCGGAGGAAAACGAGTTA 3' were followed by GCPF1 5'-GGTACACGTCTAGCTGTTGTTA 3' and GCP1 5'-CCGGAGGAAAACGAGTTATT3'. Depending on the sharpness of the primary PCR band, nested dilutions ranged from 1/10 to 1/30. All primers used in this study were designed using sequences of the LY phytoplasma obtained by 454 sequencing. Positions of these primers on the LY genome cannot yet be ascertained as the sequencing of this genome is not complete. Each PCR reaction contained 33.8 μ L H₂O; 5 μ L buffer (1.675 μ L H₂O; 1.25 μ L 1 M KCl; 1 μ L 1 M Tris; 0.5 μ L 5% Tween 20; 0.5 μ L 1% gelatin; 0.075 μ L 1 M MgCl₂); 0.1 μ g of each of the two primers; 0.04 mM of each of the dNTPs and 1 unit of Taq DNA polymerase (New England BioLabs, Waverley, MA, USA). The DNA template was between 50 and 100 ng. The total volume per PCR reaction was 50 μ L and the reaction was run for 35 cycles. Each cycle consisted of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.

The 35 thermal cycles were preceded by 1 min initial denaturation at 94°C and succeeded by 7 min final elongation at 72°C. At the end of the PCR run, 10 μ L of the PCR mixture was mixed with 7 μ L of gel loading dye, electrophoresed through 1% agarose gel using TAE buffer and visualized by UV transillumination following staining with ethidium bromide.

Cloning

Cloning instead of direct sequencing was done to ensure that the full length of the PCR-amplified regions was sequenced. PCR products were purified using Wizard PCR-preps purification kit (Promega Corp, Madison, WI) and were quantified by visualizing on agarose gel with a serial dilution of uncut lambda DNA. The PCR fragments were ligated (mixed with and incubated at 4°C overnight) with pGEM-T vector (Promega Corp, Madison, WI). The ligated PCR product was transformed into Top 10 chemically competent *Escherichia coli* cells (Invitrogen Life Technologies, Carlsbad, CA, USA). The transformed bacterial cultures were grown at 37°C on Luria-Bertani (LB) media amended with isopropyl β -D-1-thiogalactopyranoside and X-gal for blue/white colony screening. After 24 h incubation white colonies, which were regarded as carrying the cloned PCR fragment, were selected, inoculated into LB broth and incubated at 37°C with gentle shaking for 24 h. Cells

Table 1. Phytoplasma samples included in the study, listed with palm species, location strain identity and GenBank accession number of the sequenced gene fragment.

Phytoplasma sample identity	Palm species	Phytoplasma subgroup	Location ^a	GenBank accession number 16S-23S IGS ^a and <i>gcp</i> ^b gene	Year the sample was collected
* ¹ EGS1	<i>Sabal palmetto</i>	16SrIV-D	Hillsborough	HQ414247 ^a	2009
* ¹ EGS2	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414253 ^a	2009
* ¹ EGS3	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438068 ^a	2009
* ¹ EGS4	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438069 ^a	2009
* ¹ EGS5	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438070 ^a	2009
* ¹ EGS6	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438071 ^a	2009
* ¹ EGS7	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438072 ^a	2009
* ¹ EGS8	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438073 ^a	2009
* ¹ EGS9	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414258 ^a	2009
* ¹ EGS10	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414254 ^a	2009
* ¹ EGS11	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414259 ^a	2009
* ¹ Sab1	<i>S. palmetto</i>	16SrIV-D	Manatee	HQ438224 ^a	2008
* ¹ Sab2	<i>S. palmetto</i>	16SrIV-D	Manatee		2008
* ¹ Sab3	<i>S. palmetto</i>	16SrIV-D	Manatee		2008
* ¹ Sab4	<i>S. palmetto</i>	16SrIV-D	Manatee		2008
* ¹ Sab5	<i>S. palmetto</i>	16SrIV-D	Manatee		2008
* ¹ Sab6	<i>S. palmetto</i>	16SrIV-D	Manatee		2008
* ¹ Sab7	<i>S. palmetto</i>	16SrIV-D	Manatee	HQ414246 ^a	2008
* ¹ SP1	<i>S. palmetto</i>	16SrIV-D	Hillsborough		2008
* ¹ SP2	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414248 ^a	2008
* ¹ SP4	<i>S. palmetto</i>	16SrIV-D	Hillsborough		2008
* ¹ SP6	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ438074 ^a	2008
* ¹ SP7	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414250 ^a	2008
* ¹ SP9	<i>S. palmetto</i>	16SrIV-D	Hillsborough		2008
* ³ Sabal1	<i>S. palmetto</i>	16SrIV-D	Hillsborough	HQ414252 ^a ; HQ613883 ^b	2010
* ² RPA	<i>Phoenix dactylifera</i>	16SrIV-D	Hillsborough	HQ438059 ^a ; HQ613878 ^b	2007
* ² PC1	<i>P. canariensis</i>	16SrIV-D	Hillsborough	HQ438060 ^a	2007
* ² PC2	<i>P. canariensis</i>	16SrIV-D	Hillsborough		2007
* ² SEG	<i>P. canariensis</i>	16SrIV-D	Hillsborough	HQ613879 ^b	2007
* ² S5-PS	<i>P. sylvestris</i>	16SrIV-D	Manatee	HQ438065 ^a	2007
* ² S1-QP	<i>Syagrus romanzoffiana</i>	16SrIV-D	Manatee	HQ438064 ^a	2007
* ⁴ CID3	<i>P. canariensis</i>	16SrIV-D	Sarasota	HQ613886 ^b	2007
* ² PCT3	<i>P. canariensis</i>	16SrIV-D	Texas	HQ438067 ^a ; HQ613877 ^b	2001
* ⁴ PCT4	<i>P. canariensis</i>	16SrIV-D	Texas	HQ613884 ^b	2001
* ⁴ JLL	<i>Phoenix sp.</i>	16SrIV-D	Texas	HQ613888 ^b	2006
* ⁴ CLDO	<i>Cocos nucifera</i>	16SrIV-D	Honduras	HQ613887 ^b	Before 2006
* ⁴ COYOL	<i>Acrocomia aculeate</i>	16SrIV-D	Honduras	HQ613876 ^b	Before 2006
* ¹ SA1	<i>P. canariensis</i>	16SrIV-A	Manatee	HQ438063 ^a ; HQ613880 ^b	2007
* ³ LYFL	<i>C. nucifera</i>	16SrIV-A	Broward	HQ613875 ^a ; HQ613890 ^b	Before 2002
* ³ LYJAM	<i>C. nucifera</i>	16SrIV-A	Jamaica	HQ414262 ^a ; Identical to LYFL ^b	Before 2002
* ³ LYMEX3	<i>C. nucifera</i>	16SrIV-A	Mexico	HQ613881 ^b	Before 2002
* ³ LYMEX5	<i>C. nucifera</i>	16SrIV-A	Mexico	HQ441261 ^a ; HQ613882 ^b	1995

^aLocation is county within Florida, USA unless otherwise noted. *Number showing samples belong to the same set.

were lysed, using lysis buffer, to recover the ligated plasmid vectors. The plasmids were purified, resuspended in TE buffer and

submitted for sequencing. The plasmid purification kit was supplied by Promega Corp, Madison, WI. Sequencing of cloned fragments



Figure 1A. All phytoplasma strains used in this study were collected from the southern USA and Central America. States in the United States and countries in Central America from which the phytoplasma strains were collected are shown with red dots.

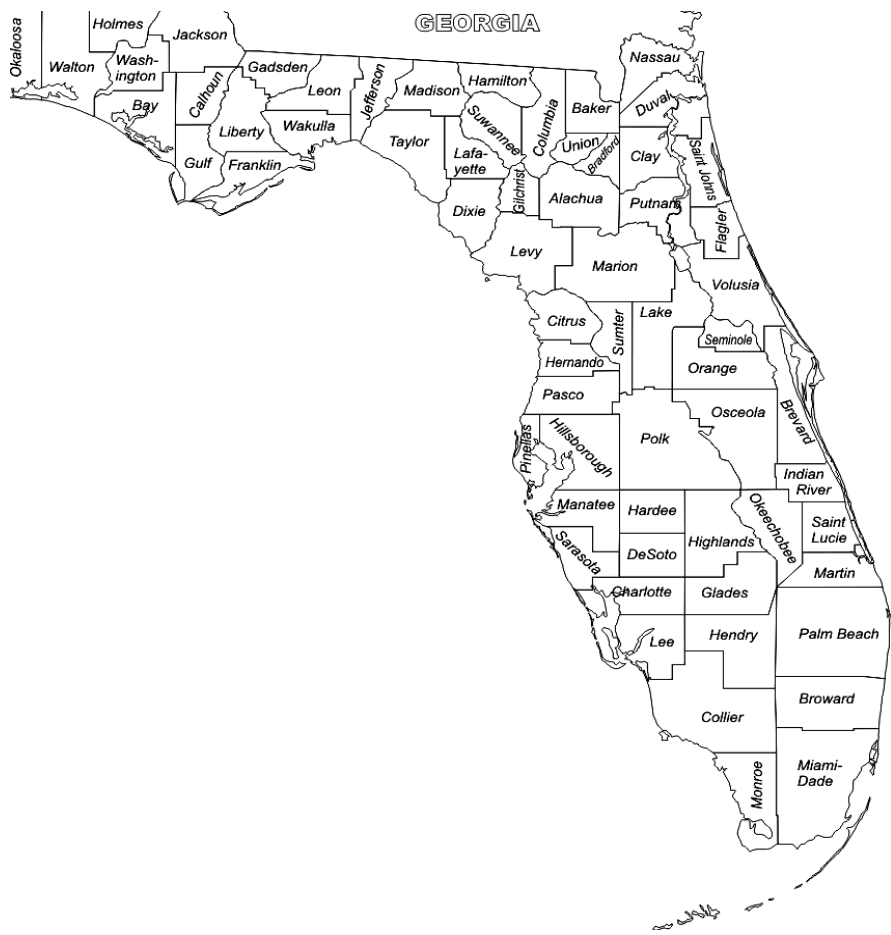


Figure 1B. A map of Florida showing counties from which most of the phytoplasma strains were obtained. The counties from which the strains were collected are shown in red dots.

was done using the M13 forward and M13 reverse primers by the University of Florida's Core DNA Sequencing Service Laboratory, Gainesville.

Sequence analysis

Sequences of the cloned fragments were assembled with SeqMan software (Lasergene™ 7.1; DNASTAR, Madison, WI, USA). Database sequence similarity searching was performed using BLAST in NCBI (website: <http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were compared pairwise using ClustalW (Larkin et al., 2007). A phylogenetic tree was constructed from the alignment by the neighbor-joining method using MEGA 4.1 software (Tamura et al., 2007). Only representative sequences were used to infer the phylogenetic trees. Sequences obtained from this study were deposited in GenBank and the accession numbers are listed in Table 1.

Restriction fragment length polymorphisms

Analysis of restriction fragment length polymorphisms was used to supplement sequencing data which may be subject to sequencing errors. Polymerase chain reaction products of the PCR amplified 16S-23S intergenic spacer region were digested separately using restriction enzymes, *Asel*, *HhaI* and *RsaI* at 37°C for a minimum of 16 h. For the *gcp* gene, *RsaI* was selected for the RFLP analysis. All the restriction enzymes used in this study were purchased from New England BioLabs, Waverley, MA, USA. These enzymes best differentiated between the phytoplasma strains as shown in a virtual test of sequence data using pDRAW32 (AcaClone, <http://www.acaclone.com>). Products of the restriction digests were separated by electrophoresis through 8% denaturing polyacrylamide gel in TBE buffer (90 mM Trisborate, 2 mM EDTA). Profiles were visualized using a UV transillumination following staining with ethidium bromide.

RESULTS

Restriction fragment length polymorphisms

It was previously determined that samples RPA, PC1, PC2, SEG, S1-QP, S5-PS and PCT3 belonged to subgroup 16SrIV-D (Harrison et al., 2008). In the current study, it was determined that all the strains from cabbage palms were subgroup 16SrIV-D, and the strains from *C. nucifera* were subgroup 16SrIV-A (except for CLDO which is a 16SrIV-B strain), as was strain SA1 obtained from *P. canariensis*. From DNA samples from 36 symptomatic plants, PCR fragments ca. 800 bp in length were amplified by nested PCR assay. This PCR fragment incorporated the entire 16S-23S intergenic spacer region. No amplification of products was observed in reactions containing DNA from the healthy palm or the water control. Similarity analysis of the assembled nucleotide sequences derived from these PCR fragments demonstrated the sequences were of phytoplasma origin, giving assurance that the PCR fragments were amplified from phytoplasmas. Based on analysis of RFLP profiles gene-

rated by digestion of each amplified PCR fragment with *Asel* restriction enzyme, 16SrIV-D strains from cabbage palms all had the same profiles, except for SP7 which had an additional band (Figure 2A to C). The 16SrIV-D strains from the *Phoenix* spp., from both Florida and Texas, and *S. romanzoffiana* had the same profiles as the 16SrIV-D strains from *S. palmetto*, which were distinct from profiles for the 16SrIV-A strains, whether from *C. nucifera* or *P. canariensis*. Further RFLP analysis based on the *HhaI* enzyme showed that all the TPD 16SrIV-D strains are similar, no matter the palm source, except for two strains (SP6 and SP7) collected from *S. palmetto*, with each containing an additional band (Figure 2D to F). Restriction enzyme *RsaI* also differentiated the cabbage palms strains similarly, with SP6 and SP7 again exhibiting additional bands (Figure 2G to I). As earlier, all 16SrIV-D strains from *S. palmetto*, *Phoenix* spp. and *S. romanzoffiana* are similar, but distinct from 16SrIV-A strains. The secondary bands in samples SP6 and SP7 in the RFLP profiles are a common occurrence resulting from digestion of non-specific PCR products.

PCR products corresponding to approximately 1.5 kb nucleotides (with about 1 kb representing *gcp* gene) were amplified using the *gcp* gene primers from fourteen of the samples listed in Table 1. No amplification was observed for the healthy palm or the water controls. The RFLP analysis of the *gcp* gene fragment, using *RsaI* restriction enzyme, shows that 16SrIV-D strains, from multiple palm hosts, are similar to each other but different from 16SrIV-A strains. The RFLP analysis performed on representative PCR amplicons is shown in Figure 3.

Molecular comparisons by phylogenetic analysis

Phylogenetic analysis using the sequence of the 16S-23S intergenic spacer region revealed that all the subgroup 16SrIV-D phytoplasmas are genetically distinct from all the 16SrIV-A phytoplasmas (Figure 4). All *gcp* sequences compared on CLUSTALW were trimmed to be of similar size, resulting in only a portion of the *gcp* gene to be analyzed. All group 16SrIV phytoplasmas in this study were distinct from other phytoplasmas with *gcp* gene sequences retrieved from GenBank. Sequence analysis of the *gcp* gene showed that the strains belonging to subgroup 16SrIV-A, namely, the Mexican strains LYMEX3 and LYMEX5, the Florida strain LYFL, the Jamaican strain LYJAM, and SA1 strain from *P. canariensis* are all similar (Figure 5). All the TPD phytoplasma strains (subgroup 16SrIV-D)—Sabal1 from *S. palmetto* in Florida; CID3, PCT3, PCT4, JLL, RPA and SEG obtained from *Phoenix* spp. in Florida and Texas; the coconut lethal decline strain CLDO from *C. nucifera* in Honduras; and COYOL strain from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. in Honduras—clustered together but were distinctly separated from the subgroup 16SrIV-A

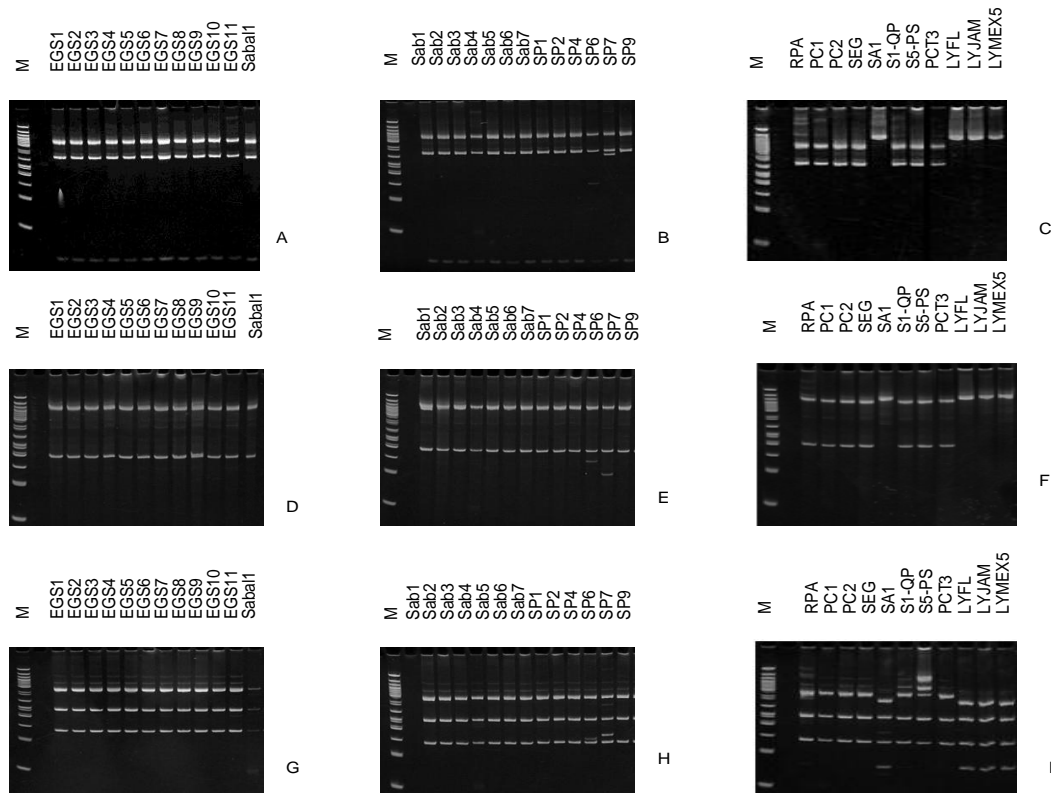


Figure 2. Restriction fragment length profiles of phytoplasma DNA (ca. 800 bp) amplified from symptomatic palms. The PCR amplification was done by primer pair 16S1064F/23SRev followed by primer pair TPD-16-23SF/TPD-16S23SR. A to C) Digestion was with *Asel*, D to F) Digestion was with *HhaI* and G to I) Digestion was with *RsaI*. M stands for the pGEM molecular size (bp) markers in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51 and 36.

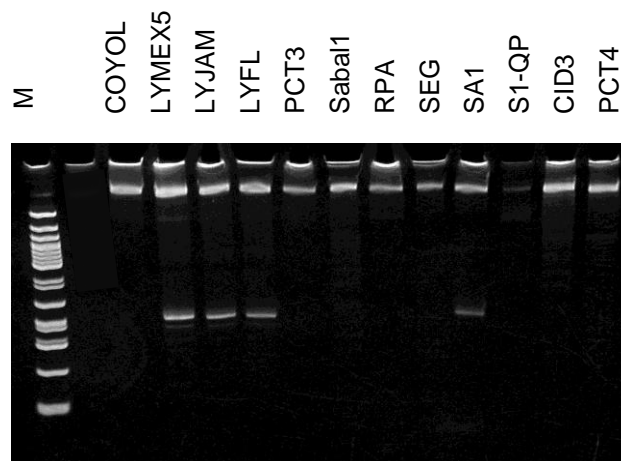


Figure 3. Restriction fragment length polymorphisms of a polymerase chain reaction (PCR) fragment amplified with nested primer pair GCPFI/GCPR1. DNA preparations were from symptomatic tissue of palms from various localities. PCR fragment was digested with enzyme *RsaI*. M on the first lane is for pGEM molecular size (bp) marker in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51 and 36.

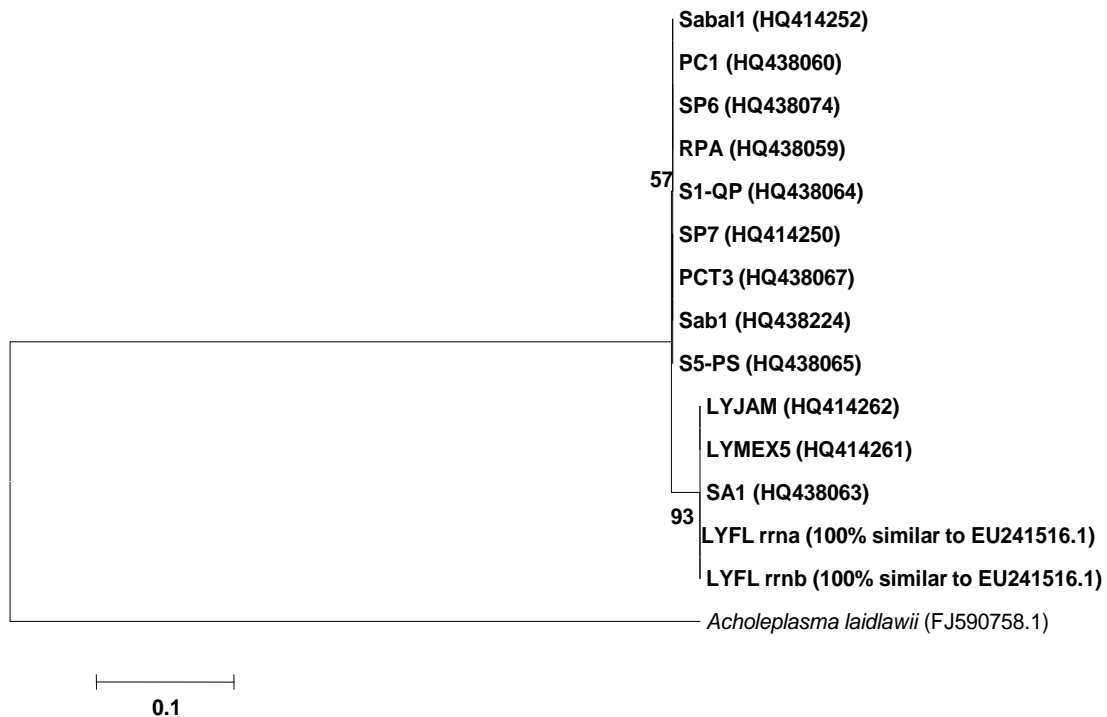


Figure 4. Inferred molecular relationships of phytoplasma strains based on the 16S-23S intergenic spacer sequence. The tree was constructed by the neighbor-joining method and bootstrap values are shown on branches. rrn means ribosomal RNA operon. The GenBank accession numbers are provided in brackets.

strains (Figure 5).

DISCUSSION

Texas Phoenix decline phytoplasma, 16SrIV subgroup D, was first reported in *S. palmetto* in west central Florida in 2008 (Harrison et al., 2008). Although, this phytoplasma subgroup had been previously reported in *P. canariensis* in Corpus Christi, Texas (Harrison et al., 2002), and in west central Florida (Harrison et al., 2008), the attack of *S. palmetto* by a phytoplasma was a surprise as no indigenous palms had been documented as being affected by phytoplasmas in Florida or Texas prior to this time, even though palm phytoplasma diseases had been active in both states since the 1970s (McCoy, 1974). *S. palmetto* is a native species that is important in the natural landscape of the state of Florida and other states in the southern USA.

Characterizing the pathogen population was important in order to understand the phytoplasma population diversity. Interestingly, while this study was in progress, 16SrIV subgroups were detected in *Sabal mexicana* Mart. (A and D), *Pseudophoenix sargentii* H. Wendl. ex Sarg. (D) and *Thrinax radiata* Lodd. ex Schult. & Schult. f. (A and D) in the Yucatan peninsula of Mexico (Vázquez-

Euán et al., 2011). All three palm species are native to that area.

Sequence analysis of the 16S-23S rRNA IGS region from the strains representing the population of the TPD phytoplasma in west central Florida showed that in this region of Florida, the phytoplasma population is probably homogenous. This apparent homogeneity of the TPD phytoplasma is found across host palm species (*S. palmetto*, *Phoenix* spp. and *S. romanzoffiana*). Sequence homogeneity of the 800 bp 16S-23S IGS region could also mean that only one strain of the phytoplasma, similar to the strain in Texas (PCT3), was introduced into west central Florida, and since its introduction, this strain has multiplied and spread throughout this part of the state. Should this be the case, prediction of the spread of this disease should be easier. However, even if the 16SrIV-D phytoplasma population is homogenous, the question still remains to the supposed sudden extension of the host range to include *S. palmetto*. Analysis of the sequence of the *gcp* gene demonstrated that the TPD phytoplasma strain infecting *S. palmetto* is not different from the TPD strains (16SrIV-D) infecting *Phoenix* spp., *A. aculeata*, and *C. nucifera*, but it is different from the LY (16SrIV-A) strains affecting *C. nucifera* and *P. canariensis*. When analyzing palm phytoplasma strains, the *gcp* gene has not proved more variable relative to the 16S rRNA gene

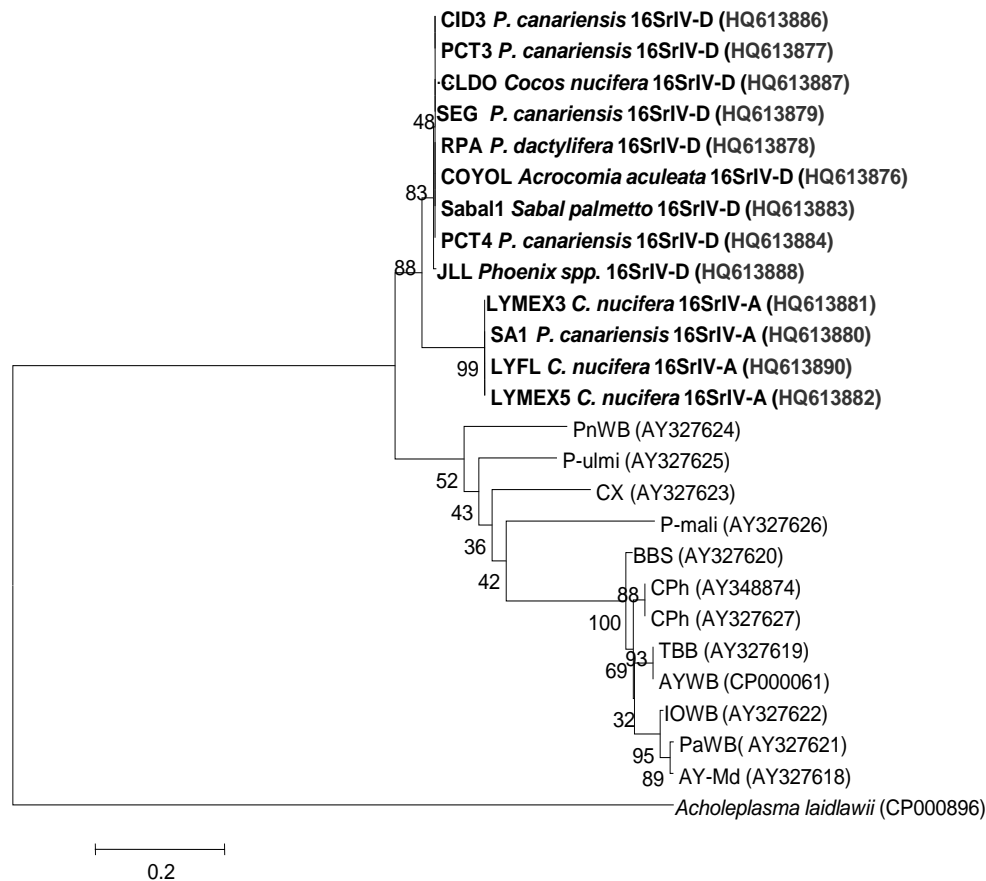


Figure 5. Molecular tree of the glycoprotease (*gcp*) gene sequences of palm lethal disease strains inferred by neighbor-joining method. Approximately, 470 bases of the *gcp* gene were used to infer the tree. Phytoplasma strains (as listed on Table 1) isolated and sequenced in this study are in bold type and the rest of the phytoplasma strain entries (not bold) were retrieved from GenBank. GenBank accession number are provided.

according to which TPD strains are 98.2 to 100% similar (Harrison et al., 2008). It was also shown that the *gcp* gene is not variable among subgroup 16SrIV-A strains.

Further characterization of the 16SrIV-D phytoplasmas using other regions of the genome is necessary to determine if there is a genetic basis for host specificity of these 16SrIV-D phytoplasmas.

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Full Length Research Paper

Genetic analysis of wild and cultivated germplasm of pigeonpea using random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers

Babasaheb Walunjkar¹, Akarsh Parihar^{2*}, Pratibha Chaurasia³, K Pachchigar¹ and R. M. Chauhan¹

¹Department of Plant Molecular Biology and Biotechnology, S. D. Agricultural University, S.K. Nagar, Gujarat, India.

²Centre of excellence, Department of Agricultural Biotechnology, Anand Agricultural University, Anand-388110, Gujarat, India.

³Department of Biotechnology, Genetics and Bioinformatics, N.V. Patel College of Pure and Applied Sciences, S.P. University, V.V.Nagar-388120, Gujarat, India.

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The reliability of the quantification of genetic diversity using only one type of marker has been questioned as compared to the combined use of different markers. To compare the efficiency of the use of single versus multiple markers, the genetic diversity was quantified among 12 diverse pigeonpea germplasm comprised of eight wild and four cultivated using both random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers, and how well these two types of markers discriminated the diverse pigeonpea germplasm was evaluated. The pigeonpea germplasm including eight wild species and four cultivated varieties was subjected to 40 RAPD and 40 microsatellite primers. The level of polymorphism as revealed by RAPD primers produced a total of 517 DNA fragments and all were found to be polymorphic that is, 100% and in SSR analysis 101 fragments were produced that too showed 100% polymorphism. The high similarity index value revealed by RAPD was 0.931 between GT-100 and ICPL-87 whereas through SSR, it was 1.00 between GTH-1 and GT-100 as well as *Rhynchosia rothi* and *Rhynchosia minima*. The least similarity index value revealed by RAPD (*R. rothi* and GTH-1) and SSR (*Rhynchosia bracteata* and ICPL-87) were 0.07 and 0.133, respectively. Using RAPD marker, the calculated arithmetic mean heterozygosity and the marker index were 0.90 and 22.47, respectively. The *R. bracteata* and ICPL-87 were found distinct from rest of other cultivars by showing only 13% similarity. Average PIC value shown by RAPD and SSR primers were found to be 0.90 and 0.18, respectively.

Key words: Pigeonpea, random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) markers.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a grain legume of the *Cajaninae* sub-tribe of the economically important leguminous tribe *Phaseoleae* (Young et al., 2003). The

genus *Cajanus* comprises 32 species most of which are found in India (18) and in Australia (13), although one is native to West Africa. Pigeon pea is the only cultivated food

*Corresponding author. E-mail: drakarsh@gmail.com. Tel: 9879442267.

Abbreviations: RAPD, Random amplified polymorphic DNA; STR, simple tandem repeats; SSR, simple sequence repeats; CTAB, cetyl trimethyl ammonium bromide; PCR, polymerase chain reaction; PIC, polymorphic information content; MI, maker index.

crop of the *Cajaninae* sub-tribe and has a diploid genome comprising 11 pairs of chromosomes ($2n = 22$) (Greilhuber and Obermayer, 1998). Wild relatives have now been reported to possess many agronomically important traits such as resistance to pests and diseases (Reddy et al., 1996). *Rhynchosia bracteata* Benth possesses resistance to pod fly damage (Sharma et al., 2003), which would be useful in cultivated pigeonpea for breeding. Genetic diversity has an important role in plant breeding programme. To test genetic resources for their productivity, quality parameters and stress tolerance, field trials are usually time consuming, therefore, molecular markers and DNA technology are used to assess diversity in the gene pool to identify genes of interest and to develop a set of markers for screening progeny (Karp et al., 1996).

Random amplified polymorphic DNA (RAPD) markers have been used for numerous applications in plant molecular genetics research despite having disadvantages of poor reproducibility and not generally being associated with gene regions (Welsh et al., 1990; Williams et al., 1990). RAPD, being a multi locus marker (Karp et al., 1997) with the simplest and fastest detection technology, have been successfully employed for determination of intraspecies genetic diversity in several grain legumes. These include *Vigna unguiculata* (Ba et al., 2004), *Vigna radiate* (Souframanien et al., 2004), *Lens* sp. (Sharma et al., 1995; Ahmad et al., 1996), *Phaseolus* sp. (Beebe et al., 2000; Chiorato et al., 2007), *Glycine* sp. (Jeffrey et al., 1998; Barroso et al., 2003), *Cicer* sp. (Ahmad, 1999), *Pisum* sp. (Cheghamirza et al., 2002; Taran et al., 2005) and *C. cajan* (Kotresh et al., 2006; Ratnaparkhe et al., 1995; Choudhury et al., 2008).

Although known by many names and acronyms, including simple tandem repeats (STR), microsatellites and simple sequence repeats (SSR), SSR have received considerable attention and are probably the current marker system of choice for marker based genetic analysis and marker assisted plant breeding (Akkaya et al., 1992). Only 20 SSRs have been previously reported in pigeonpea, of which only half were polymorphic in cultivated pigeonpea germplasm (Burns et al., 2001). In contrast, more than 1000 microsatellites have been mapped in soybean [*Glycine max* (L.)] (Song et al., 2004), and several hundred are available in chickpea (*Cicer arietinum* L.; Lichtenzveig et al., 2005), common bean (*Phaseolus vulgaris* L.; Blair et al., 2003) and groundnut (*Arachis hypogaea* L.; Ferguson et al., 2004).

SSR are generally among the most reliable and highly reproducible among molecular markers. Indeed SSR are now widely recognized as the foundation for many framework linkage maps. SSR have played a critical role even in merging disparate linkage maps, since they define specific locations in the genome unambiguously (Bell and Ecker, 1994; Akkaya et al., 1995). Hence, considering the potential of the DNA marker based genetic diversity analysis, the present study was aimed to analyze the genetic diversity among the diverse

germplasm of pigeonpea as well as to evaluate the relative usefulness of RAPD and SSR markers for diversity analysis.

MATERIALS AND METHODS

A total of 12 germplasm including eight wild viz., *Cajanus scarabaeoides*, *Cajanus platycarpus*, *Cajanus cajanifolius*, *Rhynchosia rufescence*, *Rhynchosia minima*, *R. bracteata*, *Rhynchosia canna* and *Rhynchosia rothi* and four cultivated namely GTH-1, GT-100, ICPL-87 and GT-1 were procured from the Centre for Excellence for Research on Pulses, S.D Agricultural University, S.K. Nagar (Gujarat) India. First, 25-30 seeds of each cultivar were treated with 0.1% carbendazim (fungicide), washed thoroughly with autoclaved distilled water and sown in earthen pots containing sand: soil : farmyard manure (1:1:1).

Isolation of genomic DNA

For genomic DNA isolation, about 500 mg of fresh leaf material from 4 to 5 week old seedlings of each of the 12 cultivars were ground to fine powder in liquid nitrogen and DNA was isolated following the cetyl trimethyl ammonium bromide (CTAB) method as described by Rogers et al. (1980) with some modification. The quality and concentration of the extracted DNA was estimated by spectrophotometer and the samples were diluted to a final concentration of 30 ng μ l⁻¹.

RAPD and SSR based marker analysis

The 40 primers each of RAPD (10-mer primers) and SSR procured from Bangalore Genie, Bangalore, India, were screened for the genomic DNA extracted for polymorphism survey. However, finally eight random primers viz., OPA-07, OPA-09, OPA-10, OPA-11, OPA-12, OPA-14, OPA-16 and OPA-19 and 5 SSR primers namely CCB1, CCtta001, CCat001, CCtta003 and CCtta005 were found to be polymorphic and hence, taken for further analysis (Table 1). Polymerase chain reaction (PCR) reactions were carried out in a 25 μ l reaction volume containing 1 unit of Taq DNA polymerase, 30 ng of template DNA, 0.2 μ M of primer, 2 μ l of each of dATP, dCTP, dGTP and dTTP, 1x PCR reaction buffer. Amplifications were performed in a DNA thermocycler (Eppendorf, Hamburg, Germany), programmed for initial denaturation for 3 min at 94°C, followed by 40 consecutive cycles of 30 s at 94°C, 1 min at 35°C and 72°C for 1 min and final extension at 72°C for 10 min. The annealing temperatures of the cycling parameter were readjusted for each microsatellite primers according to their calculated T_m based on the sequence composition: $T_m = 4^\circ(G+C) + 2^\circ(A+T) - 3^\circ C$. The amplified products were subjected to electrophoresis in 1.2 % agarose gel in TAE [tris acetate ethylene diamine tetra-acetic acid (EDTA)] buffer running at 80 v for 2.5 h. The gels were stained using ethidium bromide and documented using Alpha Imager-1200TM (Alpha Innotech, San Leandro, USA). Duplicated independent DNA preparations for each sample were done and only major bands consistently amplified were scored.

Data scoring and statistical analysis

Data analysis was carried out only for those primers that gave scorable patterns. Data were scored for computational analysis on the basis of the presence or absence of the PCR products. If a product was present in a genotype, it was designated as 1 and, 0 if absent. The data were maintained in the spreadsheet format for

Table 1. List of polymorphic RAPD and SSR primers along with their nucleotide sequence.

S/N	RAPD primer			SSR primer		
	Primer	Primer sequence	GC content %	Primer	Primer sequence	GC content %
1	OPA-07	5'GAAACGGGTG3'	60	CCB1	F: AAGGGTTGTATCTCCGCGTG R: GCAAAGCAGCAATCATTTTCG	55 45
2	OPA-09	5'GGGTAACGCC3'	70	CCtta001	F: TTCTGGATCCCTTTTCATTTTTC R: TGACACCCTTCTACCCCATAA	36 47
3	OPA-10	5'GTGATCGCAG3'	60	CCat001	F: CTTCCCCCACTAAGATCCA R: GTTCGTTCTCTTTAATTGACTTGC	50 37
4	OPA-11	5'CAATCGCCGT3'	60	CCtta003	F: CCAAGAAAAGGTGCTCCAAGT R: TTGCTTCTTTTCTCGCTTGC	47 45
5	OPA-12	5'TCGGCGATAG3'	60	CCtta005	F: TCTTCATTGCATGGTGT R: GCATGATATGAGATGATGACGA	42 40
6	OPA-14	5'TCTGTGCTGG3'	60			
7	OPA-16	5'AGCCAGCGAA3'	60			
8	OPA-19	5'CAAACGTCCG3'	60			

further analysis. The data were entered into binary matrix and subsequently analyzed using NTSYSpc version 2.02i (Rohlf, 1994). Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the unweighted pair group method with arithmetic mean (UPGMA) method by SAHN clustering function of NTSYS-pc. Relationships between the pigeonpea germplasm were graphically represented in the form of dendrograms. The cophenetic correlation analysis was carried out using the COPH function of NTSYS-pc. In this method, the dendrogram and similarity matrix were correlated to find the goodness-of-fit of the dendrogram constructed based on the similarity coefficients. The correspondence between the SSR and RAPD based on similarity coefficient matrices was tested based on cophenetic correlation analysis and Mantel matrix correspondence test. The Mantel matrix correspondence test was carried out using the MXCOMP function in the NTSYSpc version 2.02i. The expected heterozygosity (H_n), arithmetic mean heterozygosity (H_{av}) and marker index (MI) were calculated using the methodology of Nei (1973) and Powell et al. (1996). The expected heterozygosity, H_n for a molecular marker was calculated as, $H_n = 1 - \sum p_i^2$, where p_i is the allele frequency of the i^{th} allele. The arithmetic mean heterozygosity, H_{av} was calculated for each marker class as, $H_{av} = \sum H_n/n$, where n is the number of markers or loci analyzed. Marker index (MI) is calculated as, $MI = E(H_{av})p$, where E is the effective multiplex ratio ($E = n\beta$, where β is the fraction of polymorphic markers or loci).

RESULT

Genetic relatedness was studied among 12 germplasm of Pigeonpea using RAPD and SSR markers, so that genetically distinct germplasm showing different responses towards *Helicoverpa armigera* can be utilized in breeding for *H. armigera* for generating mapping populations to be used to identify genes or quantitative trait loci. The banding pattern thus obtained by both RAPD and SSR primers clearly distinguished cultivars into different clusters showing sufficient diversity.

Polymorphism survey using RAPD and SSR markers

In pooled RAPD analysis, all eight arbitrary oligonucleotide primers finally selected out of total 40, generated a total of 517 scorable bands with 150 loci. Among them all, 150 loci were found polymorphic, showing 100% polymorphism (Table 2). The size of the amplified products ranged from 65 to 3607 bp. A representative RAPD profile obtained by primer OPA-10 is shown in Figure 1.

Out of a total of 22 loci (289-1511bp), all 22 were polymorphic (100%). In case of SSR analysis, CC based five microsatellite (SSR) primers out of total 40, were used to analyze the genetic diversity among 12 pigeonpea germplasm (Table 3). The SSR analysis with five microsatellite primers produced a total of 12 alleles. Among these five primers, maximum amplified allele size of 250 bp was generated by CCtta-005 and minimum amplified allele size of 71 bp by CCtta001 marker. A maximum of three alleles were recorded for primers CCtta-003 and CCtta-005 (Figure 2), while primers CCB-1, CCat-001 and CCtta-001 produced two alleles each, which were the lowest in the present investigation.

Cluster analysis

The polymorphic information content (PIC) values, a reflection of allele diversity and frequency among the germplasm, were uniformly higher for all the RAPD loci tested. The PIC value ranged from 0.78 (OPA-12) to 0.94 (OPA-19) with a mean of 0.98. The similarity coefficients based on RAPD ranged from 0.070 to 0.931 with an average value of 0.501. The least similarity index value revealed by RAPD was 0.07 between *R. rothi* and GTH-1 whereas arithmetic mean heterozygosity and the marker

Table 2. Summary of genetic diversity study using RAPD analysis.

S/N	Locus name	Number of band	Total loci	Polymorphic loci	Percentage polymorphism	PIC value	Molecular weight range (bp)
1	OPA-07	63	21	21	100	0.92	147-1778
2	OPA-09	74	23	23	100	0.93	111-3204
3	OPA-10	84	22	22	100	0.93	289-1511
4	OPA-11	82	23	23	100	0.93	112-2435
5	OPA-12	28	8	8	100	0.78	65-1850
6	OPA-14	60	20	20	100	0.91	73-3607
7	OPA-16	37	11	11	100	0.86	212-1891
8	OPA-19	89	22	22	100	0.94	173-3230
	Total	517	150	150	100	7.2	-

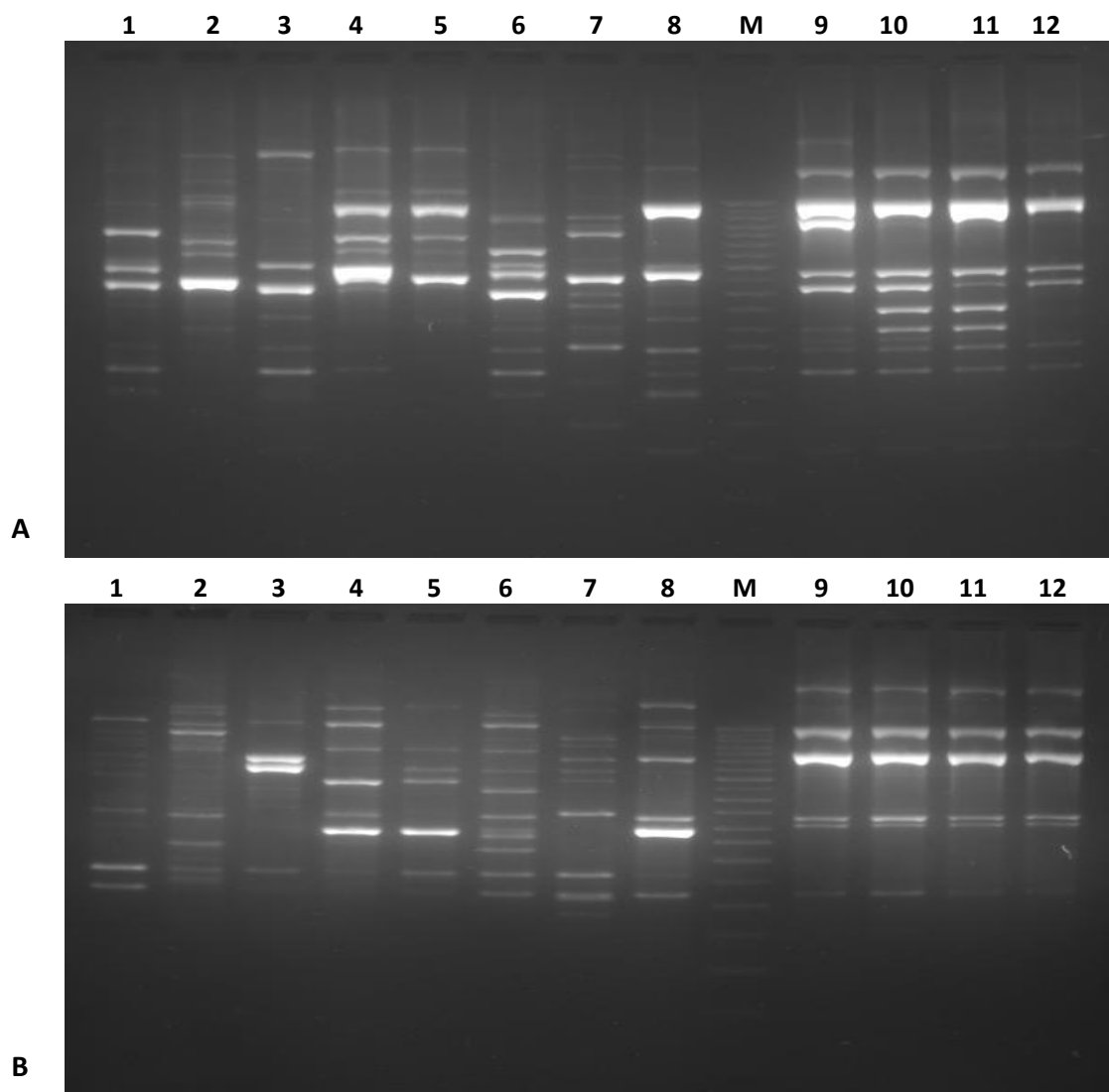


Figure 1. RAPD profiles of the eight wild and four cultivated germplasms. Lanes 1 to 8 represent wild germplasm; M, ladder and 9 to 12 cultivated germplasms; A, OPA -10; B, OPA-11; C, OPA-14; D, OPA-19. 1, *Cajanus scarabaeoides*; 2, *Rhynchosia bracteata*; 3, *Cajanus cajanifolius*; 4, *Cajanus platycarpus*; 5, *Rhynchosia rothi*; 6, *Rhynchosia canna*; 7, *Rhynchosia minima*; 8, *Rhynchosia rufescence*; M-50 bp ladder, 9-GTH-1, 10-GT-100, 11-ICPL-87, 12-GT-1.

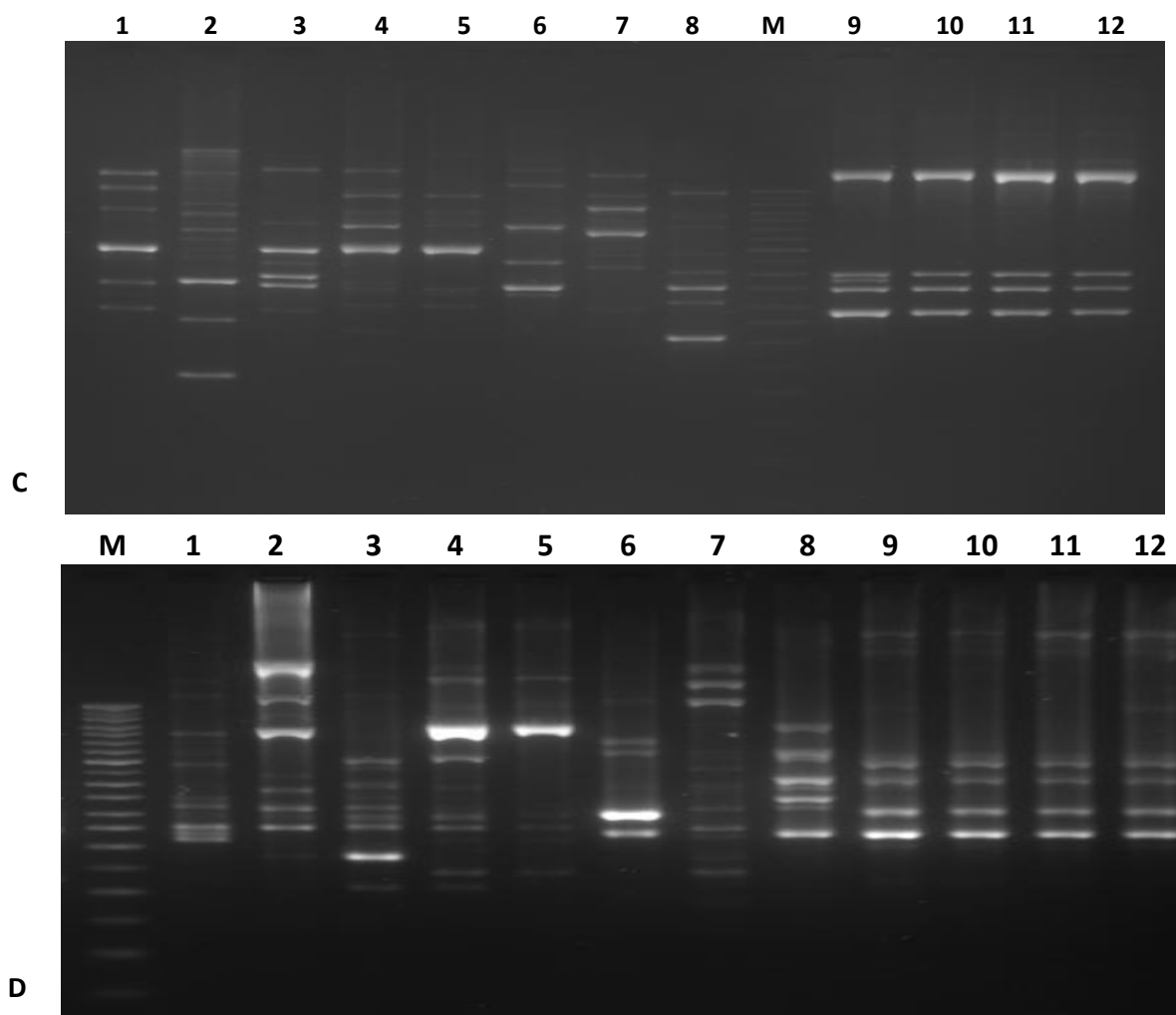


Figure 1. Contd.

Table 3. Summary of genetic diversity study using SSR analysis.

S/N	Locus name	Number of bands amplified	Molecular weight range (bp)	Difference in bp	Total number of alleles	PIC value
1	CCB-1	8	110-206	96	2	0.25
2	CCat-001	18	101-222	121	2	0.25
3	CCtta001	12	71-119	48	2	0.076
4	CCtta-003	15	87-187	100	3	0.14
5	CCtta-005	14	156-250	94	3	0.19

index were 0.90 and 22.47, respectively (Table 2).

Clustering pattern of dendrogram generated by using the pooled molecular data of eight RAPD loci indicated that two clusters namely A and B are formed at a similarity coefficient of 0.14 (Table 4). Cluster A was again divided into two sub clusters A1 and A2. First, A1 sub cluster formed two sub clusters A1a and A1b, in which cluster

A1a included *C. scarabaeoides*, *C. cajanifolius*, *R. rufescence* and *R. canna*. In this cluster, the *C. scarabaeoides* and *Cajanus cajanifolius* showed 37% similarities with the other wild germplasm of the cluster A1a. The second minor sub cluster A1b included all cultivars viz., GTH-1, GT-100, ICPL-87 and GT-1. In this cluster, GT-100 and ICPL-87 showed 93% similarities

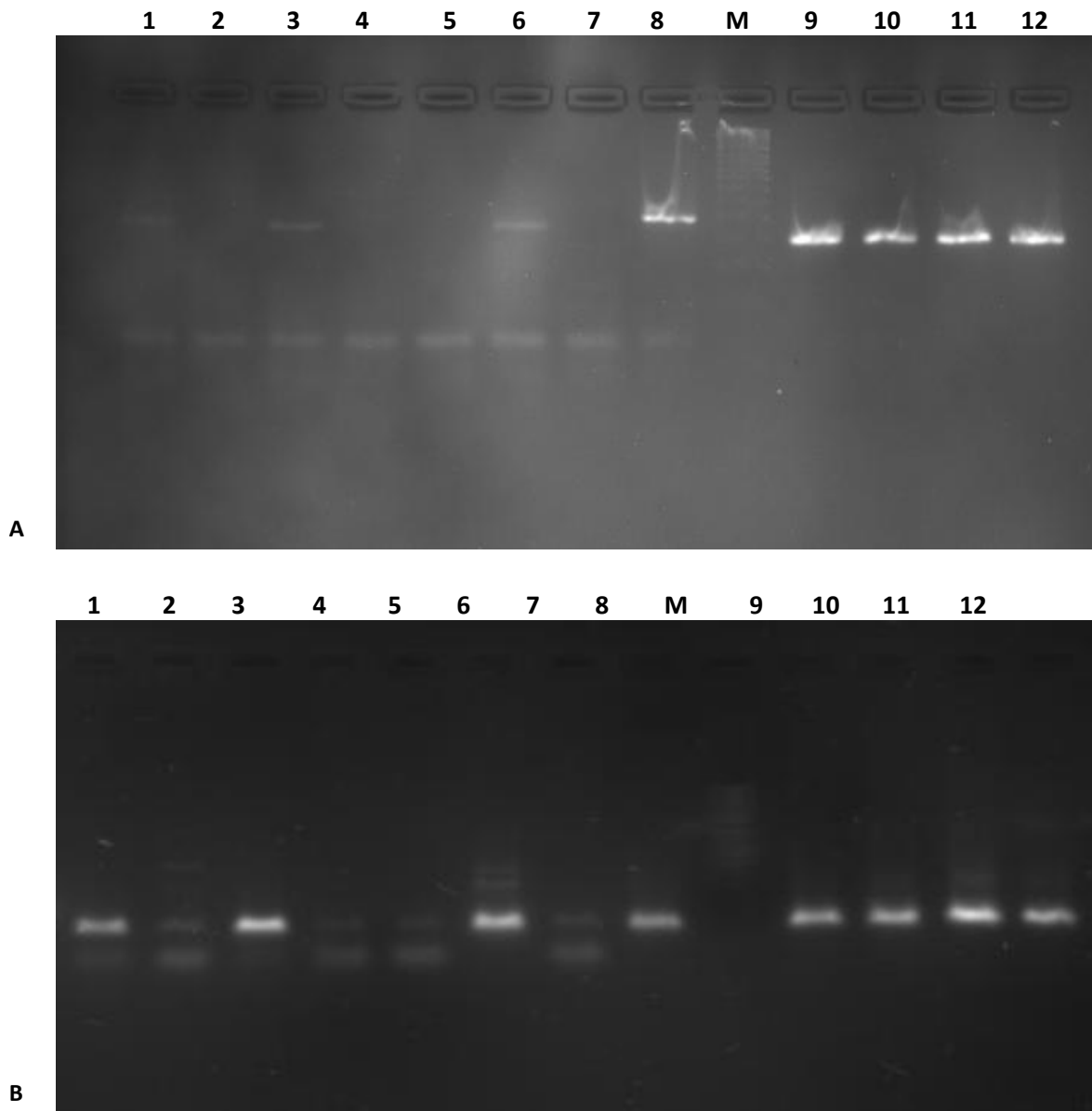


Figure 2. SSR profile of the eight wild and four cultivated germplasms. Lanes 1 to 8 represent wild germplasm; M, ladder and 9 to 12 cultivated germplasms. A, CCta-003; B, CCta005. 1, *Cajanus scarabaeoides*; 2, *Rhyncosia bracteata*; 3, *Cajanus cajanifolius*; 4, *Cajanus platycarpus*; 5, *Rhyncosia rothi*; 6, *Rhyncosia canna*; 7, *Rhyncosia minima*; 8, *Rhyncosia rufescence*; M-50 bp ladder, 9-GTH-1, 10-GT-100, 11-ICPL-87, 12-GT-1.

with the other cultivars. Sub cluster A2 included *R. minima* only. The second major cluster B formed two sub clusters in which first sub cluster B1 included *R. bracteata*. Second sub Cluster B2 contained *C. platycarpus* and *R. rothi*, which was found to be more closely related and showed 59% similarity (Figure 3).

In case of SSR analysis, the highest PIC value was recorded for CCB-1 (0.25), CCat-001 (0.25) and the lowest for CCta001 (0.076) (Table 3). The highest similarity index value of 1.00 was found between *R. rothi* and *R. minima* as well as between GTH-1 and GT-100, while the least similarity index value of 0.13 was between

R. bracteata and ICPL-87 (Table 5). The arithmetic mean heterozygosity and the marker index were 0.18 and 33.66, respectively.

Clustering pattern of dendrogram generated by using the pooled molecular data of five SSR primers indicated that two clusters namely A and B are formed at a similarity coefficient of 0.18. Cluster A was again divided into two sub clusters A1 and A2. First A1 sub cluster formed two minor sub clusters A1a and A1b. Cluster A1a was further subdivided into A1a(1) and A1a(2) in which first minor sub cluster A1a(1) included *C. scarabaeoides*, *R. rufescence* and *C. cajanifolius*. This further indicated that

Table 4. Similarity matrix for jaccard's coefficient for 12 wild and cultivated pigeonpea germplasm based on RAPD analysis.

	<i>C. scarabaeoides</i>	<i>R. bracteata</i>	<i>C. cajanifolius</i>	<i>C. platycarpus</i>	<i>R. rothi</i>	<i>R. canna</i>	<i>R. mini</i>	<i>R. rufescence</i>	GTH-1	GT- 100	ICPL- 87	GTH-1
<i>C. scarabaeoides</i>	1.000											
<i>R. bracteata</i>	0.140	1.000										
<i>C. cajanifolius</i>	0.372	0.125	1.000									
<i>C. platycarpus</i>	0.188	0.137	0.213	1.000								
<i>R. rothi</i>	0.152	0.166	0.149	0.591	1.000							
<i>R. canna</i>	0.328	0.189	0.305	0.172	0.140	1.000						
<i>R. minima</i>	0.208	0.153	0.200	0.151	0.166	0.160	1.000					
<i>R. rufescence</i>	0.359	0.116	0.315	0.197	0.136	0.298	0.170	1.000				
GTH-1	0.269	0.112	0.210	0.125	0.070	0.177	0.186	0.283	1.000			
GT- 100	0.274	0.113	0.239	0.141	0.086	0.179	0.157	0.305	0.851	1.000		
ICPL- 87	0.258	0.110	0.242	0.142	0.088	0.181	0.175	0.291	0.829	0.931	1.000	
GT- 1	0.271	0.120	0.235	0.148	0.092	0.180	0.180	0.280	0.840	0.860	0.920	1.000

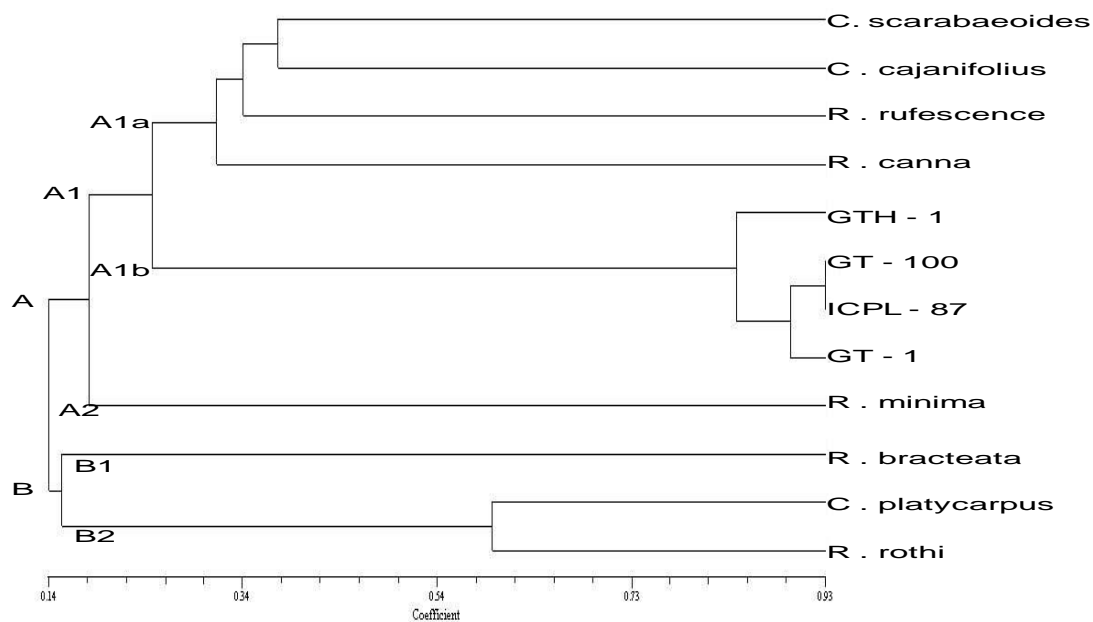


Figure 3. Dendrogram showing clustering of 12 pigeonpea germplasm constructed using UPGMA based on Jaccard's coefficient obtained from RAPD analysis.

Table 5. Similarity matrix for Jaccard's coefficient of SSR for 12 pigeonpea germplasm.

	<i>C. scarabaeoides</i>	<i>R. bracteata</i>	<i>C. cajanifolius</i>	<i>C. platycarpus</i>	<i>R. rothi</i>	<i>R. canna</i>	<i>R. minima</i>	<i>R. rufescence</i>	GTH-1	GT- 100	ICPL- 87	GT- 1
<i>C. scarabaeoides</i>	1.000											
<i>R. bracteata</i>	0.181	1.000										
<i>C. cajanifolius</i>	0.888	0.200	1.000									
<i>C. platycarpus</i>	0.666	0.250	0.750	1.000								
<i>R. rothi</i>	0.333	0.166	0.375	0.500	1.000							
<i>R. canna</i>	0.818	0.250	0.727	0.545	0.272	1.000						
<i>R. minima</i>	0.333	0.166	0.375	0.500	1.000	0.272	1.000					
<i>R. rufescence</i>	0.900	0.166	0.800	0.600	0.300	0.750	0.300	1.000				
GTH-1	0.615	0.142	0.538	0.500	0.250	0.533	0.250	0.692	1.000			
GT- 100	0.615	0.142	0.538	0.500	0.250	0.533	0.250	0.692	1.000	1.000		
ICPL- 87	0.571	0.133	0.500	0.461	0.230	0.600	0.230	0.642	0.923	0.923	1.000	
GT- 1	0.727	0.166	0.636	0.600	0.300	0.615	0.300	0.818	0.833	0.833	0.769	

two wild germplasms *C. scarabaeoides* and *R. rufescence* are more closely related and showed 90% similarity, while second minor sub cluster A1a(2) contained *R. canna* and *C. platycarpus* include sub cluster A1b. Second sub cluster A2 contained all cultivated germplasm viz., GTH-1, GT-100, ICPL-87 and GT-1. The cluster A2 contained GTH-1 and GT-100 which were highly close related and showed 100% similarity, while GT-100 and ICPL-87 showed 92% similarity. Second major Sub cluster B included *R. rothi* and *R. minima* which were grouped in one cluster and showed 100% similarity and the remaining *R. bracteata* did not form clustering (Figure 4).

DISCUSSION

The wild relatives of pigeonpea have considerable diversity. These may possess useful genes controlling economically important traits. These cultivars may serve as germplasm source of new

genes in pigeonpea for *H. armigera* resistance. In the present investigation, RAPD primers were used to study the genetic diversity of 12 pigeonpea germplasm. Out of 40, eight primers showed 100% polymorphic bands. Similar results were reported by Choudhury et al. (2008) where they found the level of polymorphism ranging from 9.1 to 100%. Lohithaswa et al. (2003) showed 63.46% polymorphism in their study to assess genetic diversity among 11 pigeonpea cultivars with RAPD markers. Malviya et al. (2010) observed the genetic diversity of 17 cultivars of pigeonpea using 17 RAPD primers. Nine out of 17 primers depicted more than 80% polymorphism.

In RAPD markers study, Jaccard similarity coefficient ranged from 0.07 to 0.93. Lakhanpaul et al. (2000) subjected 32 Indian cultivars of green gram to RAPD analysis using 21 decamer primers. Jaccard similarity coefficient values ranged from 0.65 to 0.92. *C. scarabaeoides* and *C. cajanifolius* showed highest similarities (37%) in other wild germplasm and GT-100 and ICPL-87

showed highest similarities (93%) in local cultivars. The present study revealed high level of polymorphism among wild pigeonpea germplasm as compared to cultivated germplasm. Ratnaparkhe et al. (1995) also reported low level of genetic diversity among cultivated pigeonpea as compared to the wild relatives.

In the present study, the arithmetic mean heterozygosity and the marker index in RAPD analysis were 0.90 and 22.47, respectively. Choudhury et al. (2008) found the arithmetic mean heterozygosity to be 0.48, whereas the value of the maker index (MI) was 5.027 in pigeonpea using RAPD analysis. As microsatellite or SSR markers are highly polymorphic, reproducible, co-dominant in nature and distributed throughout the genome, they have become the ideal marker system for genetic analysis and breeding application. In the present study, out of 40, five SSR markers were polymorphic in 12 pigeonpea germplasm.

The PIC value was found to be ranging from 0.076

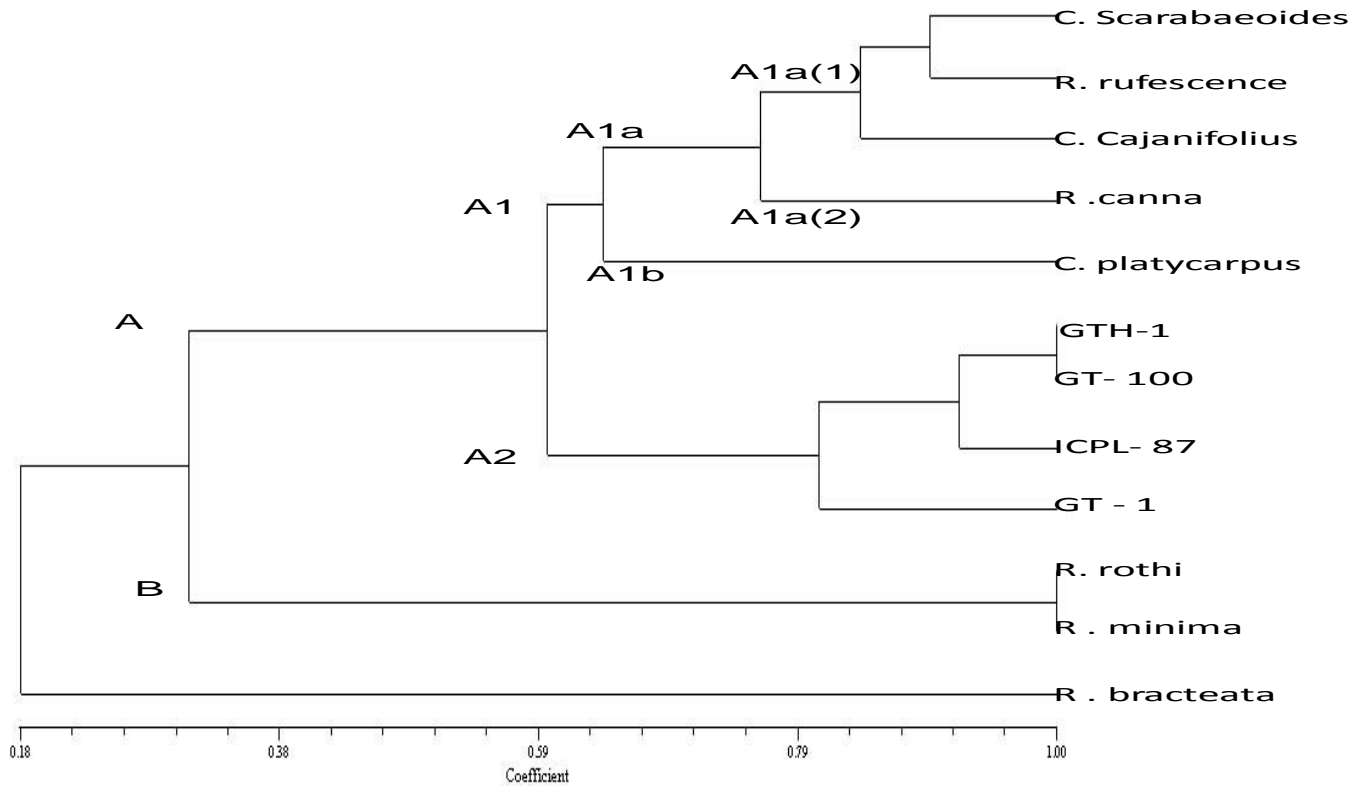


Figure 4. Dendrogram showing clustering of 12 wild and cultivated pigeonpea germplasm constructed using UPGMA based on Jaccard's coefficient obtained from SSR analysis.

to 0.25. Odeny et al. (2007) reported that 20 SSR primers were polymorphic in 15 cultivated and nine wild pigeonpea relatives. Nearly, all amplifying SSR primers detected polymorphism amongst the 24 diverse accessions. The PIC value was ranged from 0.17 to 0.80. In the results obtained by Sexena et al. (2010), 13 SSR primers were polymorphic amongst 32 cultivated and eight wild pigeonpea genotype representing six *Cajanus* species. The polymorphic information content for these markers ranged from 0.05 to 0.55.

In SSR pooled data, five polymorphic SSR primers were used for diversity analysis of 12 pigeonpea germplasm. This indicated that the cultivated GTH-1 and GT-100 are closely related and showed 100% similarity. Odeny et al. (2007) used 19 polymorphic SSR primers for diversity analysis of 24 *Cajanus* genotypes. They found that, cultivated ICP 7543 and ICP 14144 revealed the highest genetic similarity (98%). The high polymorphic results indicate a wide genetic base in pigeonpea accessions and genetic diversity may be due to their characteristics, wide distribution, amplification protocol used/selection of suitable primers.

The present analysis using pigeonpea germplasm showed that both techniques may provide consistent data and can thereby be used to study genetic diversity in pigeonpea, showing concordant values of genetic diver-

sity. This led us to generate an opinion that RAPD markers can be considered as effective as SSR markers if we succeeded to achieve the following stringency: 1, high purity of DNA; 2, selection of RAPD primes aimed at identifying DNA segment that are well separated and reproducible; 3, optimization of reagent concentrations that are critical in the amplification process, providing reliable and replicable results, and 4, identification of more strongly stained DNA segments, selecting properly different amplification programs.

Therefore, the option to analyze pigeonpea genetic diversity using RAPD markers kept track and guided genetic breeding programs can be considered an adequate strategy. On practical grounds, the OPA19, OPA10 and OPA11 primers for instance which produced the greatest number of bands and which showed the greatest potential to discriminate polymorphic DNA segments, can be recommended for future analysis of the pigeonpea genome using RAPD markers.

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Full Length Research Paper

Study of simple sequence repeat (SSR) polymorphism for biotic stress resistance in elite rice variety JGL 1798

G. Siva Kumar¹, K. Aruna Kumari^{1*}, Ch. V. Durga Rani¹, R. M. Sundaram², S. Vanisree³, Md. Jamaluddin¹ and G. Swathi¹

¹Institute of Biotechnology, College of Agriculture, ANGRAU, Rajendranagar, Hyderabad-30, India.

²Directorate of rice research, Rajendranagar, Hyderabad-30, India.

³Agricultural Research Institute, Rice Section, Rajendranagar, Hyderabad-30, India..

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To provide ready to use markers for back ground selection in marker assisted breeding of rice, we used GPP 2 as donor parent for *xa13*, *Xa21*, *Gm4* resistance to bacterial blight, gall midge and NLR 145 as another donor parent for *Pi-k^h* gene resistance to blast and JGL 1798 as recurrent parent was investigated using 128 simple sequence repeat (SSR) primers covered on chromosome number 1-12. The results reveal that 36 HRM primers showed distinct polymorphism among the donor and recurrent parents studied indicating the robust nature of microsatellites in revealing polymorphism. Based on this study, the large range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of simple sequence repeats (SSR) polymorphism.

Key words: Simple sequence repeats (SSR), parental polymorphism, JGL 1798, hyper variable markers.

INTRODUCTION

Rice (*Oryza sativa*. L), a member of the Graminae family, has a genome size of 0.45×10^9 bp (Arumunagathan and Earle, 1991), which is one tenth the size of the human genome and is a model system for cereal genome analysis. Biotic stresses, such as diseases (blast caused by the fungus *Magnaporthe grisea*, bacterial leaf blight caused by *Xanthomonas oryzae* pv. *Oryzae*, and insects (gall midge) account for significant yield losses annually. Resistance to these diseases and insect controlled either by dominant or recessive major genes (*xa13*, *Xa21*, *Pi-k^h*, *Gm4*) by QTL (Alam and Cohen, 1998; Himabindu et al., 2010). The DNA markers have been used effectively to identify resistance genes, and marker assisted selection (MAS) has been applied for integrating different resis-

tance genes into rice cultivars lacking the desired traits. Jagityal Sannalu (JGL 1798), released by Acharya N G Ranga Agricultural University (ANGRAU), Rajendranagar, Hyderabad. This variety is being widely grown in Northern Telangana region of Andhra Pradesh during both *kharif* and *rabi* seasons. The cooking quality of this variety is on par with a premium rice variety, Samba Mahsuri, one of the parents [(Samba Mahsuri (BPT 5204) × Kavya (WGL 48684)] of this variety and is susceptible to bacterial leaf blight, gall midge and blast. As on date, 100 rice blast major resistance genes (R-genes) have been identified and mapped (Sharma et al., 2012) on different rice chromosomes, and tightly linked DNA markers have been developed. The polymerase

*Corresponding author. E-mail: arunaagbsc@gmail.com.

Abbreviations: PCR, Polymerase chain reaction; MAS, marker assisted selection; BB, bacterial blight; MABB, marker assisted back cross breeding; SSRs, simple sequence repeats; PIC, polymorphism information content; CTAB, cetyl trimethylammonium bromide; EDTA, ethylenediaminetetra acetate.

chain reaction (PCR) based allele-specific and InDel marker sets are available for 9 blast resistance genes. And they provide an efficient marker system for MAS for blast resistance breeding (Hayashi et al., 2006). Eight blast resistance genes have been cloned and the genes have been used for their selective introgression into susceptible rice cultivar (Lin et al., 2007). It is imperative to use DNA markers identified within the gene or from the flanking region of the gene as a tool for an efficient MAS strategy in rice improvement (Fjellstrom et al., 2004). Additionally, several blast resistance genes could be combined using MAS in a single genetic background to develop rice cultivars with broad-spectrum durable resistance to blast. Several bacterial blight (BB) resistance genes have been associated with tightly linked DNA markers, and some of them have been cloned (*Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26*, *Xa27*) and used for breeding BB-resistant rice cultivars. Because of the availability of DNA markers derived from the resistance genes, it is now possible to pyramid several genes, into susceptible elite rice cultivars. The resistance genes *xa5*, *xa13*, and *Xa21* have been pyramided in to an indica rice cultivar (PR 106) using MAS that expressed strong resistance to BB races of India (Singh et al., 2001). It is the most effective way of transferring specific gene(s) to an elite susceptible cultivar. In rice, the feasibility of marker assisted back cross breeding (MABB) to pyramid BB resistance genes has been well demonstrated (Sundaram et al., 2008).

Resistance to gallmidge is under the control of at least 11 resistance genes (*Gm1*, *Gm2*, *gm3*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm8*, *Gm9*, *Gm10*, *Gm11*), 8 of which have been tagged and mapped. (Himabindu et al., 2010). The usefulness of resistant cultivars for protection against gall midge infestation suggests that MAS will be a highly useful tool for breeders in areas where the pest is prevalent.

According to the study of Frisch et al. (1999a), molecular markers are used in backcross breeding for two purposes: (1) To trace the presence of a target allele for which the term 'foreground selection' was suggested for this selection of target allele by Hospital and Charcosset (1997); and (2) to identify individuals with a low proportion of undesirable genome from the donor parent. This approach is called 'background selection' and was first proposed by Tanksley et al. (1989) and then by Hillel et al. (1990) and was further investigated by Hospital et al. (1992) and later reviewed by Viescher et al. (1996). The main advantage of using DNA markers is to accelerate the fixation of recipient alleles in non-target regions and to identify the genotypes containing crossovers close to target genes (Tanksley et al., 1989).

Among different classes of available molecular markers, simple sequence repeats (SSRs) are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage. SSR markers have been more useful for parental polymor-

phism study. It is the basic step for MAS. The polymorphism information content (PIC) value ranged from 0.064 (RM 274) to 0.72 (RM 580) with an average of 0.46. The Jaccard's similarity coefficient ranged from 0.42 to 0.90 reported by Seetharam et al. (2009). In rice, microsatellites have been classified into two groups based on length of SSR motif and their potential as informative markers: Class I microsatellites contain perfect SSRs >20 nucleotides in length and Class II contains perfect SSRs >12 nucleotides and <20 nucleotides in length. Class I markers are reported to be highly variable (Cho et al., 2000), whereas Class II SSRs are less variable owing to limited expansion of microsatellite repeat motif during slipped-strand mispairing over the shorter SSR template (Temnykh et al., 2001). As of now, 18 828 Class I microsatellite markers have been identified (IRGSP, 2005) and a high-density SSR map with genome coverage of approximately two SSRs per centimorgan (cM) has been constructed in rice (McCouch et al., 2002). The present study was undertaken with the objective of identifying polymorphic SSRs for further background selection of JGL 1798.

MATERIALS AND METHODS

Plant material

Three rice genotypes constituted the experimental material JGL 1798, NLR 145 collected from ARI, Rice section, ANGRAU and GPP 2 collected from DRR, Rajendranagar, Hyderabad, India.

DNA extraction and SSR analysis

Genomic DNA was extracted by modified cetyl trimethylammonium bromide (CTAB) method (Sambrook and Russel, 2001). 15 - 20 days rice leaves were extracted with DNA extraction buffer (2% CTAB, 100 mM Tris, 20 Mm ethylenediaminetetra acetate (EDTA), 1.4 M NaCl) preheated at 60 UC and 200 mg. The quality and quantity of extracted DNA was judged by comparing it with λ -DNA in agarose gel electrophoresis. DNA quantification and purity was checked by measuring the O.D at 260 and 280 nm using a UV visible spectrophotometer. For marker assisted-multiple gene introgression, 4 gene specific primers were used for foreground selection and 128 HRM primers for back ground selection. These information regarding chromosomal location and sequences of primers were obtained from www.gramene.org.

Polymerase chain reaction (PCR)

The polymerase chain reaction was carried out in Eppendorf thermal cycler using 128 primers. The PCR reaction mix includes the following: DNA 10 ng/ μ l; 10 X buffer; 10 mM dNTPs; 50 mM MgCl₂, 10 μ M each of forward and reverse primers. The PCR profile starts with initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, primer annealing 55°C for 30 s (for gene specific primers like *xa13*, *RM 547*, *RM 206* at 55°C and *pTA248* 58°C), extension 72°C for 1 min, final extension 72° C for 10 min, and cooling 4°C for α was included. These steps were repeated for 35 cycles for amplification of DNA. After completion of amplification, PCR products were stored at -20°C and the amplified products were analyzed by electrophoresis using 2% (for *xa13*, *pTA248*) and 3% (*RM 547*, *RM 206* and for background primers) agarose gels. Ethidium bromide

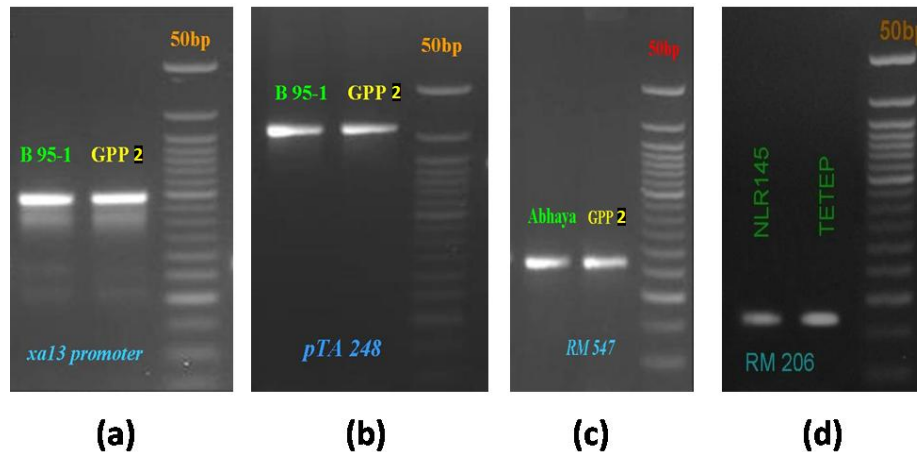


Figure 1. Validation of target genes in donor parents.

was added while pouring the gel so that the DNA fluoresces when gel was exposed to UV light. The DNA fragments were then visualized under UV transilluminator and the banding pattern was observed and recorded using gel documentation unit (Gene flash) which was stored for further scoring and permanent records.

Data analysis

The 3 genotypes were scored for the presence and absence of the SSR bands. And the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis.

RESULTS

Validation of *xa13*, *Xa21*, *Pi-k^h* and *Gm4* genes in the donor parents

In the present study, Jagityal Sannalu (JGL 1798) variety was chosen as recurrent or recipient parent, while GPP 2 was used as donor parent for *xa13*, *Xa21* and *Gm4* resistance genes. NLR 145 is another donor parent for blast resistance gene, *Pi-k^h*. Before starting marker assisted back crossing (MABC) breeding, these donors are to be confirmed for the resistance genes by comparing with their respective source materials. Once the parents were confirmed for the resistance genes, then the parental polymorphism study was carried out between recurrent and donor parental lines.

GPP 2 is the donor parent for three resistance genes viz., *xa13*, *Xa21* and *Gm4*. Hence this donor parent is to be validated for the presence of three genes by comparing with their respective check materials B 95-1 (BLB resistance genes *xa13* and *Xa21*) and Abhaya (gall midge resistance gene *Gm4*).

The results reveal that an allele of 500 bp was amplified with *xa13 promoter* in the donor parent GPP 2. This band was exactly identical to the band that was amplified in the check material, B 95-1. The marker *pTA-248* ampli-

fied an allele of 916 bp (Figure 1), which was similar with that of B 95-1 confirming that the donor parent was carrying *Xa21* gene. Huang et al. (1997) also used *pTA248*, a gene sequence based marker for *Xa21* gene in marker assisted selection. Amplification pattern of *RM547* was observed at 270 bp in GPP 2 and it was identical to the band that was observed in Abhaya. Arundathi et al., 2010 also observed *Gm4* gene in PTB10 by using gene specific primers. These results revealed that the donor, GPP 2 was carrying two BLB resistance genes and one gall midge resistance gene. These results were identical with the findings of Sundaram et al. (2008, 2009).

NLR 145, another donor parent was verified for the presence of blast resistance gene, *Pi-k^h* by using the gene specific primer, *RM206* along with check material, Tetep. The result confirmed that NLR 145 was carrying *Pi-k^h* gene since *RM206* primer pair amplified an allele of 140 bp in NLR 145 and this allele was exactly similar to that of Tetep. The result is in confirmation with the findings of Sharma et al. (2005).

Parental polymorphism for resistance genes

Study of parental polymorphism is a pre requisite to begin marker assisted selection or marker assisted back cross breeding. Unless the parents are polymorphic for the traits of interest, the further selection of plants carrying the traits of interest is not possible in the progenies. In the present study, parental polymorphism survey was taken up between JGL 1798 and GPP 2 and also between JGL 1798 and another donor parent, NLR 145. The two donor parents were tested along with the recurrent parent, JGL 1798 for all the four target genes. The results indicated that a clear polymorphism was observed between the parents, JGL 1798 and GPP 2 for *xa13* gene when the primer pair, *xa13 promoter* was used for amplification. *xa13* specific marker amplified a clear band of 260 bp in JGL 1798, while another band of 500 bp was amplified in the donor parent, GPP 2. Similarly, polymor-

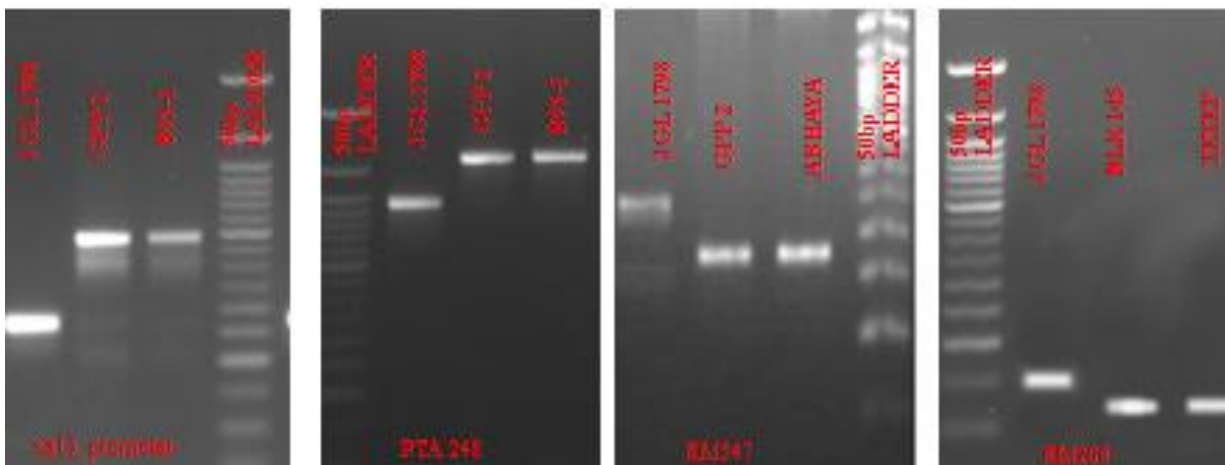


Figure 2. Polymorphism between parental lines for four target genes using gene specific primer pairs.

phism was observed between GPP 2 (916 bp) and JGL 1798 (700 bp) when *pTA-248* primer was used for *Xa21* gene. *RM547*, a gene specific primer was used for observing the parental polymorphism for the target gene, *Gm4* showed that an allele of 290 bp was observed JGL 1798, while the resistance allele of 270 bp in the donor parent, GPP 2. These results clearly showed that there was a clear polymorphism between JGL 1798 and GPP 2 for three target genes viz., *xa13*, *Xa21* and *Gm4*. Nair et al. (1996) also observed polymorphism between resistant and susceptible parents by using random amplified polymorphic DNA (RAPD) primers.

SSR primer pair of RM206 was used to study the polymorphism between JGL 1798 and NLR 145 for *Pi-k^h*, a blast resistance gene. A susceptible allele of 170 bp in JGL 1798, while the resistance allele of 140 bp was observed in NLR 145 (Figure 2). The allele that was observed in JGL 1798 was clearly different from resistance allele that was observed in NLR 145. The present investigation clearly stated that three resistance genes including one for gall midge and two for BLB were presented in GPP 2, while NLR 145 was carrying a blast resistance gene, *Pi-k^h*. The recurrent parent, JGL 1798 was carrying all the four corresponding susceptible alleles.

Since the polymorphism was very clear among the parents for all the four target genes (Table1), further selection of plants carrying the target genes in the successive backcrossing generations is referred as foreground selection in MABC (Hospital and Charcosset., 1997). Hence these markers will be used for foreground selection. Similarly Yang et al. (1994) and Mc Couch et al. (1997) used SSRs to study the polymorphism between and the rice varieties. The gene-specific markers viz., *xa13 promoter*, *pTA-248*, *RM547* and *RM206* have been reported to be tightly linked to BLB, gall midge and blast resistance genes *xa13*, *Xa21*, *Gm4*, *Pi-k^h*, respectively. Naveed et al. (2010) performed similar type of molecular survey for *xa5* gene.

Parental polymorphism survey between recipient parent, JGL 1798 and the donor parents GPP 2 and NLR 145

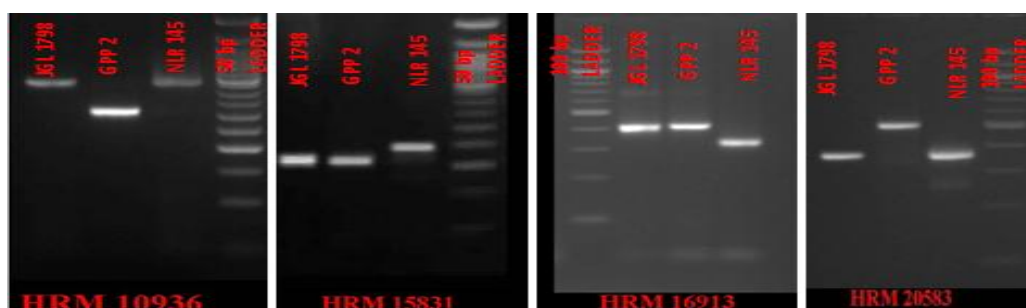
For effective use of MAS or MABB, polymorphic markers between the parents are highly useful to exercise background selection. Background selection is highly useful to identify the plants carrying desirable genome of interest. Among the 128 hyper variable primers, di nucleotide primers were maximum in number (80) followed by tri- (40) and tetra nucleotide repeats (8) (Figure 4) di nucleotide (AT) repeats were abundant on all chromosomes, followed by AAT repeats in most of the chromosomes. Among the 128 primer pairs, 82 primer pairs were recognized as monomorphic and 36 primer pairs exhibited polymorphism between recurrent parent JGL 1798 and donor parents GPP 2 and NLR 145 (Figure 3).

Twenty two primer pairs viz., HRM 10936, HRM 10167, HRM 11111, HRM 13659, HRM 14250, HRM 15855, HRM 16153, HRM 15679, HRM 17405, HRM 16652, HRM 18939, HRM 20583, HRM 20710, HRM 21881, HRM 22622, HRM 23146, HRM 23578, HRM 25310, HRM 27323, HRM 28110, HRM 28202 and HRM 28800 were detected as polymorphic in nature between recurrent parent JGL 1798 and donor parent GPP 2.

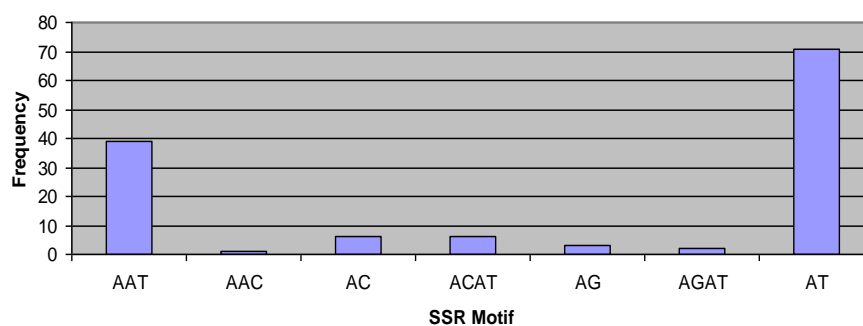
Thirty one primer pairs viz., HRM 10167, HRM 11111, HRM 13154, HRM 12469, HRM 13659, HRM14250, HRM 15626, HRM 15831, HRM 15855, HRM 16153, HRM 15337, HRM 17405, HRM 16913, HRM 16652, HRM 16649, HRM 18939, HRM 18770, HRM 19697, HRM 21539, HRM 21881, HRM 22622, HRM 23146, HRM 23578, HRM 24481, HRM 24542, HRM 24199, HRM 27323, HRM 25970, HRM 28110, HRM 28202 and HRM 28800 were observed as polymorphic markers between recurrent parent JGL 1798 and donor parent NLR 145. Five primer pairs viz., HRM 22622, HRM 23146, HRM 23578, HRM 27323 and HRM 25970 were observed

Table 1. Amplification pattern of gene linked primer pairs in donor and recipient parents.

Primer name	Linked gene	Chromosome number	Donor parent	Resistant allele size(bp)	Recipient parent	Susceptible allele size (bp)
<i>xa13promoter</i>	<i>xa13</i>	8	GPP 2	500	JGL 1798	260
<i>pTA-248</i>	<i>Xa21</i>	11		916		700
<i>RM547</i>	<i>Gm4</i>	8		270		290
<i>RM206</i>	<i>Pi-k^h</i>	11	NLR 145	140		170

**Figure 3.** Polymorphism between recipient parent JGL 1798 and donor parents GPP 2 and NLR 145 with by using HRM primers pairs.

CHROMOSOME 1 to 12

**Figure 4.** Frequencies of total 128 hyper variable microsatellite markers with different repeat motifs

on target chromosomes 8 (*xa13* & *Gm4*) and 11 (*Xa21* & *Pi-k^h*) carrying the target genes.

DISCUSSION

From application angle, DNA markers are widely used in marker-assisted breeding/selection. In MAS, markers are used at two levels, that is, foreground selection and background selection. For foreground selection, gene-specific or tightly linked markers of target traits are used. A recent review by Jena and Mackill (2008) provided the list of DNA markers that are tightly linked with some major quantitative trait loci (QTLs) or genes relating to agronomic traits. For effective use of MAB, identification of appropriate markers for background selection is equally

important. It facilitates speedy and reliable recovery of recurrent genome. One hundred and twenty eight SSR primers that are distributed all over twelve rice chromosomes were selected to assess the polymorphism between the recurrent and donor parents. These primer pairs were selected based on repeat number and location on different chromosomes. Preference was given to the primers that are with more number of repeats, tri and tetra nucleotides in addition to di nucleotides (Narasimhulu et al., 2010). Since the relative frequencies of di-, tri- and tetra nucleotide repeat motifs of the chosen markers were compared chromosome wise.

During the amplification of genomic DNA of three rice genotypes, the number of amplified fragments that are polymorphic in nature were ranged between 2 and 3, with an average polymorphic fragment per primer was 2.5, the

PIC values were ranged between 0.72 (HRM 10936) 0.88 (HRM 28800) with an average of 0.75; A high level of polymorphism was observed when compared to the low level of polymorphism in earlier studies. Similarly high PIC values were reported in barley wheat and rice (Gu et al., 2005 and Seetharam et al., 2009).

The results revealed that out of 36 polymorphic primer pairs, a maximum of seven primer pairs were observed on chromosome 3, followed by four primer pairs on chromosome 4, three each on chromosomes 1, 2, 6, 8, 9 and 12, two each on chromosomes 5, 7 and 11 and one on chromosome number 10. Among 36 primer pairs, AT repeats were maximum of 18 in number followed by 14 AAT, 2AGAT and 2 AC repeats. Similarly, Temnykh et al. (2001), Grover et al. (2007) and Narsimulu et al. (2010) observed maximum polymorphism with AT repeat primers. This richness in polymorphism is due to the presence of transposable sequences that target (AT)ⁿ repeats (Akagi et al., 1997; Grover et al., 2007).

In the present investigation, 92% of markers showed clear amplification, while 31% of markers showed polymorphism. The present study further revealed that the selected primers belongs to Class I microsatellites that are highly polymorphic in nature because of low PCR failure rate with increased probability of SSR expansion when compared to Class II microsatellites (Cho et al., 2000; Temnykh et al., 2001; Narsimulu et al., 2010).

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Full Length Research Paper

Occurrence of aflatoxin contamination in maize kernels and molecular characterization of the producing organism, *Aspergillus*

Muthusamy Karthikeyan^{1*}, Arumugam Karthikeyan¹, Rethinasamy Velazhahan², Srinivasan Madhavan² and Thangamuthu Jayaraj¹

¹Tamil Nadu Rice Research Institute, Aduthurai - 612 101, Tamil Nadu, India.

²Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India.

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Aflatoxins are toxic metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1) is a potent carcinogen, teratogen and mutagen. 660 pre- and post-harvest maize samples were collected from major maize growing areas in Tamil Nadu, India. Aflatoxin contamination was observed in 40.22% of the samples tested of which, 22.97% of pre-harvest and 53.93% post-harvest maize samples were found to be infected with AFB1 and 12.05% of the total samples exceeded WHO permissible limit of 20 µg/kg. AFB1 contamination ranged from 0 to 149.32 µg/kg. 28 *A. flavus* isolates were isolated and grouped into three sets based on aflatoxin producing potential viz., highly aflatoxin producing isolates, medium producing isolates and no aflatoxin producer or traces of aflatoxin producing isolates. The genetic coefficient matrix analysis using random amplified polymorphic DNA (RAPD) with ten random primers revealed minimum and maximum percent similarities among the tested *A. flavus* strains ranging from 35 to 89%. Cluster analysis separated the three sets of isolates into two groups (groups I and II) with each two subgroup confirming the genetic diversity among the *A. flavus* isolates from maize.

Key words: Maize, survey, *Aspergillus flavus*, aflatoxin, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Aflatoxins are a group of closely related heterocyclic compounds produced predominantly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin contaminates a vast array of food and agricultural commodities. *Aspergillus* species are capable of growing on a variety of substrates and under a variety of environmental conditions. Maize (*Zea mays* L.) is one of the most widely distributed food plants in the world and its infection by this fungus can result to mycotoxin contamination during the growing, harvesting, storage, transporting and processing stages (Bradburn et al., 1993). The economic consequences of mycotoxin con-

tamination are profound, as the crops contaminated with high levels of mycotoxin are often destroyed. The affected crops are sometimes diverted to animal feed, resulting in reduced growth rates and illness of animal consuming contaminated feeds and result in meat and milk containing toxic contaminates. Globally, high levels of aflatoxin contamination of dietary staples have been reported (Bhat et al., 1997; Setamou et al., 1997; Kpodo et al., 1996; Wild and Turner, 2002). In India, Waliyar et al. (2003) reported that 43% of maize samples were contaminated with aflatoxin with the highest AFB1 level of 806 g/kg.

Currently, there is a limited understanding of the details of the molecular variability among the isolates of *Aspergillus* spp. especially from maize. The use of non-toxicogenic strains for the biological control of toxicogenic ones has already been suggested by Dorner (2009). It is important to better understand the genetic diversity within this fungal group and the critical factors for retention or loss of characteristics such as toxicogenic capacity and virulence to plants. Variability in aflatoxin production potential of *A. flavus* isolates have been reported (Karthikeyan et al., 2009). This is why the ability to distinguish between the various *Aspergillus* species may have diagnostic value. The analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive and reliable method for determining genetic relationships among pathogenic microorganisms (Zhang et al., 2004; Khoodoo and Jaufeerally-Fakim, 2004).

The objectives of the present study was to determine the levels of aflatoxin in maize grains collected from different maize growing areas of Tamil Nadu, India, and study the genetic variability among the isolates of *A. flavus* from the maize .

MATERIALS AND METHODS

Survey and collection of samples

A survey was conducted in different agro-ecological zones comprising of 16 districts viz., Coimbatore, Dindigul, Madurai, Salem, Theni, Trichy, Namakkal, Perambalur, Erode, Thirunelveli, Thiruvannamalai, Vellore, Villupuram, Virudunagar, Sivagangai and Thoothukudi districts of Tamil Nadu, India over three years (2009 to 2011) in order to understand the magnitude of aflatoxin contamination in maize. Pre- and post-harvest maize samples were collected from farmers' fields and stores.

Detection of AFB1 by indirect competitive ELISA

Samples (2 g) were powdered in a coffee grinder and then mixed with 10 ml of solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl. The mixture was incubated on a rotary shaker for 30 min at 250 rpm at room temperature (28±2°C). The extract was filtered through Whatman No. 41 filter paper and the filtrate was used for determination of AFB1 content by indirect competitive ELISA following the method of Reddy et al. (2001). Briefly, the wells of microtiter plates were coated with 150 µl per well of AFB-BSA at a concentration of 100 µg/ml in carbonate coating buffer. The plates were washed with phosphate buffered saline (PBS) containing Tween-20 (PBST) and treated with PBST-BSA. 100 µl of sample extract or AFB1 standard was mixed with 50 µl of AFB-BSA antiserum (1:6 000) in 0.2% PBST-BSA and added into the wells. This step was followed by the addition of alkaline phosphatase labelled goat antirabbit IgG conjugate diluted to 1:4 000 in PBST-BSA. *P*-nitrophenyl phosphate prepared in 10% diethanolamine was used as substrate. The plates were incubated at room temperature and then read in an ELISA reader at 405 nm. The concentration of AFB in the samples was calculated based on the absorbance of the AFB1 standard.

Isolation of pathogen

Aspergillus sp. were isolated from the infected maize samples col-

lected from farmers fields and stored using the potato dextrose agar (PDA) medium under laboratory conditions. The *Aspergillus* cultures were identified as species using a taxonomic key and species descriptions (McClenny, 2005).

Aflatoxin (AFB1) productivity by *A. flavus* isolates

All the isolates were screened *in vitro* for their aflatoxin production potential in potato dextrose broth. The cultures were grown in 250 ml conical flasks containing 100 ml of potato dextrose broth at room temperature (28 ± 2°C) for 10 days. At the end of incubation period, culture filtrate was collected by filtering through two layers of muslin cloth. The culture filtrate was extracted with chloroform (1:1 v/v). The chloroform fraction was concentrated to 0.1 ml *in vacuo*. Further, quantification of aflatoxin contamination was carried out using ELISA method.

Molecular characterization

DNA isolation

Extraction of DNA from the aflatoxin producing *A. flavus* isolates was carried out using CTAB method (Henrion et al., 1992) with slight modification. To extract the DNA, the mycelial mat was ground to a fine powder using a pestle and mortar by the addition of liquid nitrogen. The resulting powder (200 to 300 mg) was mixed with 500 µl extraction buffer and incubated at 65°C for 30 to 60 min in water bath. Equal volume of phenol-chloroform-isoamyl alcohol was added and mixed well by inversion and centrifugation at 13000 rpm for 5 to 10 min and the supernatant was transferred into a new tube. The solution was then mixed with equal volume of isopropanol, followed by gentle mixing. The mixture was incubated for 1 h at -70 or -20°C for 2 h. DNA was then pelleted by centrifugation at 13000 rpm for 10 min, washed with 70% ethanol, pelleted again and dried at room temperature. Finally, the DNA pellet was rehydrated in 30 to 50 µl of 10 mM TE buffer and stored at -20°C until use. RAPD work was performed with Opron Primers viz., OPR2 (5'CACAGCTGCC3'), OPR8 (5'CCCGTTGCCT3'), OPR13 (5'GGACGACAAG3'), OPP12 (5'AAGGGCGAGT3'), OPP17 (5'TGACCCGCCT3'), OPP19 (5'GGGAAGGACA3'), OPC 02 (5'GTGAGGCGTC3'), OPC 08 (5'TGGACCCGTTG3'), OPC 11 (5'AAAGCTGCGG3'), OPC 15 (5'GACGGATCAG3') and the resulted gels were scored via computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as '1' and if absent, it was designated as '0'.

The RAPD data were analysed using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) computer package18. The data were used to generate Jaccard's similarity coefficients for RAPD bands. The Jaccard's coefficients between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA). The amplifications were carried out twice to check for reproducibility.

RESULTS AND DISCUSSION

Survey and collection of *Aspergillus* isolates

The results shown in Tables 1 and 2 indicate that aflatoxin contamination in maize samples was observed in 40.22% of the samples tested. AFB1 was detected in 22.97% of pre-harvest and 53.93% post-harvest maize

Table 1. Level of aflatoxin contamination ($\mu\text{g}/\text{kg}$) in maize samples in different districts of Tamil Nadu.

S/N	District	Pre-harvest		Post-harvest	
		Total sample	Aflatoxin infected	Total sample	Aflatoxin infected
1	Coimbatore	35	10	40	24
2	Dindigul	27	9	35	18
3	Madurai	25	7	37	26
4	Erode	18	4	26	12
5	Salem	20	6	28	16
6	Namakkal	19	7	24	17
7	Karur	22	6	24	13
8	Virudhunagar	29	7	19	11
9	Sivagangai	18	2	27	14
10	Thirunelveli	25	2	25	10
11	Thiruvannamalai	22	2	23	12
12	Vellore	12	2	23	10
13	Villupuram	11	1	25	9
	Total	283	65	356	192

Table 2. Aflatoxin B1 content in maize samples assessed by indirect competitive ELISA.

Sample	Number of sample analyzed	Number of samples with aflatoxin B1 ($\mu\text{g}/\text{kg}$)		
		0	1-20	>20
Pre-harvest maize kernels	283	218	43	22
Post-harvest maize kernels	356	164	137	55
Sub total	639	382	180	77

samples and 12.05% of the total samples exceeded 20 $\mu\text{g}/\text{kg}$. In total, AFB1 was detected in 257 out of 639 samples with amounts ranging from 0.4 to 149.32 $\mu\text{g}/\text{kg}$. It was evident from this study that post-harvest stage is the favorable stage for infection by *Aspergillus* spp. and aflatoxin production.

The occurrence of high levels of AFB1 in food and feed stuffs has been reported by several workers (Reddy et al., 1984; Singh et al., 1984; Balasubramanian, 1985; Selvasubramanian et al., 1987; Dhavan and Chaudary, 1995; Dutta and Das, 2001). Dutta and Das (2001) reported that AFB1 content in feed samples collected from different parts of Northern India were very high with an average of 0.412 to 0.514 ppm. It is well known that growth of *Aspergillus* spp. and subsequent production of aflatoxins in maize is dependent on a number of factors such as temperature, humidity, insect injury, handling during harvest and storage (Hell et al., 2003). A significant positive correlation between moisture content of maize seeds and *A. flavus* population and aflatoxin production has been reported (Oyebanji and Efiuvwevwere, 1999). Karthikeyan et al. (2009) reported wide variability in aflatoxin production potential of different isolates of *A. flavus* from maize. Vijayasamundeeswari et al. (2009) reported that AFB1

was detected in 61.3% of the maize kernel samples and the levels of AFB1 in 26% of the pre- and post-harvest maize kernels exceeded 20 $\mu\text{g}/\text{kg}$. Hence, the variations in aflatoxin content among different samples may have been as a result of unsatisfactory storage conditions, collection of samples from different regions, high moisture content, stages of samples, seasons of collection and the occurrence of aflatoxigenic fungi.

Aflatoxin (AFB1) productivity by *A. flavus* isolates

All *Aspergillus* fungi isolated from infected maize seed were found to be *A. flavus*. When they were tested for their ability to produce aflatoxin, all the isolates varied in their level of toxin production. The amount of AFB1 produced by the toxigenic isolates of *A. flavus* ranged from 0 to 58.53 ng/ml. Among the various isolates of *A. flavus*, the isolate AFM2 produced the highest amount of (58.53 ng/ml) AFB1 (Table 3).

Based on these results, the isolates were grouped into three categories viz., no aflatoxin producer or traces of aflatoxin producer (0 to trace ng/ml), medium aflatoxin producer (1 to 10 ng/ml) and high aflatoxin producer (>10 ng/ml).

Table 3. Aflatoxin (AFB1) productivity by *A. flavus* isolates.

<i>A. flavus</i> isolate	Locality	Aflatoxin B1 (ng/ml)
AFM1	Coimbatore	0
AFM2	Coimbatore	58.53
AFM3	Coimbatore	9.02
AFM4	Erode	0
AFM5	Erode	0
AFM6	Erode	5.5
AFM7	Salem	79.5
AFM8	Salem	0
AFM9	Salem	08.48
AFM10	Dindigul	3.65
AFM11	Dindigul	68.48
AFM12	Dindigul	5.45
AFM13	Madurai	0
AFM14	Madurai	7.23
AFM15	Madurai	7.90
AFM16	Namakkal	9.58
AFM17	Namakkal	77.95
AFM18	Namakkal	0
AFM19	Karur	9.95
AFM20	Karur	0
AFM21	Karur	0
AFM22	Virudhunagar	52.15
AFM23	Sivagangai	0
AFM24	Thirunelveli	44.75
AFM25	Thiruvannamalai	53.46
AFM27	Vellore	59.84
AFM28	Villupuram	0

Molecular characterization

Molecular characterization was performed on AFM2, AFM7, AFM11, AFM17 (high aflatoxin producing isolates), AFM3, AFM6, AFM10, AFG12 (medium aflatoxin producing isolates) and AFM1, AFM4, AFM5, AFG8 (no aflatoxin or traces of aflatoxin producing isolates). A total of 71 bands were amplified with the 10 primers and 12 isolate. The pairwise Jaccard's coefficients for the genetic similarities among the 12 isolates are presented in Table 4. The cluster analysis of the distribution of 71 RAPD bands is shown as a dendrogram (Figure 1). All 12 accessions were distinguished from each other. The distribution of the bands isolates of three different aflatoxin producing groups merged together.

Other workers (Bayman and Cotty, 1993; Croft and Varga, 1994; Jovita and Bainbridge, 1996; Tran-Dinh et al., 1999; Lourenco et al., 2007) have reported no correlation between DNA band profiles and toxin production using RAPD. Egel et al. (1994) grouped strains with similar toxigenic capacities, in a more subtle differentiation than the simple classification of toxin producers and non producers. Other researchers working

with different mycotoxin producing fungi have reported that no correlation could be observed between clustering of the isolates of these fungi based on RAPD and their mycotoxin-producing abilities or aggressiveness (Toth et al., 2004). The total sizes of the restriction fragments in each enzyme digest in the present study exceeded the apparent size of the ITS-PCR product. This difference in size of the fragments could be attributed to the presence of multiple forms of the rDNA-ITS gene cluster in single isolates. This phenomenon is common in fungi and has been reported in *Sclerotium rolfsii* (Harlton et al., 1995), *Ascochyta* sp. (Fatehi and Bridge, 1998) and *Phytophthora* sp. (Appiah et al., 2004).

Aflatoxin affects the quality of the commodities and there by hitting the export trade in the international market. The present study reveals that the level of AFB1 contamination is more than 26% of the pre- and post-harvest maize kernel samples and exceeded the permissible level of 20 µg/kg, the tolerance level fixed by the World Health Organization (WHO, 1991) and US National Grain and Feed Association (US NGFA, 2009). The presence of aflatoxin in maize kernels and feeds presents a risk for human and animal health. Therefore, proper

Table 4. Similarity matrix for Jaccard's coefficients for 12 *A. flavus* isolates.

	AFM1	AFM 4	AFM5	AFM8	AFM3	AFM6	AFM10	AFM12	AFM 2	AFM7	AFM11	AFM17
AFM 1	1.00											
AFM 4	0.83	1.00										
AFM 5	0.68	0.76	1.00									
AFM 8	0.62	0.70	0.67	1.00								
AFM 3	0.57	0.70	0.61	0.72	1.00							
AFM 6	0.59	0.67	0.55	0.57	0.72	1.00						
AFM 10	0.48	0.60	0.52	0.48	0.53	0.56	1.00					
AFM 12	0.35	0.50	0.39	0.46	0.51	0.42	0.62	1.00				
AFM 2	0.38	0.50	0.46	0.52	0.50	0.41	0.54	0.73	1.00			
AFM 7	0.47	0.61	0.48	0.51	0.49	0.41	0.58	0.63	0.58	1.00		
AFM 11	0.58	0.68	0.57	0.47	0.47	0.58	0.62	0.54	0.47	0.71	1.00	
AFM 17	0.52	0.62	0.49	0.43	0.43	0.52	0.62	0.55	0.48	0.65	0.89	1.00

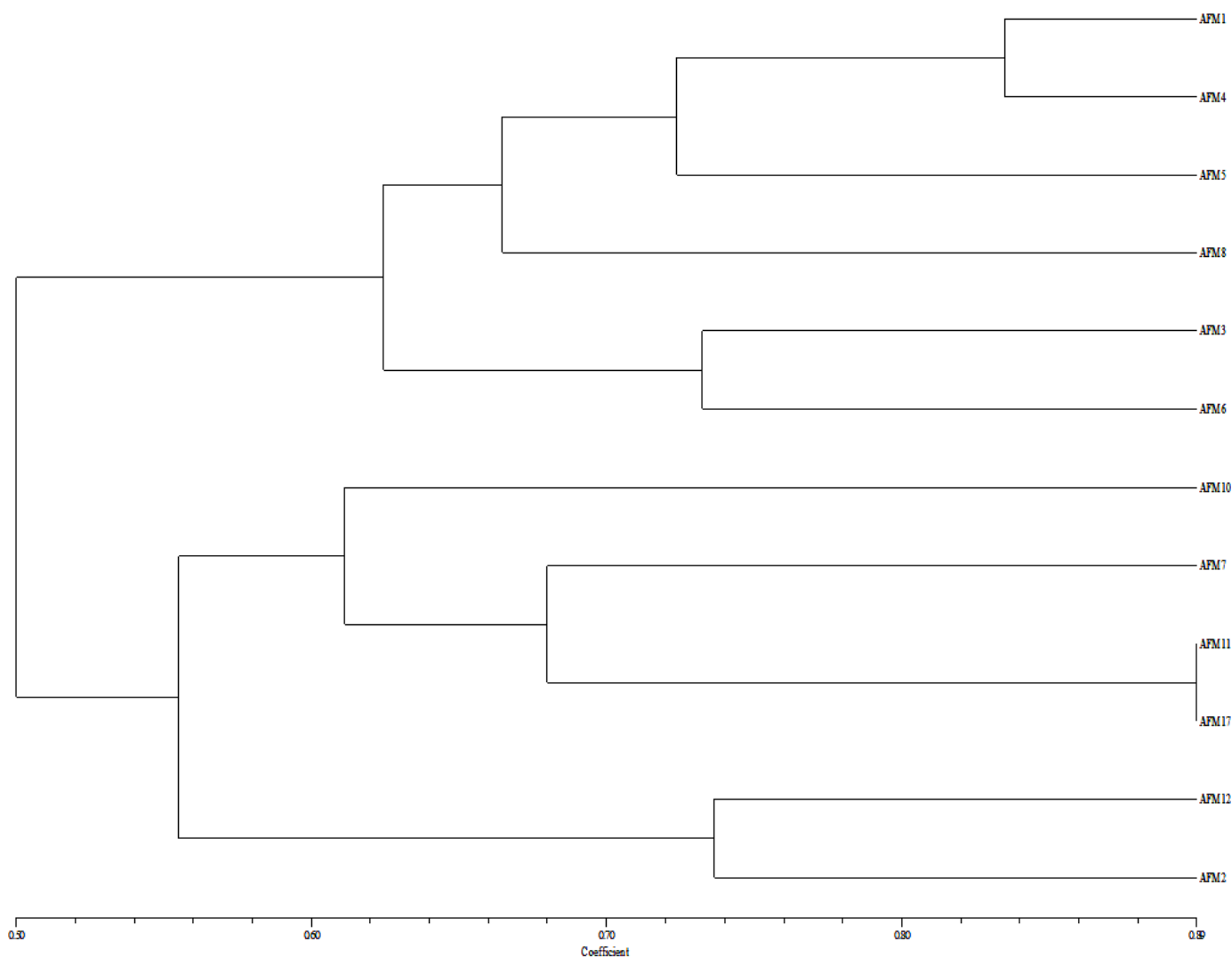


Figure 1. Dendrogram of *A. flavus* isolates based on 71 PCR bands amplified by 10 RAPD primers. The bar on the bottom represents similarity index based on Jaccard's coefficients.

post-harvest handling of maize and proper storage of feeds can greatly help in reducing infection by *Aspergillus* spp. and subsequent contamination with aflatoxins and the variability among *A. flavus* isolates in aflatoxin production potential, and genetic makeup may present a problem for the control of *A. flavus* infection.

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Full Length Research Paper

Estimation of genetic diversity of the Kenyan yam (*Dioscorea* spp.) using microsatellite markers

Z. K. Muthamia^{1*}, F. E. Morag, A. B. Nyende², E. G. Mamati² and B. W. Wanjala¹

Kenya Agricultural Research Institute (KARI), Jomo Kenyatta University of Agriculture and Technology (JKUAT), International Institute of Tropical Agriculture (IITA), Nairobi, Kenya.

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Yam landraces in Kenya have not been fully characterized both at morphological and molecular level. Application of molecular markers can overcome this bottleneck. 187 accessions comprising of 166 yam landraces and 21 Yam DNA samples from IITA, Nigeria were extracted from leaf samples grown at Muguga and genotyped at BeCA. DNA was extracted using CTAB method. Twelve (12) primer pairs were used for genotyping and PCR products detected on ABI-3730 capillary system. Data was analyzed for genetic diversity, ordination and analysis of molecular variance with GenAlEx software. A total of 131 alleles were amplified with a minimum of two alleles and a maximum of 13 alleles per primer with a minimum allele size of 64 bp and a maximum of 368 bp. Accessions from Eastern province had the highest number of unique alleles. Shannon's information index (I) was 0.1444 for West African samples and 0.2366 for Central province. Accession dispersion revealed four clusters with no distinct geographical pattern. Dense clustering of accessions was an indication of genetic relatedness. Analysis of molecular variance revealed that most variation of 88% ($P < 0.010$) was found within populations or provinces. The simple sequence repeats (SSR) markers were polymorphic and were able to discriminate local yam landraces.

Key words: Genetic diversity, microsatellite, yam, Kenya.

INTRODUCTION

Dioscorea spp., belong to the family Dioscoreaceae and the genus *Dioscorea*. The genus had been reported to have 600 species but has recently been estimated as comprising less than 200 species (Ayensu and Coursey, 1972). Classifications by Hutchinson (1959), Burkill et al. (1960), and Ayensu and Coursey (1972) place the family under the order Dioscoreales.

The taxonomy of yams is complex and further groupings could emerge based on recent molecular biology techniques. For example, controversy exists in the relationships among various species (Terauchi et al., 1992). Kenya's yam diversity is represented by a number of species including *Dioscorea minutiflora* Engl, *Dioscorea bulbifera* L., and *Dioscorea dumetorum* (Kunth) Pax. that

are grown for food by mainly elderly farmers in the Eastern, Central, Western and Coastal regions of the country (Maundu et al., 1999).

There is need for a concise phylogeny investigation to fully understand the local landraces in Kenya vis vis those in the region (Wilkin, P, personal communication). Mwirigi et al. (2009), used morphological markers on 43 Kenyan local landraces and identified four major clusters. Morphological and agronomic features cannot be relied upon and is the major cause of inconsistency in identification. An understanding of the diversity of the local yam germplasm provides a good baseline for further improvement of the crop.

Molecular markers are useful for cultivar identification,

*Corresponding author. E-mail: zkmathamia@yahoo.com. Tel: +254722352305.

because they are not influenced by variable environmental conditions or plant phenology, and are a basis for discriminating among cultivars with similar morphological characteristics (Beebe et al., 2000). Genetic markers are also valuable for determining the phylogenetic relationships among accessions and for true-to-type plant identification (Aggarwal et al., 2008). Examples of molecular markers that have been utilized in phylogenetic analysis include: restriction fragment length polymorphism (RFLPs), and PCR-based markers such as random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLP), microsatellites/simple sequence repeats (SSRs) and sequence characterized amplified regions (SCARs).

Good DNA markers should be: highly polymorphic, co dominant, abundant in the genome, evenly distributed throughout the genome, easy and fast to assay, high reproducibility and easy to exchange data between laboratories (Weising et al., 1995). No DNA marker technology currently fulfills all of these criteria. However, amplified fragment length polymorphism (AFLP) and microsatellites or simple sequence repeats (fulfill most of these requirements. Use of molecular markers not only help in genetic diversity and phylogenetic analysis but also enhance genetic gain in selection in a plant breeding program (Spooner, 2005).

Molecular markers such as RFLP, RAPD, AFLP and SSR have been applied in yams for taxonomic, phylogenetic, diversity and mapping studies (Terauchi et al., 1992; Terauchi and Kanoma, 1994; Asemota et al., 1996; Mignouna et al., 1998, 2002a, 2003; Egesi et al., 2006; Tostain et al., 2007).

A study of 45 yam cultivars from eight West African countries using nine SSR loci showed that all the cultivars could be distinguished with three SSR loci (Mignouna et al., 2003). These authors found that the SSR-based classification differed from that based on morphotypes and no geographical structure was observed. In this study, the objective was to describe the diversity of the yam landraces in Kenya using SSRs.

MATERIALS AND METHODS

DNA extraction

Young leaf tissues of 5 g were harvested from 166 yam landraces and yam wild relatives planted at Muguga field genebank. In addition, 21 DNA samples from IITA, Nigeria were sourced making a total of 187 accessions. These were transported in cool boxes with dry ice to BecA for molecular analysis. The accessions from IITA represented *D. alata* L., *D. rotundata* Poir., *D. cayenensis* L., *D. esculenta* (Lour.), *D. bulbifera* and *D. dumetorum*. DNA was extracted following the CTAB method (Doyle and Doyle, 1987) with some modifications (Wanjala et al., 2013).

Optimization of PCR conditions

A total of 12 primer pairs were used (Table 1). PCR amplifications were performed to a final volume of 10 µL consisting of 1X buffer,

2 mM MgCl₂, 0.2 mM dNTPs, 0.08 pmoles /µl each of the forward and reverse primers, 0.375U Taq Polymerase, 4.525 µl of dd H₂O and 25 ng of DNA. GeneAmp PCR system 9700 thermal cycler was used with the following programme: denaturation at 94°C for 3 min, followed by 35 cycles of primer extension stage of 94°C for 30 s, 56°C for 1 min (annealing temperature depending on marker) and 72°C for 2 min, with final extension step of 72°C for 10 min with a final hold at 4°C. PCR optimization was done for all markers and the optimum conditions identified.

Fragment analysis

The PCR products were detected on ABI-3730 capillary system using the LIZ-500 as internal size standard. Data capture was done using the genscan[®] software (Applied Biosystems) and the resulting fragments analysed the alleles scored using the Genemapper[®] software ver4.1. Size matching/ calling was based on Local Southern Method algorithm with reference to a defined standard range, GS75-500 (-250) Liz base pairs. Alleles were sorted according to size and tolerance level of 0.4 base pairs as minimum distance between adjacent bins in base pairs. This process was repeated for each marker until all alleles were binned, from the smallest allele size to the largest sized alleles. A new bin was created each time the threshold tolerance between two sequentially sized alleles was attained. The mean and ranges for all bins were calculated and bin labels rounded up to the nearest whole number was assigned to each group. This data was then fed to the software for bin adjustment. Scored results were exported to an Excel matrix.

SSR data analysis

Alleles were scored and converted to Binary data by use of ALS Binary software. Data was analyzed on 187 accessions for genetic diversity, ordination analysis and Analysis of Molecular Variance (AMOVA) with GenAlEx software (Peakall and Smouse, 2009). In addition, cluster analysis of the cultivars was performed using population genetic analysis software PopGen32 (Yeh et al., 1997). Diversity among and within populations was determined. Genetic distance, matrix dendrogram, F statistics, gene diversity over loci, proportion of polymorphic loci, Shannon index and gene frequency were analyzed (Nei, 1987). To show the relationship between populations and individuals, Principal Co-ordinate Analysis (PCoA) was generated.

RESULTS

Allele frequency

A total of 131 alleles were amplified by 12 SSR primer pairs analyzed in 187 accessions with a minimum of two alleles (Da1F08) and a maximum of 13 alleles (Da1A01 and Dab2D08) and minimum allele size of 64 bp and a maximum of 368 bp. Eastern population had the highest number of private bands (bands unique to a single population) while Coast population had none (Figure 1).

Population genetic diversity

Percentage of polymorphic loci of genotypes from Eastern province was 76.3%, while those from West

Table 1. Yam microsatellite markers and allele sizes detected in 187 genotypes.

Marker name	Min size detected (bp)	Max size detected (bp)	Number of alleles detected	Allele sizes detected	Forward primer sequence (5'-3')	Dye	Reverse primer sequence (5'-3')
YM13	113	254	9	113,184,188,193,209,215,232,235,254	TATAATCGGCCAGAGG	VIC	TGTTGGAAGCATAGAGAA
Dpr3F04	64	148	10	64, 66,71, 127,130,132,135,138,145,148	CCCATGCTTGTAGTTGT	PET	TGCTCACCTCTTTACTTG
Da1A01	141	221	13	141,147,152,155,157,160,164,170,172,180,188,202,221	TGTAAGATGCCACATT	VIC	TCTCAGGCTTCAGGG
Dab2D08	124	368	13	124,146,162,224,236,259,268,279,287,296,338,349,368	ACAAGAGAACCGACATAGT	6-FAM	GATTTGCTTTGAGTCCTT
YM15	180	298	12	180,201,205,208,212,219,221,230,232,234,236,298	TTGAACCTTGACTTTGGT	PET	GAGTTCCTGTCCTTGGT
Dab2DO6	123	180	6	123,129,132,148,162,180	AACATATAAAGAGAGATCA	PET	ATAACCCTTAACTCCA
Dpr3B12	127	170	8	127,133,135,138,142,147,159,170	CATCAATCTTTCTCTGCTT	NED	CCATCACACAATCCATC
Dpr3F12	130	190	11	130,135,141,148,151,153,156,161,165,172,190	AGACTCTTGCTCATGT	PET	GCCTTGTTACTTTATTC
Dab2CO5	103	221	11	103,146,151,163,173,179,187,194,201,217,221	TCCCCATAGAAACAAAGT	NED	TCAAGCAAGAGAAGGTG
Dab2EO7	104	183	12	104,108,111,128,137,139,142,144,151,162,168,173,183	TTCCCTAATTGTTCTCTTGTG	PET	GTCCTCGTTTTCCCTCTGTGT
Dab2EO9	97	144	9	97, 105,110,115,119,121,124,127,144	TACGGCCTCACTCCAACACTA	VIC	AAAATGGCCACGTCTAATCCTA
Da1FO8	152	198	2	152,198	AATGCTTCGTAATCCAAC	PET	CTATAAGGAATTGGTGCC

Africa had 32.8% (Table 2). The genetic diversity coefficients based on Nei's genetic diversity (H) was lowest for West African populations at 0.0872 while those from Central province had 0.1551. Shannon's information index was 0.1444 for West African samples and 0.2366 for Central province.

To construct the genetic relationships between the six regions, the values of genetic distance were subjected to hierarchical clustering by

unweighted paired-grouping method with arithmetic averages (UPGMA). The cluster analysis separated the sub-populations into two distinct groups A and B which did not reflect their geographic origins (Figure 2). This was corroborated by dendrogram (figure not shown) that gave four clusters.

The accessions dispersion (Table 2) revealed four groups. Clade A was made up of accessions

from all the six regions, with most accessions from Meru in Eastern region and the Central regions clustering together. Five accessions from West Africa representing *D. cayenensis*, *D. esculenta*, *D. rotundata*, *D. alata* and *D. bulbifera* formed part of this clade. Clade B was dominated by accessions from Embu in Eastern province. Clade C represented accessions from the six regions with dense clustering in the upper part of the

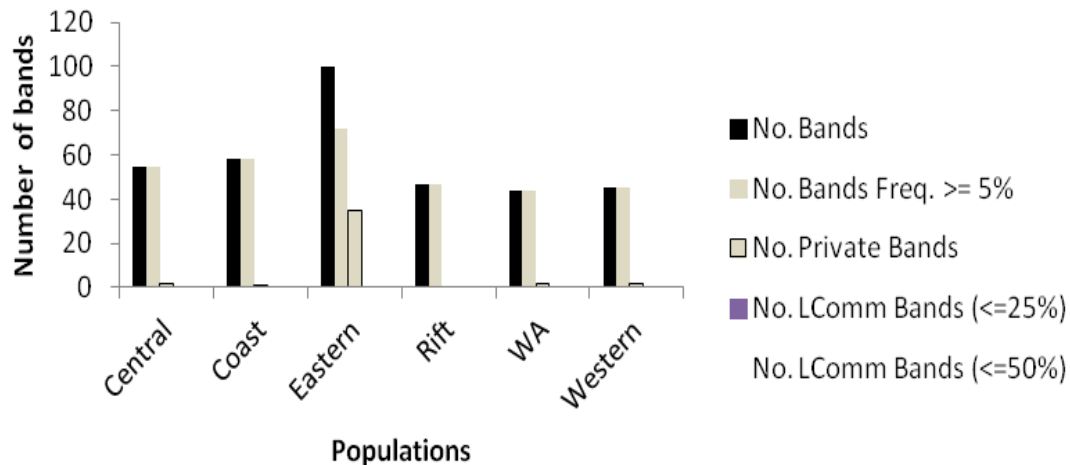


Figure 1. Allele frequency in six regions. No. Bands = number of different bands; No. Bands Freq. $\geq 5\%$ = number of different bands with a Frequency $\geq 5\%$; No. Private Bands = number of bands unique to a single population; No. LComm Bands ($\leq 25\%$) = number of Locally Common Bands (Freq. $\geq 5\%$) Found in 25% or Fewer Populations; No. LComm Bands ($\leq 50\%$) = number of locally common bands (Freq. $\geq 5\%$) Found in 50% or fewer populations.

Table 2. Genetic diversity indices.

Population	H	I	Number of polymorphic loci	Percent polymorphic loci
Central	0.1551	0.2366	54	41.2
Coast	0.1112	0.1870	58	44.2
Eastern	0.1232	0.2086	100	76.3
Rift Valley	0.1407	0.2142	47	35.8
West Africa	0.0872	0.1444	43	32.8
Western	0.1174	0.1824	44	33.5

Mean diversity estimates (H) and Shannon's information index (I).

clade.

Three West African accessions clustered in this clade. Eight accessions from West Africa were represented in clade D together with local landraces. Accessions from West Africa were found in all the four clades.

In clade A, Ac 3079 (*D. bulbifera* from West Africa) clustered closely with Ac 10 and Ac 23 which are both local accessions of species *D. bulbifera*. The clustering of Ac 164 (*D. odoratissima*) with West African *D. rotundata* and *D. cayenensis* suggests close relationship between the three species. Accessions from West Africa were distinct and did not form strong clusters in the four clades as compared to most of the local landraces. Due to the large number of genotypes used, individual groupings within the clades were not properly visualized. There was no clear geographical separation of accessions.

Analysis of molecular variance

Analysis of molecular variance (AMOVA) based on six

populations indicated that most variation was found within populations (88% $P < 0.010$) (Table 3). On the other hand, variation among populations was 12% ($P < 0.001$) among all the major populations studied.

DISCUSSION

A total of 131 alleles were detected using 12 SSR markers. Kolesnikova (2007) reported 281 alleles in 391 accessions of six economically important yam species using 23 SSR markers. Otoo et al. (2009) detected 27 loci using 13 SSR markers in a study of pona complex yam in Ghana. The higher number of alleles detected with some of the markers means they can be used more universally than the ones with low allele detection. The range of allele sizes detected is similar to that obtained by Otoo et al. (2009) and Kolesnikova (2007). From the current study, alleles were detected outside the range suggested by Otoo et al. (2009) and Kolesnikova (2007). This is new information reported in this finding.

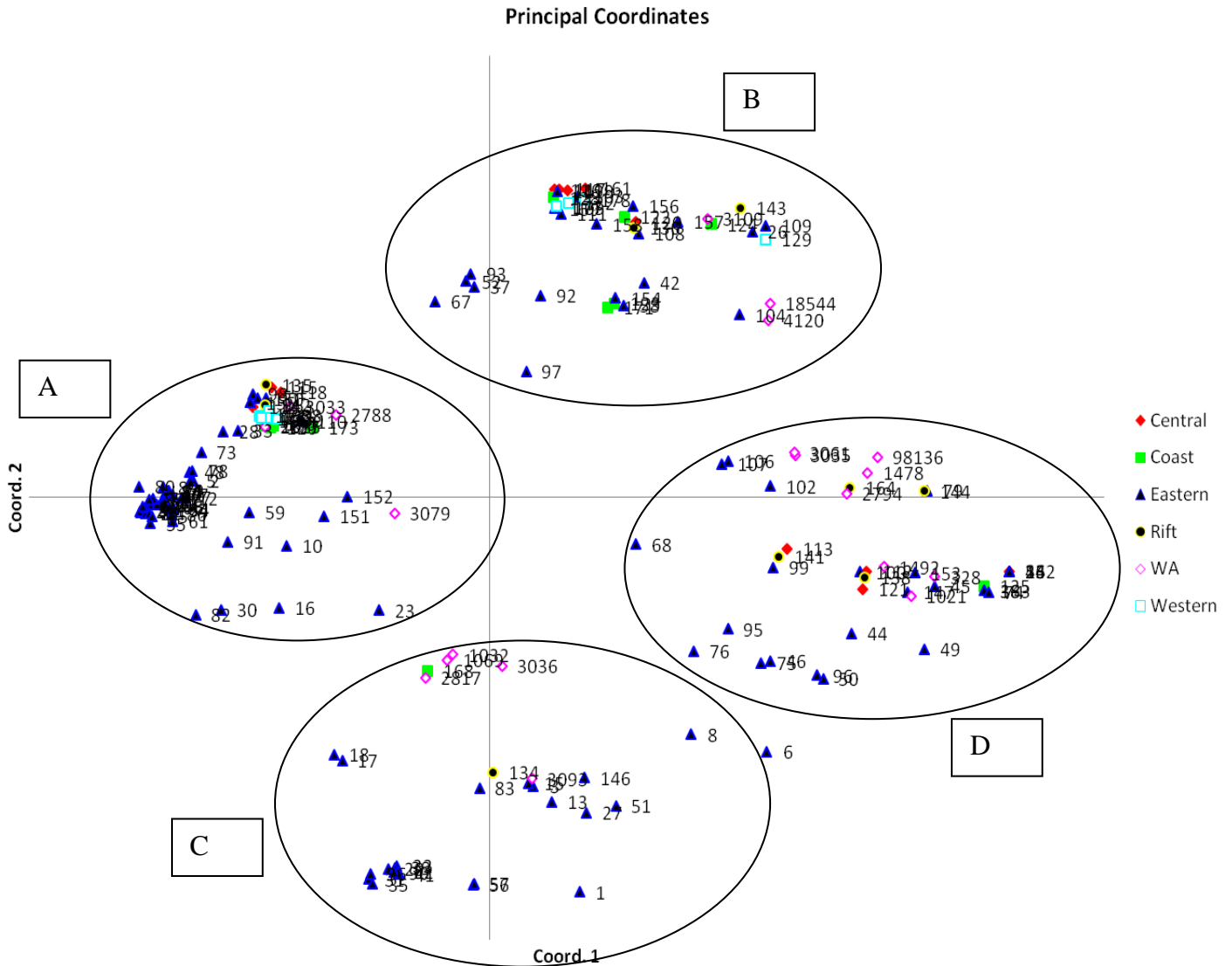


Figure 2. PcoA 1 and 2 showing 187 yam accessions dispersion in six regions.

Table 3. Analysis of Molecular Variance.

Source	Df	SS	MS	Estimated variance	Percent
Among Pops(province)	5	593.487	118.697	4.290	12
Within Pops(province)	181	5586.347	30.864	30.864	88
Total	186	6179.834		35.154	100

Stat	Value	P(rand >= data)
PhiPT	0.122	0.001

Df = Degrees of freedom; SS = Sum of squares; MS = Mean square; PhiPT = P(rand >= data) = Probability value.

Phylogenetic studies on the Kenyan Yam sp would shed more light on this.

The SSR markers used in the study were polymorphic

and were able to discriminate the yam landraces in Kenya. Tostain et al. (2006) found SSR markers to be discriminatory enough in diversity studies in yam. The percen-

tage of polymorphic loci ranged from 32.8% for West Africa to 76.3% for Eastern population. PIC is a measure of marker informativeness related to expected heterozygosity and is calculated from allele frequencies (Bolstein et al., 1980; Hearne et al., 1992). The lower percent of polymorphic loci for West Africa, western Kenya and Rift Valley have to do with the small number of samples obtained from these regions. The genetic diversity coefficient for West Africa population was 0.0872 and that for the Central population 0.1551 while the Shannon's information index, (I) for West Africa 0.1444 and for the Central population 0.2366. Eastern region had the highest percent of unique bands suggesting that some of these landraces may have been domesticated from wild species. Tostain et al. (2007) observed some unique alleles in some local landraces of *D. rotundata* suggesting a possible domestication from *D. burkilliana* or *D. mangenotiana*.

The principal coordinate analysis revealed wide scattering of accessions from the six populations. Accessions from West Africa did not cluster together but scattered among the local landraces in the four clades. The clustering pattern suggests a genetic relationship among the accessions collected from the same regions. Mignouna et al. (2003) reported no geographical structure in a collection of cultivars from eight West African countries but Tostain et al. (2007) reported genetic diversity organized according to cropping regions. The clustering of the local *D. alata* and *D. bulbifera* with similar known checks from West Africa means that the two are correctly identified.

The study indicates that among the local yam landraces there are those closely related to the West Africa yam species and yet there are others that are distantly related. The close clustering of most accessions collected from the same locality suggests possible duplication. Muluneh (2006), using AFLPs showed that Ethiopian accessions were closer to *D. cayenensis* and *D. rotundata* than all the other species studied suggesting involvement of wild species in the process of domestication of yams in Ethiopia. Their finding also showed some clustering together of local landraces distinct from the West African materials. The relationship between *D. cayenensis* and *D. rotundata* has been a subject of debate for close to half a century. On the basis of phylogenetic studies using RFLP analysis in chloroplast and nuclear ribosomal DNA, it was suggested that *D. cayenensis* is a variety of *D. rotundata* (Terauchi et al., 1992). More recent studies based on isozyme (Dansi et al., 2000a) and molecular markers (Ramser et al., 1997; Mignouna et al., 1998, 2005) support the separate identity of the two species.

Though there was no general geographical pattern of accessions dispersion there was evidence of genetic relatedness of many landraces collected from Eastern region. Other workers have not reported geographical relationships with diversity (Muluneh, 2006; Mignouna et

al., 2005). The high diversity within the regions (88%, $P < 0.010$), suggest little or no germplasm exchange between regions. There was low variation among the populations studied (12%, $P < 0.001$). This finding is supported by the fact that yams are exclusively transplanted using corms which are usually bulky and cumbersome to transport thus limiting transfer of propagation material from one region to another.

Conclusion and recommendation

The SSR markers used were polymorphic and were able to discriminate the landraces. More taxonomic work needs to be done to clearly place the local landraces and especially *D. minutiflora* which seems to have many related cultivars. The SSR markers did not group together all the West African species as expected but there was dispersion among the accessions. Landraces from the eastern part of the country presented the widest diversity and highest number of private alleles suggesting a possible centre of dispersal and domestication of yams in Kenya. The study will help eliminate duplicates in the genebank collections thereby cutting maintenance costs.

It is recommended that more phylogeny studies be conducted to elucidate the taxonomy of *D. minutiflora* and its related cultivars.

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Full Length Research Paper

Isolation and characterization of altered root growth behavior and salinity tolerant mutants in rice

K. Ashokkumar^{1*}, M. Raveendran¹, N. Senthil¹, D. Vijayalaxmi¹, M. Sowmya¹, R. P. Sharma²
and S. Robin³

¹Department of Plant Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore - 641 003, TN, India.

²National Research Centre for Plant Biotechnology, IARI, New Delhi-12, India.

³Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-3, India.

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Generation, screening and isolating mutants for any developmental and adaptive traits plays a major role in plant functional genomics research. Identification and exploitation of mutants possessing contrasting root growth behavior and salinity tolerance in rice will help us to identify key genes controlling these traits and in turn will be useful for manipulating abiotic stress tolerance through tilling and genetic engineering in rice. In this study, we have screened about 1500 mutants (M_2 generation) generated by treating an upland drought tolerant genotype Nagina 22 with Ethyl Methane Sulfonate (EMS), for their root growth behavior and salinity tolerance under hydroponic conditions. Six independent mutant lines possessing significantly shorter roots and three mutant lines exhibiting greater degree of salinity tolerance than the wild type plants were identified. The identified mutant lines were advanced to M_5 generation to allow the mutants to reach homozygosity, and the fixed mutants were confirmed for their phenotype. One mutant namely N22-C-241-5-6 was found to possess significantly shorter roots than wild type N22, and it was also noticed that the mutant was devoid of root cap. Among the three salinity tolerant mutant lines identified, N22-C-334-3 was found to possess a greater degree of tolerance upto 250 mM NaCl stress at germination stage. These identified mutant lines can be used for further physiological, biochemical and molecular biology experiments to identify candidate gene(s) controlling root growth behavior and salinity tolerance in rice.

Key words: Rice, mutation, EMS, altered root growth and salinity tolerant mutant.

INTRODUCTION

Abiotic stresses such as drought and salinity are major yield limiting factors for upland and rainfed lowland rice ecosystems. Improving rice yield with no additional lands available for cultivation depends mainly on the development of drought and salinity resistant rice genotypes suitable for these marginal environments. Efforts through conventional breeding approaches are resulting in slow

progress in developing drought and salinity resistant rice genotypes mainly due to the complexity of mechanisms controlling these traits.

Developing rice genotypes with the right combination of characters for specific drought and salinity prone environments therefore require an understanding of the physiological processes of the plant, environment and interaction

*Corresponding author. E-mail: biotech.ashok@gmail.com.

between them (O'Toole and Chang, 1979). In this context, biotechnology offers us a powerful means of manipulating drought and salinity tolerance in rice through various approaches. Among various biotechnological means, functional genomics helps us to generate knowledge on networks of stress perception, signal transduction and defensive responses.

Nguyen et al. (1997) suggested that drought resistance in rice depends mostly on one or more of the following components: ability of the roots to exploit deep soil moisture to meet evapotranspirational demand; moderated water use through reduced leaf area and shorter growth duration; capacity for osmotic adjustment and the control over non-stomatal water loss from leaves. Among the above traits, root system architecture that is, root length, volume and thickness determines the efficiency of water and nutrient absorption ability of a plant. Although rice is generally adapted to well water or irrigated environments, there is genetic variation for root traits. Some of the traditional upland cultivars are having deep roots whereas most of the cultivated semi-dwarf varieties are of shallow rooted types.

By exploiting this genetic variation, few studies have been carried out to identify quantitative trait loci (QTLs) controlling root traits, which need confirmation across the genetic background before putting them into use. Difficulty in precise phenotyping and tissue sampling of rice roots prevents the progress in generating knowledge on the gene(s) controlling root traits. Identification and characterization of suitable single gene root mutants possessing contrasting root traits will serve as near isogenic lines for functional genomics studies. In rice, only very few attempts have been made to isolate and characterize root system mutants.

Rice is moderately sensitive to salinity (Akbar et al., 1972) and the most practical approach to overcome the problem of salinity is development of tolerant varieties. The basic requirements are genetic variability, screening techniques and understanding of genetics and physiological mechanism of tolerance. The progress in developing salinity tolerant rice genotypes through conventional approaches is limited due to non-availability of rich genetic diversity in rice germplasm for salinity tolerance. Some traditional cultivars and landraces of rice are more tolerant than many elite cultivars to salinity stress. However, they generally have poor agronomic traits, such as tall plant stature, photosensitivity, poor grain quality, and low yield which limit their exploitation in conventional breeding programs. In addition, polygenic nature of tolerance mechanisms slows down the rapid generation of knowledge on its genetic mapping and molecular basis (Bohnert and Jensen, 1996). Genetic analysis of salt sensitive rice mutant increase the Na^+ in rice salt sensitive2 (*rss2*) controlled single recessive gene (Zhou et al., 2013). Therefore, isolation of single gene-mutants exhibiting contrasting degree of salinity tolerance will be an ideal material for functional genomics studies

which will help us to manipulate salinity tolerance in rice through genetic engineering.

By keeping the above in mind, the present study was formulated with an aim of identifying single-gene mutants in rice possessing contrasting root morphology and salinity tolerance. Ethyl methane sulfonate (EMS) induced Nagina 22 mutants were screened under hydroponic conditions and putative independent mutants possessing significantly shorter roots and gain-of-function mutants for salinity tolerance were identified.

MATERIALS AND METHODS

Screening for mutants was performed using M_2 seedlings derived from single M_1 plants rice (*Oryza sativa* L. cv. Nagina 22; indica type) derived by treating the seeds with 0.8% EMS.

Screening for altered root growth behavior mutants

About 1500 M_2 lines were screened for identifying putative mutants possessing short roots and enhanced level of tolerance against salinity. Ten seeds per each M_2 line were germinated in a germination paper medium. Seven day old seedlings (seven seedlings per line) were transferred onto a styro-foam (with holes) floated on plastic container containing Yoshida nutrient solution. The pH of the nutrient solution (0.18 mM $(\text{NH}_4)_2\text{SO}_4$, 0.27 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.09 mM KNO_3 , 0.18 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.09 mM KH_2PO_4 , 0.04 mM K_2SO_4 and 0.02 mM $\text{NaEDTAFe} \cdot 3\text{H}_2\text{O}$) was adjusted to 4.5, and the solution was changed once in a week. Plants showing significantly shorter root than the wild-type Nagina 22 were selected on the 15 day after transplanting. Selected lines/plants were transferred to pots containing soil mixture and advanced to further generations. The selected lines were retested at M_5 stage for the uniformity in phenotype.

Screening for salinity mutants

In order to identify mutants exhibiting enhanced level of tolerance against salinity stress, the Yoshida nutrient solution was salinized on the 22nd day after observing the root characters by adding NaCl up to required concentration (100 mM). The pH of the solution was adjusted to 4.5, and monitored daily. Putative gain-of-function mutants exhibiting enhanced tolerance against 100 mM NaCl stress was identified based on the development of leaf rolling, wilting and drying symptoms. Identified mutant lines were maintained under salt-free conditions up to M_5 generation and the mutants were again tested for their salinity tolerance along with the salinity tolerant rice genotype FL 478. The mutant lines were also tested for their ability to germinate under high salinity conditions by germinating 25 seeds (3 replications) of each mutant line in the presence of 100, 150, 200 and 250 mM NaCl in Petri dishes. Emergence of radicle and plumule was considered as a criterion for germination.

Statistical analysis

All data were analyzed by statistical software SAS 9.1 Versions. Where appropriate, Duncan's multiple range test (DMRT) at the 0.05 probability level was used to group means. Standard error were calculated by manually using the formula, $SE = SD / \sqrt{(N)}$; where, SD = standard deviation of mean; N= number of observation of sample.

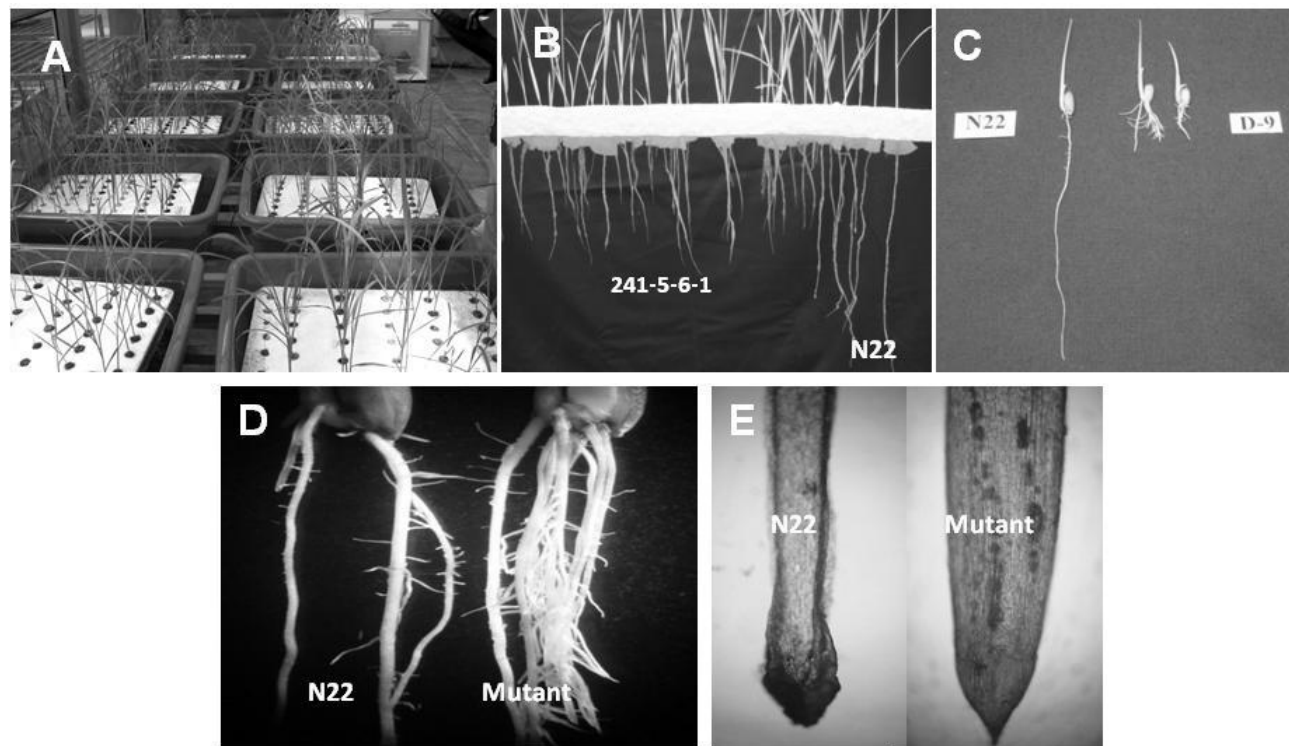


Figure 1. Identifying putative root mutants in EMS induced Nagina 22 mutant lines. A, Screening for root traits under hydroponic conditions; B, homozygous short root mutant (N22-C-241-5-6) showing significantly shorter root length than wild type Nagina 22; C, other two short root mutants; D, a mutant possessing more number of lateral roots than Nagina 22; E, putative mutant showing defect in the root cap.

Table 1. Root length and plant height of putative short root mutants.

Genotype	Root length (cm) (mean \pm 2SE)	Plant height (cm) (mean \pm 2SE)
Nagina 22	20.0 \pm 1.14	99.8 \pm 2.95
N22-C-241-5	9.6 \pm 0.53	70.6 \pm 6.52
N22-C-241-5-6-1	8.5 \pm 0.30	61.6 \pm 1.52
N22-D-8	10.1 \pm 1.06	76.2 \pm 2.80
N22-D-9	8.8 \pm 0.83	70.4 \pm 2.65
N22-D-105	8.0 \pm 0.38	78.4 \pm 3.25
N22-D-106	7.9 \pm 0.45	79.0 \pm 3.50

Root length was measured at 22 days after germination. Plant height was measured at maturity. Each value is a mean of 7 plants.

RESULTS

Isolation of altered root growth behavior mutants

Isolation of short root mutants

In order to identify rice plants showing abnormalities in morphogenesis and growth of root system, 1500 M_2 mutant lines (seven seedlings per line) were germinated in papers and transferred onto nutrient solution filled trays and maintained hydroponically (Figure 1A). A total of 12 M_2 lines were found to possess significantly shorter roots

than the wild type. Out of the 12 lines, six of them had uniformly shorter roots than the wild type and they did not exhibit segregation for root length. In all the six mutant lines, root length was more than 50% less than the wild type (Table 1 and Figure 1B). In the remaining six mutant lines, single plants possessing significantly shorter roots that were 50% shorter than those of the wild type were identified (Figure 1C). These M_3 lines were found to show segregation for root length and are being forwarded to next generations to fix the mutation. Further studies on these identified mutants helped us to identify putative

Table 2. Observations on number of lateral roots present in seminal root and crown roots of wild type Nagina 22 and putative short root mutants.

Genotype	Number of lateral roots in seminal roots			Number of lateral roots in crown roots		
	5 DAS	10 DAS	15 DAS	5 DAS	10 DAS	15 DAS
N22	38.4 ^a	220.4 ^a	326.6 ^a	10.4 ^a	46.0 ^a	90.4 ^b
N22-C-241-5-6-1	4.0 ^c	29.0 ^d	51.6 ^e	2.0 ^c	4.8 ^b	63.6 ^{bc}
N22-C-12-1	36 ^a	56.4 ^d	92.6 ^{de}	3.8 ^{bc}	25.6 ^{ab}	96.0 ^b
N22-C-241-5	17.2 ^b	154.4 ^b	182. ^{bc}	3.2 ^{bc}	35.8 ^a	84.0 ^b
N22-D-8	38 ^a	184.4 ^{ab}	217.4 ^b	7.2 ^{ab}	45.0 ^a	93.4 ^b
N22-D-9	3 ^c	149.0 ^{bc}	180.6 ^{bc}	4.8 ^{bc}	46.2 ^a	122.0 ^b
N22-D-105	0.0 ^c	0.0 ^e	108.8 ^d	0.0 ^c	0.0 ^c	222.0 ^a
N22-D-106	15.8 ^c	107.0 ^c	157.6 ^c	2.2 ^c	24.0 ^{ab}	73.2 ^b

Means followed by same letter are not significantly different at the 5% level by Duncan's Multiple Range Test. Plants were germinated and transferred onto hydroponic culturing and observations were made on 5, 10 and 15 DAS. Each values is mean of 8 plants.

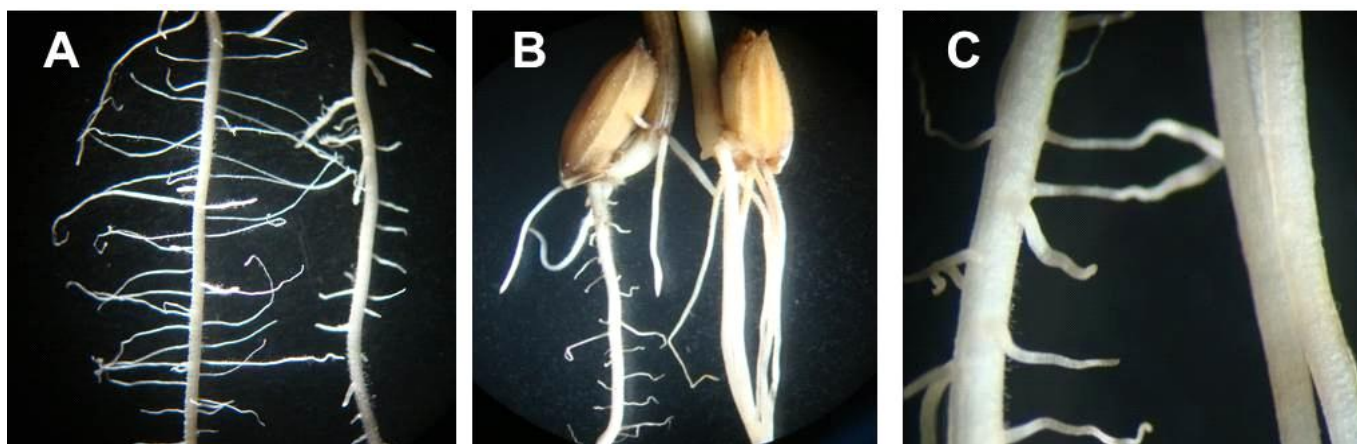


Figure 2. Mutants exhibiting variation in lateral root morphology. A, Mutant (N22-C-241-5-6) possessing significantly lesser number of lateral roots than the wild type Nagina 22; B, mutant (N22-D-105) showing absence of lateral roots at 7 DAS; C, close-up view of lateral root-less mutant.

mutants for other root growth traits also. One of the six short root mutants was found to possess significantly more number of lateral roots than the wild type (Figure 1D). A putative root mutant showing an abnormality in root cap structure was also identified (Figure 1E). This mutant was found to be devoid of root cap when compared to the wild type which had a well-developed root cap at the same growth stage.

Isolation of mutants showing variation in number of lateral roots

Number of lateral roots present in both seminal root and crown roots were observed at different growth stages of seedlings. One of the short root mutants namely, N22-C-241-5-6-1 was found to possess significantly lesser number of lateral roots in both seminal and crown roots than wild type Nagina 22 (Table 2). Moreover, it was

observed that the length of lateral roots was found to be significantly shorter than the wild type Nagina 22 (Figure 2A). Another short root mutant N22-D-105 was found to possess no lateral roots at 7 DAS (Figure 2B and C). But interestingly, it was observed that this mutant started developing large number of lateral roots after 10 DAS (Table 2). At 15 DAS, this mutant N22-D-105 was found to possess significantly greater number of lateral roots in both seminal and crown roots (Table 2). All the mutants were found to possess significantly lesser number of lateral roots in the seminal root.

Isolation of mutants showing variation in number of crown roots

All the seven short mutants were characterized for the number of crown roots along with the wild type Nagina 22. The wild type Nagina 22 was found to possess a

Table 3. Observations on number of crown roots per plant.

Genotype	Number of crown root		
	5 DAS	10 DAS	15 DAS
N22	2.2 ^{bcd}	3.2 ^b	4.4 ^b
N22-C-241-5-6-1	0.0 ^d	1.4 ^c	3.0 ^c
N22-C-12-1	2.8 ^{bc}	3.4 ^b	4.6 ^b
N22-D-7	0.0 ^d	0.3 ^c	0.8 ^d
N22-D-8	4.0 ^a	3.2 ^b	3.6 ^c
N22-D-9	0.0 ^d	0.0 ^d	0.0 ^e
N22-D-105	3.2 ^{ab}	6.4 ^a	7.0 ^a
N22-D-106	1.8 ^c	3.0 ^b	3.2 ^c

Plants were germinated and transferred onto hydroponic culturing and observations were made on 5, 10 and 15 DAS. Each values is mean of eight plants. Means followed by same letter are not significantly different at the 5% level by Duncan's multiple range test.

average of five crown roots at around 10 -15 DAS. The present study resulted in the identification of few interesting mutants showing variation for number of crown roots. A mutant N22-D-105 was found to possess significantly greater number of crown roots (7) than the wild type (4.4) at 15 DAS (Table 3). This mutant was found to exhibit another interesting developmental variation for the growth of lateral roots also as described in the previous section. Another mutant namely N22-D-9 was found to possess no crown roots even up to 15 DAS (Table 3). This mutant was designated as *crown rootless* mutant (*crl mutants*). The mutant N22-D-7 was found to possess significantly lesser number of crown roots than the wild type Nagina 22 (Table 3).

Isolation of gain-of-function mutants for salinity tolerance

In order to screen plants showing altered response against salinity stress, 1500 M₂ mutant lines (seven seedlings per line) were germinated in papers and transferred onto nutrient solution filled trays and maintained hydroponically. Salinity stress was imposed to 25-day's old seedlings by adding NaCl to 100 mM concentration and tolerance level of the mutants was assessed based on the development of wilting and drying symptoms. The wild-type Nagina 22 was found to be completely dried within seven days after imposition of salinity stress and most of the mutant lines were also found to be susceptible. Three single plants from three independent mutant lines namely N22- SPS-5, N22-334-3, and N22-293-1, exhibited the very high degree of tolerance against salinity stress. All the three mutants were found to remain green even after 15 days of salinity stress when all other mutants and wild type plants were dried completely. These three single plants were transferred onto pots and maintained under greenhouse con-

ditions normally. Seeds were collected from all the three mutant plants and forwarded to further generations till M5 stage. Fixed mutants were tested for their degree of salinity tolerance at both germination and vegetative stage. The germination of wild type Nagina 22 was found to be affected significantly beyond 100 mM NaCl stress. Of the three mutants, N22-334-3 mutant exhibited very high degree of salinity tolerance and it was found to germinate even at 250 mM NaCl stress (Figures 3 and 4). During vegetative stage, all the three mutants were noticed to exhibit tolerance against salinity stress same as that of tolerant check FL 478 (Figure 5).

DISCUSSION

Mutants for root morphological traits have been reported in crops like maize (Miller and Moore, 1990), barley (Tagiliani et al., 1986) and Arabidopsis (Benfey et al., 1993). However only few mutants exhibiting altered root growth behaviour in rice have been reported (Ichii and Ishikawa, 1997; Wang et al., 2006). Reduction in root length was found to be associated with reduction in plant height as in the case of semi-dwarfing genes. But there are reports where reduction in root length was independent of shoot length (Ichii and Ishikawa, 1997). In this study, it was observed that the plant height of the six homozygous short root mutants was not affected significantly by the reduced root length at seedling stage. But there was a reduction in the final plant height at maturity stage. Thorough analysis of the anatomical differences in roots of the mutants and genetic complementation analysis may help us to categorize the mutants before getting into functional genomics studies. The six homozygous short root mutants identified in this work showed approximately 50% reduction in root length over the wild type and exhibited segregation for root length. Several researchers have identified and characterized rice mutants with reduced seminal root length (Liang and Ichii, 1996; Ichii and Ishikawa, 1997; Inukai et al., 2001). Mutants possessing uniform short roots were further confirmed by growing in large numbers along with Nagina 22. In the present study, one of the short root mutant, N22-C-241-5-6-1 was found to have significantly fewer lateral roots in both seminal and crown roots than the wild type Nagina 22 (Table 1). Moreover, it was observed that the length of lateral roots was found to be significantly shorter than the wild type Nagina 22. Isolation of lateral rootless (*lrt*) rice mutants were done recently by Wang et al. (2006) rice (*Oryza sativa* L. cv. Nipponbare). In our study, the six mutants were found to possess significantly less number of lateral roots in the seminal root. The developmental mechanisms of lateral root formation in rice isolation of mutants exhibiting either loss-of-function or gain-of-function property in development of lateral root formation is important. Debi et al. (2003) reported the isolation of a novel lateral root mutant that is specifically affected in lateral root elongation and root hair formation.

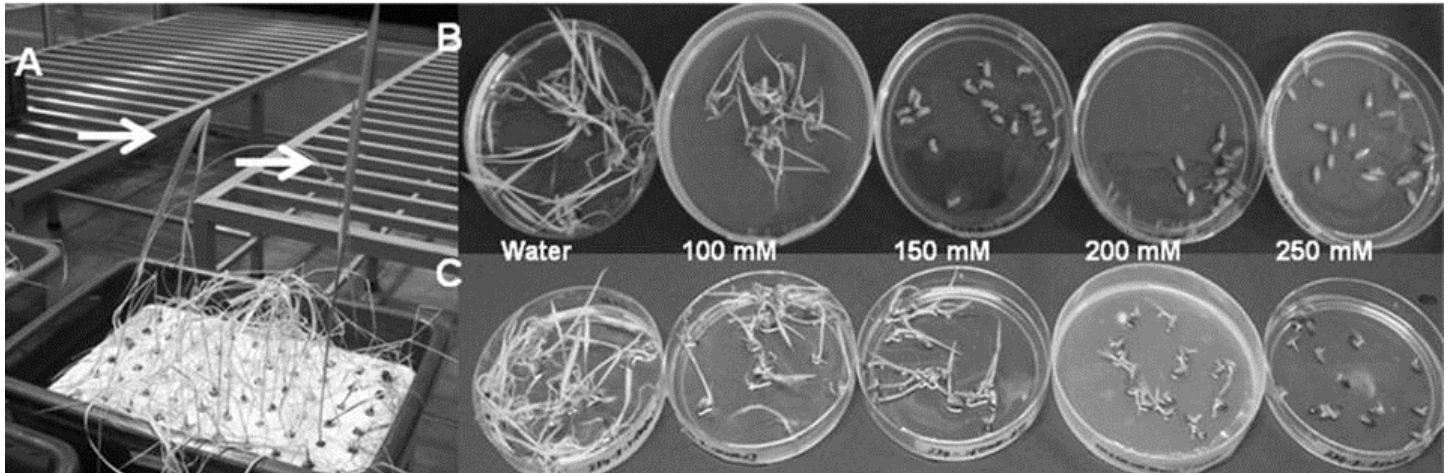


Figure 3. Identification of putative salinity tolerant mutants in rice. A, Survival of two independent mutants under salinity stress (two weeks under 100 mM NaCl stress); B, germination of wild type Nagina 22 at various levels of NaCl and it showed susceptibility at 150 mM NaCl stress; C, germination of a putative salinity tolerant mutant N22-C334-4 under various levels of NaCl which showed tolerance upto 250 mM NaCl stress.

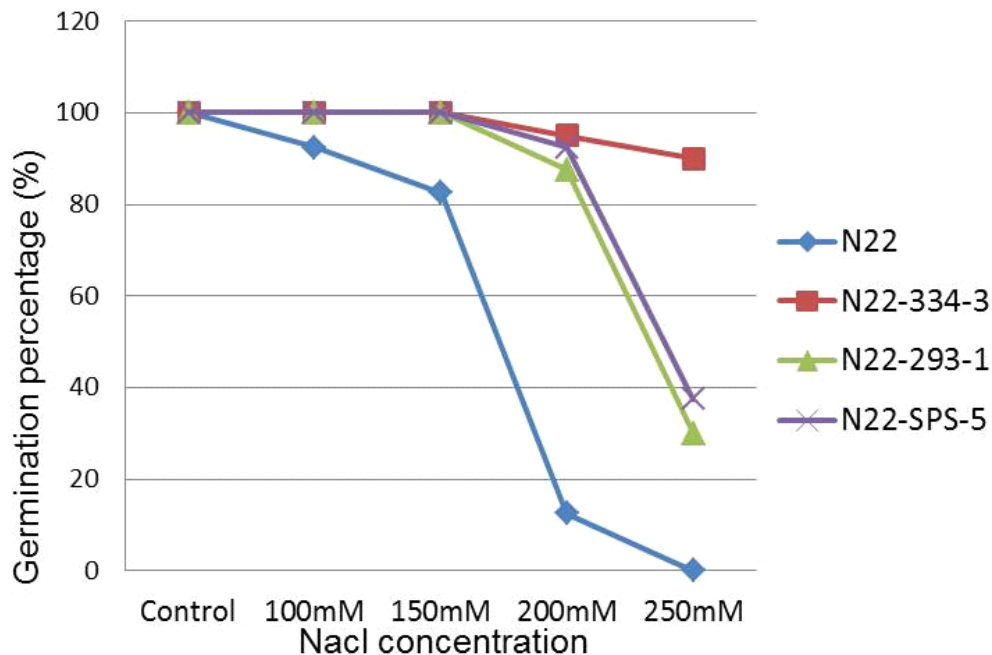


Figure 4. Observation on salinity tolerant mutants performance in germination percentage at 7 days after induced various levels of salinity stress. Each percentage value is mean of $n = 75$ from 3 replications.

Lateral rootless mutants such as *Lrt1* have been previously isolated in rice (Hao and Ichii, 1999). Crown rootless mutant is unable to initiate the crown root primordia and *crl* mutant have been isolated in previous studies in rice (Coudert et al., 2011; Kitomi et al., 2011). In our study, the mutant N22-D-9 was found to possess no crown roots even up to 15 DAS. This mutant was

designated as *crl* mutants. The mutant N22-D-7 was found to possess significantly fewer crown roots than the wild type Nagina 22 (Table 3). Yao et al. (2003) reported such mutants showing extreme inhibition of elongation of seminal root, crown roots and lateral roots and altered root hair formation at the seedling stage or early growth stage.

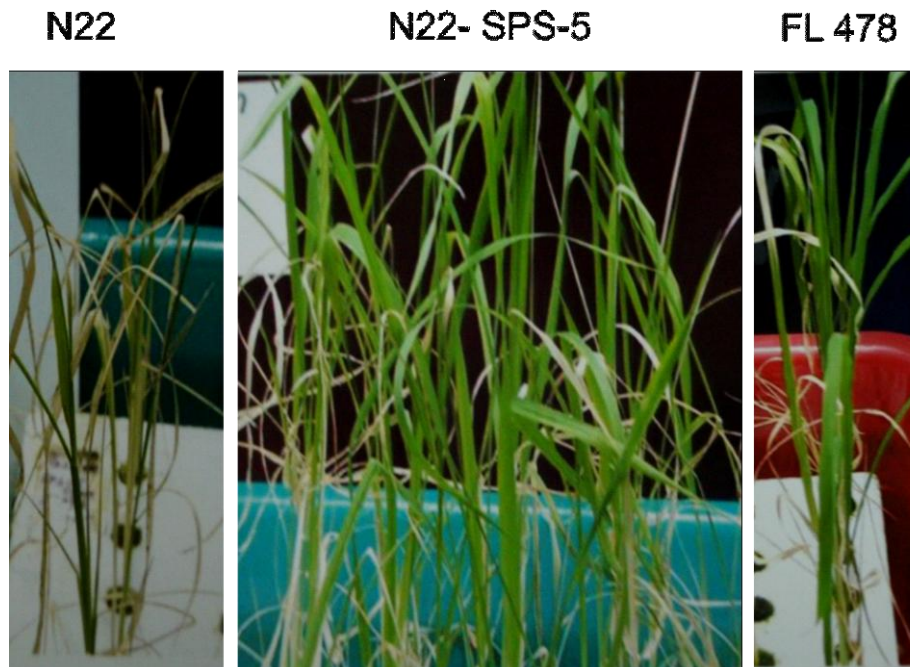


Figure 5. Confirmation of putative salinity tolerant mutants in rice. Confirmation of salinity tolerant ability in identified N22-SPS-5 mutant under salinity stress (two weeks under 100 mM NaCl stress); with Wild type N22; and check variety FL 478.

Genetically to improve the morphogenesis and function of roots in higher plants, it is important to collect mutants associated with various root growth traits. Availability of rice mutants exhibiting either loss-of-function or gain-of-function phenotype for salinity tolerance is limited especially in *indica* type (Miah et al., 1996; Sathish et al., 1997). In this study, among the 1500 mutants, three mutants exhibited significantly greater degree of tolerance to 100 mM NaCl stress at seedling stage. The level of tolerance was almost comparable to the tolerant check FL 478, a derivative of the salinity tolerant traditional cultivar Pokkali.

To improve rice genotypes for their tolerance against drought and salinity stresses, it is imperative to understand the physiological, genetic and molecular mechanisms underlying various components controlling these complex phenomena. In this context, it is important to collect various mutants associated with these traits contributing to drought and salinity tolerance in rice. There has been a long history of mutation breeding in the cereals with several important varieties resulting from selection of mutant phenotypes (Ahloowalia and Maluszynski, 2001). However, systematic development of mutant populations as genomic resource has only commenced recently. We used a large collection of EMS induced Nagina 22 mutants for identifying lines exhibiting contrasting behavior for root growth traits and salinity tolerance. As a result of our investigation, we have identified six homozygous mutants possessing significantly shorter roots than the wild type and three mutants exhibiting enhanced level of tolerance against salinity

stress than the wild type. These mutants can now be used for further genetic, functional genomics and molecular marker studies.

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Full Length Research Paper

Assessment of microbial diversity under arid plants by culture-dependent and culture-independent approaches

Nimisha Sharma^{1*}, Govind Singh² and Yemmanuar Sudarsan²

¹Indian Agricultural Research Institute, New Delhi, Pusa, India, 110 012.

²College of Agriculture, SKRAU, Bikaner, Rajasthan 334 006.

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In this work, microbial community structure of two distinct arid plants like ker (*Capparis deciduas*) and pearl millet (*Pennisetum glaucum*) was assessed and defined by culture-dependent and culture-independent approaches on the basis of 16S rRNA and random amplified polymorphic DNA (RAPD) analysis. The average Jaccard's similarity coefficient values for cultivated bacteria that is within ker and pearl millet rhizosphere were 0.701 and 0.707, respectively, for non-rhizosphere of ker and pearl millet 0.739 and 0.762, respectively, and for non-cultivable bacteria under ker (0.519) and under pearl millet (0.534). Both culture-dependent and culture-independent methods indicated that in arid crops, microbial diversity is more influenced by soil type rather than plant type and lower Jaccard value for metagenome showed that whole community harbours more diversity because of different microflora than cultivated only. Salinity and temperature tolerance study of bacteria indicated that ker rhizosphere harbours more salinity and temperature tolerant bacteria.

Key words: Random amplified polymorphic DNA (RAPD), ribotyping, Thar Desert, microbial diversity, 16S rRNA.

INTRODUCTION

Soil is a complex habitat where a large number of different microorganisms interact. Soil microbial diversity is an important index of agricultural productivity. Both the plant and soil types influence the microbial diversity of the rhizosphere. Interaction of plants and microorganisms is a result of co-evolution and their balance is important for sustainable agriculture (Smith and Goodmann, 1999; Lau and Lennon, 2011). The influence of perennials as well as annuals in microbial diversity is expected to be more pronounced in harsh climates of the desert. Such studies are scanty for the Thar Desert and most of them pertain to cultivable types, which represent only 1% of the total microbial diversity in the soil and their population may be

greatly affected by changes in environmental conditions, hence, failed to envisage the entire population. Until recently, investigators had no idea how accurately cultivated microorganisms represented the overall microbial diversity. The cultivation-dependent approach is limited by the fact that the overwhelming majority of microorganisms present in soil cannot be cultivated under laboratory conditions.

The development of molecular phylogenetics has recently enabled characterization of naturally occurring microbial biota without cultivation. There is a vast amount of information held within the genomes of cultivable and non-cultivable microorganisms, and new methods based

*Corresponding author. E-mail: nims17sharma@gmail.com. Tel: +91-011-25843214. Fax: +91-011-25843214.

Abbreviations: RAPD, Random amplified polymorphic DNA; PM, pear millet rhizosphere; OP, near pearl millet field; KR, ker rhizosphere; OK, near ker plantation; CTAB, cetyl trimethyl ammonium bromide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; NB, nutrient broth.

on analysis of DNA allow investigation of this potential. In recent decades, a number of biological methods based on characterization of microbial DNA or RNA have been developed for reasons of better characterization. These methods are used to identify bacteria and describe bacterial DNA diversity in the case of individual bacteria, or DNA of entire microbial communities. In practice, the use of molecular biological methods includes isolation of the total DNA, amplification and analysis of 16S *rRNA* genes to get material for further analysis. Molecular techniques over the last few decades have revealed an enormous reservoir of unexplored microbes. Among these, 16S *rRNA* gene sequencing has been primarily responsible in revealing the status of our lack of knowledge of microbial world such that half of the bacterial phyla recognized so far consist largely of these yet uncultured bacteria (Lozupone and Knight, 2007).

The soil gains importance, especially in saline and drought prone areas. This effect is always more pronounced in the rhizosphere. Hence, the rhizobacteria form a group of the best adapted micro organisms (Hartmann et al., 2008). Soil bacterial communities and the soil processes mediated by bacteria are critical for ecosystems functioning and productivity in arid lands. There is a need to integrate the soil bacterial community into our understanding of ecosystem interactions. The arid-ecosystem inhabiting hardy and woody plants like ker and pearl millet. Ker is distributed as natural wild in the arid and semi-arid regions of north-west India which mainly covers the parts of western Rajasthan and could be improved as a potential plant for horticultural and industrial uses in future (Paroda, 1979).

Pearl millet is used for the present study owing to its increasing worldwide importance as a food and forage crop (Vania et al., 2006). Selection of these crop species for the present study is to help understand if there are some essential rhizobacteria required for its establishment and growth in nature and may also lead to isolation of novel or efficient plant growth promoting rhizobacteria under extreme drought and heat that may further be utilized for enhancing the yield. A very few attempts have been made to study the genetic diversity of the rhizospheric and non-rhizospheric bacteria of pearl millet and ker.

Therefore, the objectives of present study are the isolation and characterization of both culturable and non-culturable microbial diversity under ker and pearl millet through random amplified polymorphic DNA (RAPD) and ribotyping as well as the study of the morphological and physiological properties of isolated bacteria.

MATERIALS AND METHODS

Sample collection

Three soil samples each from rhizosphere of ker and pearl millet along with adjacent non-rhizospheric areas of different location of SKRAU, Bikaner, Rajasthan (71° 54', 74°12' E longitudes and 27°

11', 29° 3'N latitudes) were collected in the month of September that have similar soils and vegetation. The samples from ker rhizosphere represented the area that was under cultivation once (about 10 years ago), while the pearl millet rhizospheric samples were from cultivated lands. Cultivated land was included to find out if certain bacterial types are supported by pearl millet even with drastic disturbance in soils. Samples from each site were well-mixed, air-dried, ground and allowed to pass through a 2.0 mm sieve and stored separately at -80°C for further analysis. Physiochemical parameters of soil were analyzed which showed the soils have electrical conductivity (ECe) 0.30-1.25dS/m, pH ranging from 7.5-8.0 and organic carbon from 0.18-0.45%. All the experiments repeated thrice.

Metagenome profiling

Soil microbial DNA was extracted from 15 g of soil sample using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) originally suggested for plants, with some modifications. Next 15 g soil sample was well homogenized with 15 ml DNA 2X CTAB DNA extraction buffer (100 mM Tris, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 2% CTAB and 2µl/ml β-mercaptoethanol) supplemented with 1% sodium dodecyl sulfate (SDS), in capped polypropylene tubes. A brief sonication treatment (30s at 15 W (4/10) for 50% active cycles) with titanium microtip was also given using BRAUN LABSONIC U sonicator, followed by incubation at 60°C for 1 h, deproteination by chloroform-isoamylalcohol and precipitation with one-third volume of propanol.

The DNA thus obtained was further purified with fast DNA spin kit (Obigene). Since desert soils are poor in microbial population, this kit recommended for isolation of DNA from small amounts of soil (1 g) directly, was used to purify DNA isolated from larger amounts of soil. In order to develop RAPD profiles, ten arbitrary primers (OPG-2, 3, 11, 12 and so on) obtained from Operon Technologies Inc. (Alameda, California) were used. Polymerase chain reaction (PCR) was performed in a final volume of 25 µl containing 10X assay buffer, 1.0 unit of *Taq* DNA polymerase (Bangalore Genei), 200 µM each of dNTPs (Fermentas), 10 pmol/ reaction of random primers and 50 ng template DNA. A thermal cycler (Biometra) was programmed for the initial denaturation step (94°C) of 5 min, followed by 44 cycles of 1 min denaturation along with 1 min primer annealing (37°C) and 2 min primer extension (72°C), followed by the 7 min primer extension (72°C) step. Amplicons were resolved by electrophoresis on 1.2% agarose gel (Himedia) containing 0.5 µg/ml ethidium bromide and run for 3-3.5 h at 100 V with cooling.

The 16S *rRNA* gene was amplified with bacterial specific universal primers P1 (5' AGAGTTTGATCCTGATCCTGGCTCAG 3') and P2 (5' TACCTTGTACGACTT 3'). PCR reaction was performed in final volume of 25 µl containing 2.5 µl 10X assay buffer with MgCl₂ (Bangalore Genei), 0.19 µl of *Taq* polymerase (3 U/µl), 2.0 µl dNTPs, 1 µl primer-1 (10pM/µl) (OPERON TECHNOLOGIES), 16.3 µl deionised water and 2.5 µl template DNA (25 ng/µl). The PCR was performed for 35 cycles in 'Thermocycler' (Bio metra). The PCR was set with initial denaturation step of 5 min at 94°C, followed by 35 PCR cycles (denaturation at 94°C for 1 min, a primer annealing at 48°C for 1min, and primer extension at 72°C for 2 min) with a final extension of 7 min at 72°C. The PCR products, were analyzed on 1% agarose gel (Himedia, molecular grade), prepared in 1X TBE buffer containing 0.5 µg/ml of ethidium bromide. The gel was viewed under UV trans-illuminator and photographed by digital camera (Gel Doc S- Mini Bis Bioimaging. System, USA). Amplified PCR products (5-10 µl) were digested singly overnight at 37°C with 5 U of two different restriction enzymes viz; *DpnII* and *AluI* (New England Biolabs). Restricted fragments (10 µl) were analyzed on horizontal gel electrophoresis in 3.0% agarose (Sisco Research laboratories

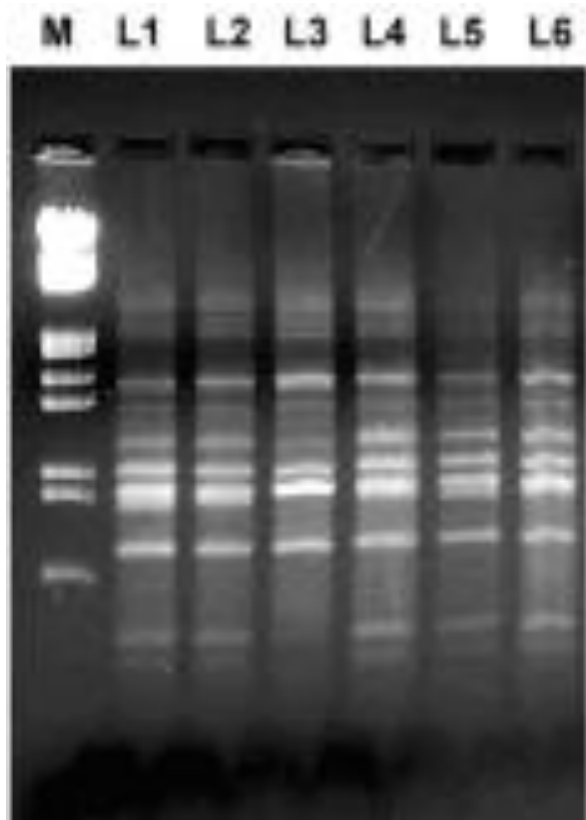


Figure 1. RAPD profile generated by primer OPG-11 using metagenome for rhizosphere of Ker and Pearl millet. M, Marker- Lambda DNA/*Eco R I* digest; L1-L3, pearl millet rhizospheric metagenome; L4-L6, Ker rhizospheric metagenome.

Pvt. Ltd) in 1X TBE buffer and electrophoresed at 75 volts for 3 h and visualized after ethidium bromide (0.5 µg/ml) staining.

The presence or absence of particular DNA fragment was converted into binary data and the pair wise genetic similarities among two genotypes under study was determined using Jaccard's coefficient (Jaccard, 1908). Cluster analysis for the genetic distance was then carried out using UPGMA (unweighted pair group method with arithmetic mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the genomes using computer program NTSYS pc version 2.02 (Rohlf, 1998).

Morphological and physiological properties of bacterial isolates

For the isolation of rhizospheric and non-rhizospheric bacteria, 10 g soil from each sample was added to 90 ml of 0.85% saline water and vortexed for 30 min on a mechanical rotary shaker and serially diluted up to 10^{-4} to 10^{-6} and 100 µl of each suspension were plated on plain nutrient agar (NA) and incubated at 37°C temperature for 24 to 48 h. Bacterial cultures were maintained in paired nutrient slants and stored at 4°C till further use (Rouatt and Katznelson, 1961). These bacterial isolates were examined under the stereo microscope (Leicca Int., Germany) for their shape and colony colour and tested for gram staining. The intrinsic resistance

of the rhizospheric and non-rhizospheric bacterial isolates against salinity and temperature was evaluated by observing the growth on nutrient agar medium (Yildirim et al., 2008).

Genetic diversity of bacterial isolates

Genomic DNA of the sixty four rhizospheric and non-rhizospheric bacterial isolates was isolated from 24 h old culture grew in nutrient broth (NB) medium using a modified method of DNA isolation (Hill et al., 1972; Pierre et al., 1991). PCR and ribotyping analysis were done as described above.

RESULTS AND DISCUSSION

Standardization of DNA isolation from soil

Genomic DNA of pure quality is the prime requisite for molecular studies. Soil community DNA isolation is cumbersome because of organic matter and humus present in the soil. They also co-precipitate with DNA in any standard protocols and interfere with PCR reactions. Initially, standard CTAB method resulted in lower yield (0.53 and 1.26 µg/g) and inferior quality DNA ($A_{260/280}$ 1.0 and 1.36). Enzymatic lysis involving lysozyme also yielded low quantity (0.73 and 0.86 µg/g) and quality DNA ($A_{260/280}$ 1.2 and 1.3) for two samples. Addition of anionic detergent, SDS along with CTAB yielded maximum DNA (1.53 and 1.4 µg/g) in both the samples but was of inferior quality ($A_{260/280}$ 1.35 and 1.4). A sonication step was incorporated in this method to increase the yield and to improve the quality of DNA. Even though, the sonication treatment increased the yield, the quality remained inferior which was evident from ratio of $A_{260/280}$ values ranging from 1.06 to 1.22. Therefore, an additional purification step using Fast DNA Soil Kit (Qbiogene Fast DNA spin kit) was attempted. After this purification, the DNA yield declined and ranged from 3.45 to 4.05 µg/g of soil, but the quality improved ($A_{260/280}$ ranged from 1.56 to 1.72). Incorporation of SDS along with CTAB resulted in higher yield and relatively moderate purity (1.4 to 1.53). Similar results have been reported by several workers (Chen et al., 2010; Siddhapura et al., 2010).

Metagenome profiling using PCR and 16S rRNA

Molecular characterization of the soil samples from rhizospheric regions of pearl millet and ker with ten RAPD markers was done. The total number of bands amplified varied from 11 (OPG- 16) to 21 (OPG-2). The size of amplified fragments ranged from 100 to 3530 bp (Figure 1) and predominantly within the range of 325 to 3500 bp. Average number of bands per primer were 14.3. The number of polymorphic bands ranged from 10 (OPG- 16) to 19 (OPG-2) producing a total of 86 polymorphic bands which accounted for 92.4% average polymorphism and an average amplification of 14.3 bands per primer.

Table 1. Jaccard's average similarity coefficient generated by UPGMA analysis for different soil metagenome.

Parameter	KR1	KR2	KR3	PMR1	PMR2	PMR3
KR1	1.000					
KR2	0.494	1.000				
KR3	0.505	0.602	1.000			
PMR1	0.462	0.473	0.483	1.000		
PMR2	0.483	0.602	0.473	0.526	1.000	
PMR3	0.516	0.548	0.483	0.623	0.559	1.000

KR, ker rhizosphere; PMR, pearl millet rhizosphere.

The Jaccard's similarity coefficient ranged from 0.462 [PMR-1 (BKN) and KR-1 (BKN)] to 0.623 [PMR-1 (BKN) and PMR-3 (BKN)] with an average of 0.522 for RAPD patterns (Table 1). The average similarity within ker (0.519) and pearl millet (0.534) rhizosphere was almost the same. The clustering pattern also revealed that the two major clusters had 49.8% similarity.

Metagenome from pearl millet rhizosphere presented higher genetic similarity (54.5%) that could be expected from complex metagenome. One major cluster comprising of three pearl millet samples (PMR-1, PMR-2 and PMR-3) and two of ker samples (KR-2 and KR-3) with 51.4% similarity coefficient indicated that there could be some common microflora in the metagenome. KR-1 branched as separate shoot from the major cluster with 50.2% dissimilarity. The conserved 16S *rRNA* gene analysis is also a preferred molecular tool to elucidate the phylogenetic relationship among bacteria as it provides unambiguous data which is reproducible across laboratories (Drancourt et al., 2000). The 16S *rRNA* gene was amplified with bacterial specific universal primers and gel electrophoresis of undigested PCR products revealed that all isolates produced a single band of about 1500 bp (Figure 5). Same results were obtained by Sudhir et al. (2009). Single digestion of this fragment with tetra-cutter *Alu*I resulted into distinct banding pattern including four to six bands with molecular sizes ranging from 130 to 450 bp with six distinct restriction pattern in all the samples analyzed. The application of UPGMA clustering has shown the existence of one major cluster when restriction profiles of amplified 16S rRNA obtained with *Alu*I. The correlated profiles have been grouped together with different soil samples exhibiting identical ARDRA profiles. Major cluster consisted rhizospheric soil metagenome of both the plant species *viz*; ker and pearl millet and non-rhizospheric soil metagenome of pearl millet. Major cluster further sub divided into a number of sub clusters, within rhizospheric soil metagenome of pearl millet 100% similarity was found due to the closeness in the banding pattern obtained after restriction with *Alu*I and it showed 75% similarity with non-rhizospheric soil metagenome of pearl millet. Both rhizospheric and non-rhizospheric soil metagenome of ker showed 80% percent similarity (Figure 2).

Morphological and physiological properties of bacterial isolates

Morphological study of bacterial isolates indicated that out of 64 isolates, 53 were found to be gram negative whereas, 11 stained were gram positive (Table 2). Salinity and temperature tolerance study of bacterial isolates showed that most of the bacterial isolates (rhizospheric as well as non-rhizospheric) were less halophilic. Bacterial population was similar in both rhizospheric and non-rhizospheric soil samples at 37°C without supplementation of NaCl in the medium but highest bacterial population was found under ker rhizosphere when nutrient agar medium was supplemented with 10% percent NaCl at 50°C temperature. It indicated that ker rhizosphere harbours more salinity and temperature tolerant bacteria (Table 3). The phylogeny of bacterial cultures has been explored mainly using morpho-logical characters especially to define and identify bacterial taxa but only morpho-logical characters could not be reliable attributes to all members of the same morpho-logical species (Lakshmanan et al., 2003).

Bacterial diversity study using RAPD and 16S rRNA from rhizosphere of pearl millet and ker along with respective non-rhizospheric areas

Molecular characterization of the bacterial isolates from rhizosphere and non-rhizosphere regions of pearl millet with 10 RAPD markers indicated that 8 primers produced 74 scorable amplicons of sizes ranging between 350 to 2700 bp with average polymorphism 82.4% and resolving power of 9.25 bands per primers. The Jaccard's similarity coefficient values within pearl millet rhizospheric bacterial isolates varied from 0.318 [PM-10 (bacterial isolate from pearl millet rhizosphere) and PM-16 (bacterial isolate from pearl millet rhizosphere)] to 0.954 [PM-8 (rhizospheric) and PM-9 (rhizospheric)] with an average of 0.707. Within non-rhizospheric bacterial isolates, it varied from 0.500 [OP-5, OP-15; OP-1, OP-5 (bacterial isolate from near pearl millet field)] to 1.000 [OP-7 and OP-10 (non-rhizospheric isolates)] with an average of 0.762. The average similarity between two groups was 0.650. Cluster

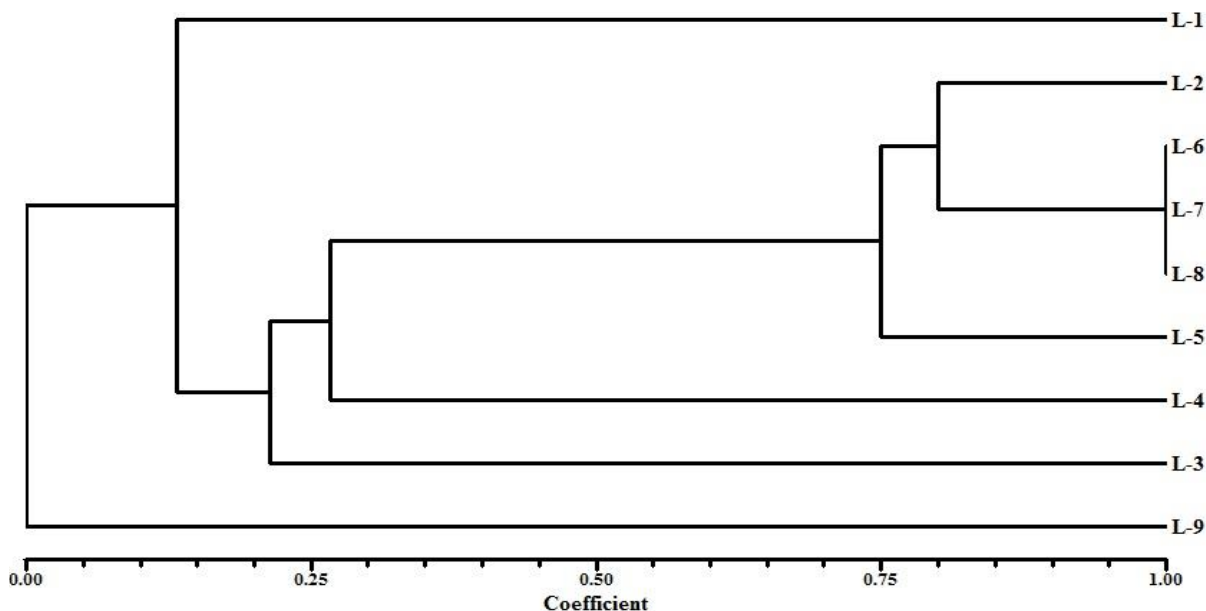


Figure 2. Cluster analysis of rhizospheric and non-rhizospheric community genome using restriction enzyme *Alu I*. L1, OK ; L2- L4, KR; L5, OP; L6-L8, PMR; L9, control; KR, ker rhizosphere; PMR, pearl millet rhizosphere; OP, non-rhizospheric area near pearl millet; OK, non rhizospheric area near ker.

analysis of thirty two bacterial isolates of pearl millet resulted in a distinct clustering of isolates into two major clusters with 57% genetic similarity (Figure 3).

Genetic diversity study of the bacterial isolates from rhizosphere and non-rhizosphere regions of ker with 10 RAPD markers indicated that 8 primers produced 63 scorable bands. The size of amplified fragment ranged from 300 to 3500 bp most lying between the size ranges of 400 to 3000 bp with 71.4% average polymorphism, with an average amplification of 7.9 bands per primer. Genetic similarity estimates based on RAPD data by Jaccard's coefficient analysis generated similarity coefficient matrix for ker samples, which ranged within rhizospheric bacterial isolates of ker from 0.370 [KR-13 (bacterial isolate from pearl millet rhizosphere) and KR-15 (bacterial isolate from ker rhizosphere)] to 0.926 [KR-3, KR-4; KR-5, KR-6; KR-6, KR-7; KR-5, KR-11; KR-6, KR-11 and KR-7, KR-11 (ker rhizospheric)] with an average of 0.701 for RAPD. Within non-rhizospheric bacterial isolates, similarity coefficient varied from 0.370 [OK-9 and OK-13 (isolate near ker plantation)] to 1.000 [OK-7 and OK-10 (non-rhizospheric isolates)] with an average of 0.739. The average similarity coefficient between two groups was 0.628. Cluster analysis resulted in a dendrogram comprising of two clusters with 49.2% genetic similarity (Figure 4).

RAPD based detection of genetic polymorphism has been successfully used for inter and intra-specific genetic diversity of microbial communities of the soil across the tropics and sub tropics (Harry et al., 2001; Patel and Behra, 2011; Sharma et al. 2008)). Results of molecular characterization revealed that there is considerable varia-

tion between the isolates of rhizospheric and non-rhizospheric region and also, within group variation were evinced. These results are at par with the results earlier reported in the genetic diversity studies of bacterial isolates of rhizospheric regions (Babalola et al., 2002; Gajbhiye et al., 2010).

The bacterial isolates were clustered into two distinct clusters with few exceptions based on the habitat as rhizospheric and non-rhizospheric which substantiates the moderately broad distribution of genetic variability. Similarity indices of high values were obtained within rhizospheric samples invariably in pearl millet and ker samples. This also suggests that there are similar organisms in the rhizosphere regions of pearl millet and ker which could be probably because both crops belong to arid-ecosystem. The study was in concordance with the reports on rhizospheric bacterial isolates of ker, maize, sorghum and cotton (Singh et al., 2004; Sharma et al., 2005).

Both culture- dependent and culture-independent methods indicated that in arid crops, microbial diversity is more influenced by soil type rather than plant type and lower Jaccard value for metagenome showed that whole community harbours more diversity because of different microflora than cultivated only.

Conclusion

The analysis of the community by comparing the isolates not only contributes to the general understanding about the diversity but also provides a large collection of organisms that could be further characterized and utilized.

Table 2. Morphological characteristics of bacterial isolates, isolated from rhizosphere of pearl millet (*Pennisetum glaucum*), ker (*Capparis deciduas*) and their respective non-rhizospheric areas.

Isolate	Gram stain	Shape of bacteria	Colony colour on nutrient agar	Isolate	Gram stain	Shape of bacteria	Colony colour on nutrient agar
PM-1	-ve	Rods	Yellow brown	KR-1	-ve	Rods	Creamy
PM-2	-ve	Rods	Light pink	KR-2	-ve	Short rods	Yellow
PM-3	-ve	Rods	Off white	KR-3	-ve	Rods	Off white
PM-4	-ve	Rods	White	KR-4	+ve	Rods	White
PM-5	-ve	Rods	Light green	KR-5	-ve	Short rods	Yellow brown
PM-6	-ve	Rods	Yellow	KR-6	-ve	Rods	Yellow
PM-7	-ve	Rods	Off white	KR-7	-ve	Rods	Off white
PM-8	-ve	Rods	Yellow	KR-8	-ve	Rods	Yellow
PM-9	-ve	Rods	White	KR-9	+ve	Rods	White
PM-10	-ve	Rods	Creamy	KR-10	-ve	Curved rods	Creamy
PM-11	+ve	Rods	Creamy	KR-11	+ve	Rods	Creamy
PM-12	-ve	Cocci	Creamy	KR-12	-ve	Cocci	Off white
PM-13	-ve	Rods	Yellow	KR-13	-ve	Rods	Yellow
PM-14	-ve	Rods	Off white	KR-14	-ve	Rods	White
PM-15	-ve	Cocci	Yellow	KR-15	-ve	Cocci	Yellow
PM-16	+ve	Rods	Yellow brown	KR-16	-ve	Rods	Creamy
OP-1	+ve	Rods	White shiny	OK-1	+ve	Rod	Off white
OP-2	+ve	Rods	White	OK-2	-ve	Short rods	Light pink
OP-3	-ve	Cocci	Yellow	OK-3	-ve	Short rods	White
OP-4	-ve	Rods	Light pink	OK-4	-ve	Cocci	Yellow
OP-5	-ve	Rods	White	OK-5	-ve	Rods	Creamy
OP-6	-ve	Rods	Yellow	OK-6	-ve	Curved rods	Yellow brown
OP-7	-ve	Rods	Light green	OK-7	-ve	Rods	Light brown
OP-8	-ve	Rods	Creamy	OK-8	-ve	Rods	Creamy
OP-9	+ve	Rod	Off white	OK-9	+ve	Rods	White shiny
OP-10	-ve	Short rods	Light pink	OK-10	+ve	Rods	White
OP-11	-ve	Short rods	White	OK-11	-ve	Cocci	Yellow
OP-12	-ve	Cocci	Yellow	OK-12	-ve	Rods	Light pink
OP-13	-ve	Rods	Creamy	OK-13	-ve	Rods	White
OP-14	-ve	Curved rods	Yellow brown	OK-14	-ve	Rods	Yellow
OP-15	-ve	Rods	Light brown	OK-15	-ve	Rods	Light green
OP-16	-ve	Rods	Creamy	OK-16	-ve	Rods	Creamy

Pm, Pearmillet rhizosphere; op, near pearl millet field; kr, ker rhizosphere; ok, near ker plantation.

Table 3. Physiological characteristics of bacterial isolates, isolated from rhizosphere of pearl millet, ker and their respective non-rhizospheric areas.

Isolate	Effect of varying NaCl conc.			Effect of varying temp.			Isolate	Effect of varying NaCl conc.			Effect of varying temp.		
	5%	10%	15%	40°C	45°C	50°C		5%	10%	15%	40°C	45°C	50°C
PM-1	+	+	+	+++	+	+	KR-1	+	+	+	++	+	+
PM-2	+	+	+	++	+	+	KR-2	+	+	+	++	+	+
PM-3	+++	+	+	++	+	+	KR-3	+++	+	+	+++	+	+
PM-4	+	+	+	++	+	+	KR-4	+	+	+	++	+	+
PM-5	+++	+	+	++	+	+	KR-5	+++	+	+	+++	+	+
PM-6	+	+	+	++	+	+	KR-6	+	+	+	++	+	+
PM-7	+++	+	+	++	+	+	KR-7	+++	++	+	+++	+	+
PM-8	++	+	+	++	+	+	KR-8	+	+	+	++	+	+

Table 3. Contd

PM-9	++	+	+	++	+	+	KR-9	+	+	+	++	+	+
PM-10	+++	+	+	++	+	+	KR-10	+++	+	+	+++	+	+
PM-11	+++	++	+	+++	++	+	KR-11	+++	+++	+	+++	+++	++
PM-12	+++	++	+	+++	++	+	KR-12	+++	+++	+	+++	++	+
PM-13	+++	+	+	+++	++	+	KR-13	+++	+	+	+++	++	+
PM-14	+++	+	+	++	+	+	KR-14	+++	+	+	++	+	+
PM-15	+++	++	+	+++	++	+	KR-15	+++	+++	+	+++	+++	+
PM-16	+++	++	+	+++	++	+	KR-16	+++	+++	+	+++	++	+
OP-1	+++	+++	++	+++	++	++	OK-1	++	+	+	+++	+	+
OP-2	++	+	+	++	+	+	OK-2	++	+	+	+++	+	+
OP-3	++	+	+	++	+	+	OK-3	+	+	+	++	+	+
OP-4	+	+	+	++	+	+	OK-4	+	+	+	++	+	+
OP-5	++	+	+	++	+	+	OK-5	++	+	+	+++	+	+
OP-6	+++	+++	+	+++	+++	+	OK-6	+	+	+	++	+	+
OP-7	++	+	+	++	+	+	OK-7	++	+	+	+++	+	+
OP-8	+++	+++	+	+++	+++	+	OK-8	+	+	+	++	+	+
OP-9	++	+	+	+++	+	+	OK-9	+++	+++	++	+++	++	++
OP-10	++	+	+	+++	+	+	OK-10	++	+	+	++	+	+
OP-11	+	+	+	++	+	+	OK-11	++	+	+	++	+	+
OP-12	+	+	+	++	+	+	OK-12	+	+	+	++	+	+
OP-13	++	+	+	+++	+	+	OK-13	++	+	+	++	+	+
OP-14	+	+	+	++	+	+	OK-14	+++	+++	+	+++	+++	+
OP-15	++	+	+	+++	+	+	OK-15	++	+	+	++	+	+
OP-16	+	+	+	++	+	+	OK-16	+++	+++	+	+++	+++	+

+. No growth; ++, slight growth; +++, good growth.

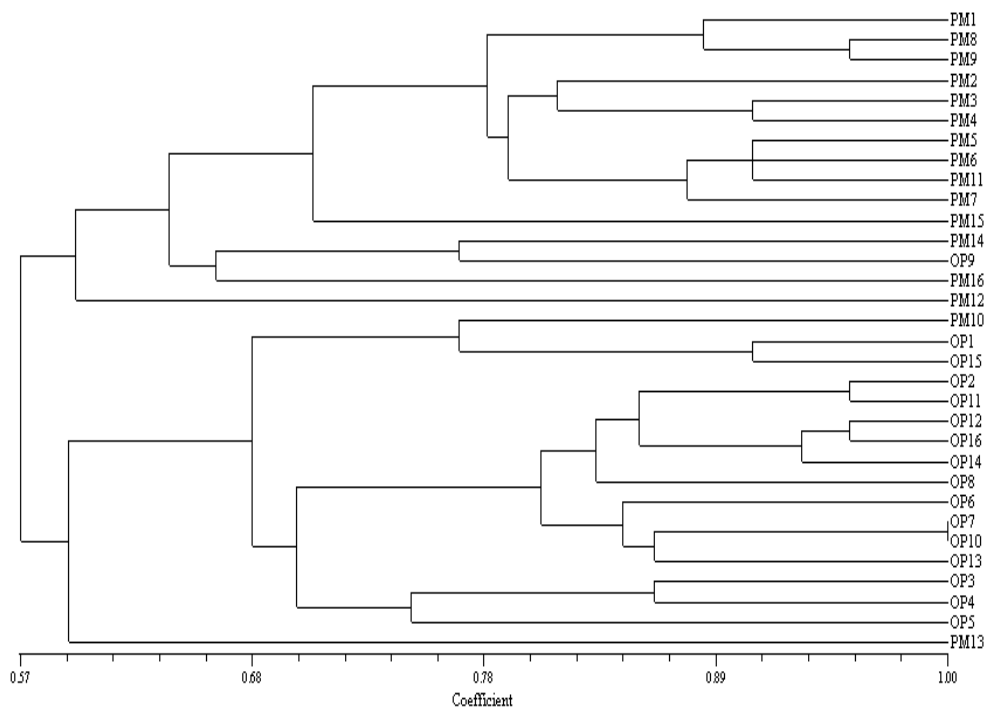


Figure 3. Dendrogram showing relationship among thirty two bacterial isolates from rhizospheric and non-rhizospheric pearl millet soil samples generated by UPGMA analysis based on RAPD.

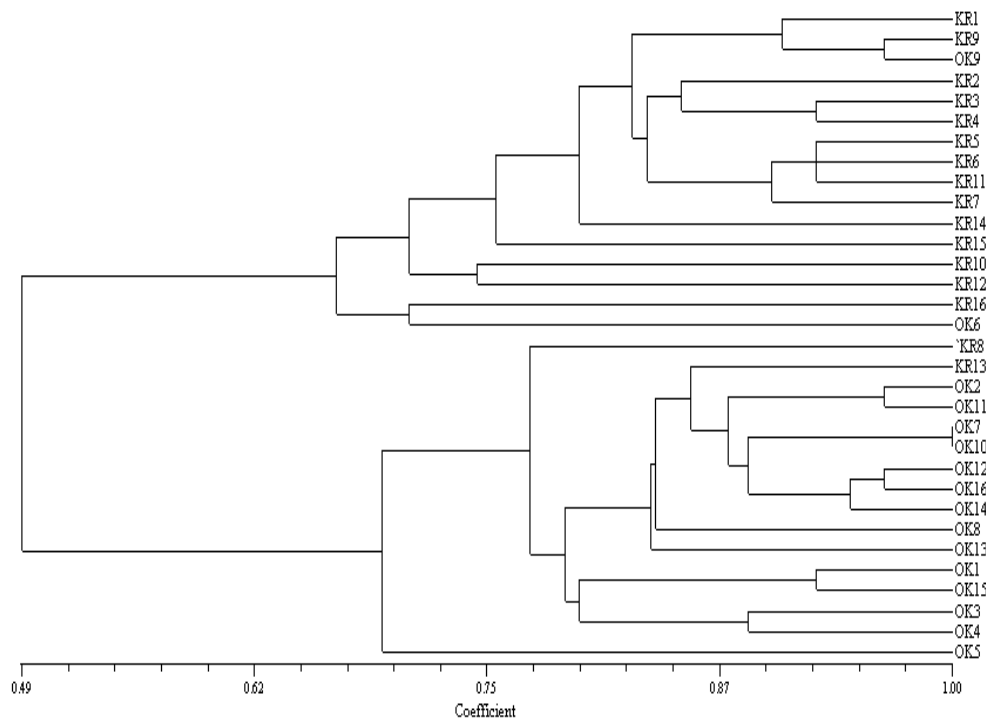


Figure 4. Dendrogram showing relationship among thirty two bacterial isolates from ker rhizosphere and non-rhizosphere soils generated by UPGMA analysis based on RAPD.

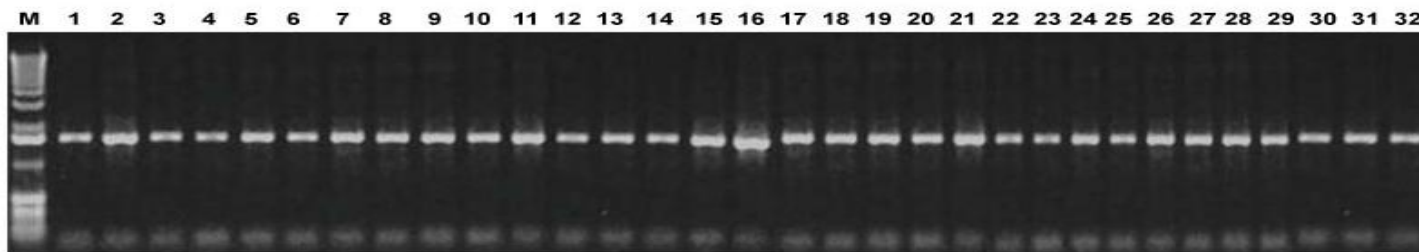


Figure 5. Agarose gel profile of 16S rDNA amplification of pearl millet and ker rhizospheric bacterial isolates. M, Marker-Lambda DNA/*Eco R* I digest; Lanes 1- 16: rhizospheric soil bacterial isolates of pearl millet (*Pennisetum glaucum*) field; 17-32, rhizospheric soil bacterial isolates of ker (*Capparis deciduas*).

Both culture- dependent and culture-independent methods indicated that in arid crops, microbial diversity is more influenced by soil type rather than plant type. Further, this research covered a small area of arid region but could be utilized for whole desert region crops and is suitable to assess microbial diversity.

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Full Length Research Paper

Application of *Cry1Ab/Ac Bt* strip for screening of resistance for *Maruca vitrata* in cowpea

Mohammed, B. S. *, Ishiyaku, M. F. and Sami, R.A.

Department of Plant Science, Ahmadu Bello University, Zaria. Nigeria.

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Maruca vitrata is a significant constraint to cowpea production in most cowpea growing areas of sub-Saharan Africa. Yield losses caused by *M. vitrata* in these regions are estimated in millions of tons annually and the prevalence of *M. vitrata* infestation is steadily increasing. Recombinant DNA technology have led to development of some cowpea lines with *Maruca* resistance as well as other important agronomic traits but it is time-consuming and difficult to screen for the resistant trait especially in the segregating populations using conventional screening techniques, which will lead to delay in the development of *Maruca* resistant cowpea varieties. The use of allele-based selection tool will make it easier to select plant traits and reduce the time needed to develop new *Maruca* resistant cowpea varieties. In this study, the efficacy of using *Cry1Ab/Ac Bt* strip for detecting *Maruca* resistant transgene in transgenic cowpea was systematically investigated for the first time through field derived progenies. The results show that the *Cry1Ab/Ac Bt* strip was effective for detecting the presence of the resistant gene in cowpea genome. *Maruca* resistant plants were successfully screened from the segregating cowpea plants and the genetics of the gene was monitored. The *Cry1Ab/Ac Bt* strip was found to be suitable for genetic analysis of the *Maruca* resistant transgene in cowpea. This study has demonstrated the precision of using *Cry1Ab/Ac Bt* strips as a screening tool of transgenic lines containing *Cry1Ab* gene, this has an importance in the hybridization programme where genotypes having *cry* gene can be distinguished at seedling stage at lesser time, with the potential of putting the breeding process on a fast track and increase the efficiency of breeding activities.

Key words: *Bacillus thuringiensis*, *Cry1Ab/Ac Bt* strips, transgenic cowpea, *Maruca vitrata*.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is considered the most important food grain legume in the dry savannas of tropical Africa (NGICA, 2002). It is the most important indigenous African legume for both home use and as a cash crop and especially important for the Sahel because of its drought tolerance (Kushwaha et al., 2004). It is rich in quality protein and has energy content almost equivalent to that of cereal grains, it is a good source of quality fodder for livestock and also provides cash income (Davis et al., 1991). Nearly 200 million people in Africa

consume the crop (AATF, 2010; NGICA, 2002). Cowpea is consumed in many forms; the young leaves, green pods, and green seeds are used as vegetables, dry seeds are used in various food preparations, the haulms are fed to livestock as nutritious supplement to cereal fodder and being a fast growing crop, cowpea curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contribute to soil fertility (Singh et al., 2002).

The overall productivity of its existing traditional geno-

*Corresponding author. E-mail: binsaba@yahoo.co.uk. Tel: +2348059285409.

types are low due to their prominent susceptibility to insect pests (Darshana et al., 2007) and among the most damaging insects are aphids, flower thrips, cowpea pod borer, pod sucking bugs and the cowpea weevils (Darshana et al., 2007). The cowpea pod borer (*Maruca vitrata*) is a serious lepidopteran pest that inflicts severe damage to cowpea on farmers' fields (Figure 3). In severe infestations, yield losses of between 70-80% have been reported (AATF, 2010). Control through spraying with insecticide has not been fully adopted by farmers due to the prohibitive costs, causing resource-poor farmers to opt for cheaper but more toxic alternatives that impact their health (AATF, 2010).

Breeding for insect resistance with the aid of phenotypic selection is time consuming, laborious and relatively expensive (Xu and Crouch, 2008). In addition, most crops have a high level of heterozygosity that makes visual selection difficult but selection based on allele composition will avoid this problem (Ibitoye and Akin-Idowu, 2010). Ability to select breeding progeny early at the seedling stage is another advantage of using allele-based selection tools (Ibitoye and Akin-Idowu, 2010). The number of plants that are needed to be maintained in a crop breeding programme can be reduced by eliminating progenies that do not carry the desirable allele at the seedling stage, saving space, time, labor and other resources (Ibitoye and Akin-Idowu, 2010). The present study was designed and conducted in order to understand the efficacy of using *Cry1Ab/Ac Bt* strips for detecting *Maruca* resistant transgene in transgenic cowpea through field derived progenies.

MATERIALS AND METHODS

The Research was conducted under the confined field trial site (CFT) between July, 2011 to August, 2012 at the Institute for Agricultural Research (IAR), Samaru-Zaria, Nigeria. Two genetically engineered cowpea lines: Transgenic cowpea line TCL-709 and TCL-711, and three non-transformed cowpea genotypes: IT97K-499-35, IT93K-693-2 and IT86D-1010, were used in this study. Data were collected as scores of *Cry1Ab Bt* strip test.

Cry1Ab inheritance studies

To establish the potency of *Cry1Ab/Ac Bt* strips as a screening tool for *Maruca* resistant transgene, the inheritance of *Cry1Ab* gene was monitored with the aid of *Bt* strips in filial generations.

Development of the genetic population

The transgenic cowpea lines TCL-709 and TCL-711 along with three non-transgenic genotypes: IT97K-499-35, IT93K-693-2 and IT86D-1010 (the original parent of the transformed lines having the same genetic architecture except the *Cry1Ab* gene) were crossed using biparental mating as described by Sharma (2006) to generate F_1 population. Some F_1 seeds were advanced to second filial generation (F_2) populations by self pollination. The following six combinations of crosses were produced: IT97K-499-35 x TCL-709, IT97K-499-35 x TCL-711, IT93K-693-2 x TCL-709, IT93K-693-2 x TCL-711, IT86D-1010 x TCL-709 and IT86D-1010 x TCL-711.

Field evaluation

The parents, F_1 and F_2 generations were evaluated under field conditions during the 2012 cowpea growing season at CFT Samaru-Zaria between June to August, 2012. The trial was planted using randomized complete block design with three replications. The plant to plant and row to row spacing was kept at 30 by 75 cm, respectively. The plot size was 3 x 5 m for all entries except F_2 plants which were 6 x 5 m. No insecticidal spray against lepidopteran insects was applied.

Test procedure

The screening was carried out with the aid of *Cry1Ab/Ac Bt* strips to check for the presence of *Cry1Ab* gene in the genetic populations (P_1 , P_2 , F_1 and F_2) of transgenic cowpea. Detection of *Cry1Ab* proteins on cowpea involved assaying plant leaves for expression of the *Cry1Ab* gene. A quick *Bt* strip test was used to confirm the expression of the *Cry1Ab* protein in cowpea transgenic lines. This was achieved by placing leaf discs in test tubes containing buffer and then slowly inserting *Bt* strips into the buffer. Then, formation of a single line in the test tube proved that the test was working while the appearance of a second lower line showed that *Cry1Ab* protein was present (Envirologix, 2008). Figures 1 and 2 illustrate a typical type of *Cry1Ab Bt* strip test. In these figures, the appearance of two lines on the test membrane indicates the presence of the *Cry1Ab Bt* gene, while the appearance of only the top (control) line indicates a negative response.

Cry1Ab gene screening in F_1 generation

The plants were screened with the aid of *Cry1Ab/Ac Bt* strips and the transfer of *Cry1Ab* gene from a transgenic cowpea plant to a non-transgenic cowpea plant was checked. The number of positive and negative plants indicating presence and absence of the transgene respectively, were taken to infer the behaviour of the transgene whether dominant or recessive and establish the efficacy of the *Cry1Ab/Ac Bt* strips.

Cry1Ab gene screening in Segregating Generations

Adequate sample size was taken from each F_2 family and analyzed with the aid of *Cry1Ab/Ac Bt* strips. Since the gene is expected to segregate in F_2 generations, the plants were clearly classified as *Cry1Ab*-positive or *Cry1Ab*-negative regarding the *Cry1Ab* expression where *Cry1Ab* positive plants indicates resistance to *M. vitrata* while *Cry1Ab* negative plants indicates susceptibility to *M. vitrata*. Envirologix (2008) procedures for *Cry1Ab/Ac Bt* strip test was carefully followed. The data was subjected to Chi-square goodness of fit test against the Mendelian ratio 3:1 for the F_2 generations (Kiani et al., 2009).

Statistical analysis

Data recorded for the genetic segregation of *Cry1Ab* transgene were analyzed with the help of Chi-square (χ^2) goodness of fit test, to determine whether the observed data conforms to the expected Mendelian 3:1 ratios for F_2 segregating populations of each cross. The following formula was used using a *Proc Frequency* for a chi-square test of goodness of fit by McDonald (2009):

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$



Figure 1. *Cry1Ab/1Ac Bt* strips showing positive, negative and invalid result (Enviroligix, 2008).

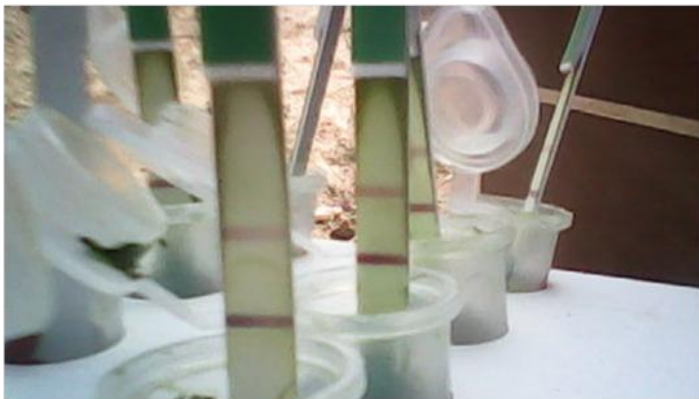


Figure 2. *Cry1Ab/1Ac Bt* strips in test tubes showing positive results.

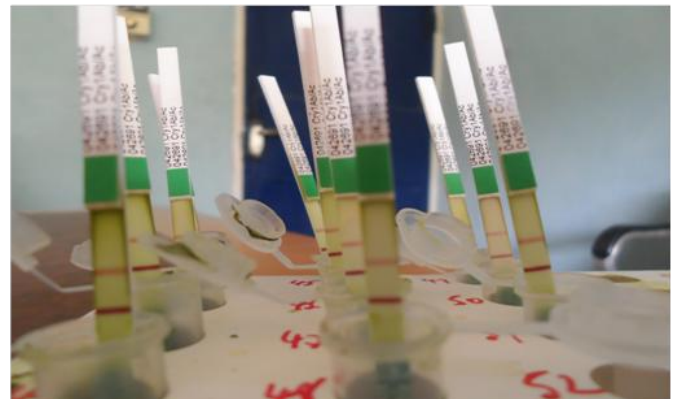


Figure 3. Showing larvae and adult *Maruca vitrata* (legume pod borer) pest.

Table 1. Detection of *Cry1Ab* gene in Parents and F₁ populations of transgenic cowpea.

Genotype	Number of plants tested	Positive	Negative	Expected ratio
TCL-709	50	50	0	1:0
TCL-711	50	50	0	1:0
IT97K-499-35	25	0	25	0:1
IT93K-693-2	25	0	25	0:1
IT86D-1010	25	0	25	0:1
IT86D-1010 x TCL-709	23	23	0	1:0
IT86D-1010 x TCL-711	23	23	0	1:0
IT97K-499-35 x TCL-709	30	30	0	1:0
IT97K-499-35 x TCL-711	28	28	0	1:0
IT93K- 693-2 x TCL-709	25	25	0	1:0
IT93K-693-2 x TCL-711	23	23	0	1:0

Positive, *Cry1Ab* gene is present, that is, resistant to *M. vitrata*; negative, *Cry1Ab* is absent, that is, susceptible to *M. vitrata*.

Table 2. Detection of *Cry1Ab* gene in F₂ populations of transgenic cowpea crosses.

Cross (female x male)	No. of plants tested	Positive	Negative	Expected ratio	Chi-square	DF	Probability
IT86D-1010 x TCL-709	105	81	24	3:1	0.26	1	0.61 ^{ns}
IT86D-1010 x TCL-711	81	61	20	3:1	0.004	1	0.95 ^{ns}
IT97K-499-35 x TCL-709	89	65	24	3:1	0.18	1	0.67 ^{ns}
IT97K-499-35 x TCL-711	111	85	26	3:1	0.15	1	0.70 ^{ns}
IT93k- 693-2 x TCL-709	75	58	17	3:1	0.22	1	0.64 ^{ns}
IT93k- 693-2 x TCL-711	131	101	30	3:1	0.31	1	0.58 ^{ns}

Positive, *Cry1Ab* gene is present, that is, resistant to *M. vitrata*; negative, *Cry1Ab* is absent, that is, susceptible to *M. vitrata*; ns, not significant at p=0.05.

Where, O, Observed value; E, expected value; \sum , summation.

RESULTS

Screening for *Cry1Ab* transgene in F₁ generations

The results of the six set of F₁ plants analyzed with the aid of *Cry1Ab Bt* strips to study the efficacy of *Bt* strips for detecting the transgene's presence through transmission and expression of the transgene are given in Table 1. It was found that all the F₁ plants were positive to *Cry1Ab Bt* strip test. It thus means that the gene was successfully transferred from *Bt* lines to non-*Bt* lines and the *Cry1Ab Bt* strips were potent as detecting tool for the target gene.

Screening for *Cry1Ab* transgene in segregating populations

As shown in Table 2, it reveals that the Mendelian segregation ratios (3:1) existed in all the six cross combinations for the F₂. The F₂ populations of these crosses segregated into plants with positive and negative *Cry1Ab* gene indicating the presence and absence of the *Maruca*

resistant gene, respectively, with a good fit to the Mendelian ratio of 3:1 with non significant Chi-square values (X^2) for F₂ plants of the following crosses; IT97K-499-35 x TCL-709 ($X^2 = 0.18$; P = 0.67), IT97K-499-35 x TCL-711 ($X^2 = 0.15$, P = 0.70), IT93K-693-2 x TCL-709 ($X^2 = 0.22$; P = 0.64), IT93K-693-2 x TCL-711 ($X^2 = 0.31$; P = 0.58), IT86D-1010 x TCL-709 ($X^2 = 0.26$; P = 0.61), IT86D-1010 x TCL-711 ($X^2 = 0.0041$; P = 0.95) (Table 2). This has demonstrated the potency of the *Bt* strips for detecting the presence of the transgene in the segregating populations of transgenic cowpea crosses. The strip screening clearly grouped the F₁ plants as resistant plants just like the transgenic parents and the segregating progenies of F₂ were seen clearly behaving as hypothesized into 3:1 Mendelian test ratio.

DISCUSSION

Detection of *Cry1Ab* with *Bt* strips

The genetic segregation and pattern of inheritance of *Cry1Ab* gene in the genetically modified cowpea were monitored in six crosses of cowpea involving transgenic and non-transgenic lines. In the present study, the segre-

gation of *Cry1Ab* gene was found to be in Mendelian fashion in all the six cowpea crosses, the results indicate that the resistant trait was controlled by a single dominant gene in the crosses that were examined. The transgenic lines carried the dominant gene while the recessive allele resides in the susceptible genotypes. In the F_1 generation studies, the *Cry1Ab* gene was found to be successfully transferred from transgenic to non-transgenic and it was dominant. These results are in agreement with earlier research works on genetically modified *Bt* crops with *Cry1Ab* transgene: *Cry1Ab* transgene is inherited as single dominant gene, in *Bt* corn where the *Cry1Ab* conferred resistance to stem borer (*Ostrinia nubilalis*) (Murenga et al., 2012), in *Bt* Rice containing resistant gene to striped stem borer (*Chilo suppressalis*) (Kiani et al., 2009; Wang et al., 2012), in crosses of transgenic Rojolele Rice (Sulistyowati et al., 2008) and in *Bt* Cotton where Khan (2008) and Zhang et al. (2000) studied the inheritance and segregation of foreign *Bt* (*Bacillus thuringiensis* toxin) and *tfdA* genes. The ability to obtain 3:1 segregation in F_2 generations using the *Cry1Ab Bt* strips means that these tests could be employed for wide-scale studies in the field to enhance cowpea breeding for resistance to *M. vitrata*.

The results obtained here indicate that it is possible to use this technology to select for *Maruca* resistant genotypes in cowpea. Similar results have been reported in other crops (corn, soybean, cotton and canola) using *Bt* strips technology to select plants carrying *Cry1Ab* transgene (Stave, 2002; USDA/GIPSA 2006) and had proven to be effective in detecting the presence of the transgene in these crops. *Cry1Ab Bt* strip tests for genetically engineered crops are currently being used on a large scale in the United States to manage the sale and distribution of grains that are genetically transformed (Stave, 2002). In several of these applications, it is important to get a result rapidly in the field, and in these situations strip tests are particularly useful.

Using the *Cry1Ab Bt* strips, the screening were done at seedling stage with good precision, this saves time and resources. The use of *Cry1Ab Bt* strips as a screening tool of transgenic lines containing *Cry1Ab* gene is strongly recommended, this has an importance in the hybridization programme where genotypes having the transgene can be distinguished at seedling stage at lesser time. The benefits of this technology have important implications for improving the efficiency of the characterization of cowpea genotypes for resistance to *Maruca* in the laboratory, especially when working in remote areas and in developing countries where access to laboratory facilities, chemicals, and equipment for polymerase chain reaction (PCR) procedures are limiting. The *Cry1Ab Bt* strip test was found to be the most suitable in order to rapidly analyze large number of plants in lesser time and to differentiate between the two groups. Elite and promising plants can be faithfully screened and selected at seedling stage particularly during the development of backcross

population, aimed towards development of transgenic cowpea varieties. Results obtained from *Bt* strips sampled materials were effective and reproducible in our hands from the six F_2 populations used. The studies described here that the *Bt* strips screening offers a simple, sensitive and specific tool appropriate for identifying *Maruca* resistant transgene. We conclude that the application of this technology has the potential to significantly enhance the *Maruca* resistant cowpea breeding program, and the efficiency of breeders to speed-up the process of developing and deploying *Maruca* resistant cowpea varieties to farmers. This study demonstrates that *Bt* strip is an effective, economic and sensitive method for sampling and identifying resistant cowpea plants using leaf tissues.

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Full Length Research Paper

Upshot of the ripening time on biological activities, phenol content and fatty acid composition of Tunisian *Opuntia ficus-indica* fruit

Amel Rabhi^{1*}, Hanen Falleh², Ferid Limam³, Riadh Ksouri², Chedly Abdelly² and Aly Raies¹

¹Laboratoire de Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis (FST), Campus Universitaire 2092 El-Manar II, Tunis- Tunisia.

²Laboratoire des plantes extrêmophiles, Centre de Biotechnologie à la Technopole de Borj-Cédria (CBBC), BP 901, 2050 Hammam-lif, Tunisia.

³Laboratoire des Substances Bioactives, Centre de Biotechnologie à la Technopole de Borj-Cédria (CBBC), BP 901, 2050 Hammam-lif, Tunisia.

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Total phenol content and the antioxidant activities of three cultivars of *Opuntia ficus-indica* (L.) Mill. were evaluated. The results show that the ecotypes were significantly different according to their fatty acid composition, antioxidant and antibacterial activity, as well as their polyphenol profiles. Rossa fruit collected in August exhibited the higher phenolic content (15.48 mg GAE g⁻¹ DW) with a stronger antioxidant activity. Bianca fruit collected in August exhibited a lower phenolic content (3.13 mg GAE g⁻¹ DW) together with the stronger antiradical activity (96.14%). The advantage of this ecotype was more discernible as compared to positive controls butylated hydroxytoluene (BHT, 79.75%). Likewise, CpG chromatogram identification revealed also an important difference between the ecotypes on oleic and linoleic acids.

Key words: *Opuntia ficus-indica*, variability, phenol content, fatty acid, antioxidant activity.

INTRODUCTION

Opuntia ficus-indica are an important source of alimentary nutrients and vitamins (Sawaya et al., 1983; Teles et al., 1984). The fruits are eaten fresh, dried or preserved in jams, syrups or processed into candy-like products (Hoffman, 1980). Their juices are sometimes fermented, either into ethanol or wine and other beverages or used in food flavourings and colourings (Bustos, 1981; Retamal et al., 1987; Saenz, 1996; Gurrieri et al., 2000). Cactus pear could have a double application, both becoming an option for obtaining natural colouring features and providing health benefits through its antioxidant function (Stintzing and Carle, 2004;

Tesoriere et al., 2005). It has been reported that the extracts of fruits and stems exhibits hypoglycemic (Ibañez-Camacho and Roman-Ramos, 1979; Trejo-Gonzalez et al., 1996) anti-ulcer, (Galati et al., 2001; Lee, 2002), and anti-allergic actions (Lee et al. (2000). In addition, Park et al. (1998) reported analgesic and anti-inflammatory actions of the fruit and stem extracts. Recently, the methanol extract of *O. ficus-indica* fruits was shown to inhibit free radical-induced neuronal injury in mouse cortical cultures (Wie, 2000).

Furthermore, it was shown that cactus pear seed is rich in oleic acid (C18:1) and linoleic (C18:2) acids (16.7 and

*Corresponding author. E-mail: espoirs@yahoo.fr. Tel: +216 93218013.

Abbreviations: BHT, Butylated hydroxytoluene; BHA, butylated hydroxyanisole; RFA, Rossa Fruit of August; GFA, Gialla Fruit of August; BFA, Bianca Fruit of August; RFN, Rossa Fruit of November; GFN, Gialla Fruit of November; BFN, Bianca Fruit of November.

70.3%, respectively), which representing 87% of the total fatty acids (Ennouri et al., 2005). At the beginning of the 19th century, commercial populations were established on the island of Sicily to meet the increasing demand for late-ripening fruits, produced through "scozzolatura". This technique consists of removing the flowers and cladodes spring flush to get a second bloom, causing the fruits to ripen in October to November, 2 to 3 months later than the summer crop (Barbera 1992; 1993). It has been demonstrated that the great biodiversity in fruit colours (red, orange, purple, pink, yellow, and lime-green), results in great potential for the flat stemmed *Opuntias* in arid lands (Griffiths and Hare, 1907; Pimienta, 1990; Barbera et al., 1995; Inglese et al., 1995; Parish and Felker, 1997). Cultivars for fruit production can be distinguished by the colour of the fruit, peel and the ripe flesh, which can be red-purple, yellow-orange, white-cream, or greenish-white flesh. Cultivars also differ in plant shape, vigour, fertility, cladode and fruit size, fruit ripening time, seed count and ability to reflow (Wessels, 1988; Pimienta, 1990; Barbera, 1993). Interestingly, some works reported the presence of phenolic compounds in fruit samples of cactus pears (Kuti, 2000; Lee and Lim, 2000; Morales et al., 2007). Polyphenols constitutes the main powerful compound, on account of their multiple applications in food industry, cosmetic, pharmaceutical and medicinal materials (Maisuthisakul et al., 2007).

Structurally, phenolics embrace an aromatic ring, bearing one or more hydroxyl substituent, and range from simple phenolic molecule to highly polymerised compounds (Bravo, 1998). In addition to their role as antioxidant, these compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects (Balasundram et al., 2006). The literature reports few scientific studies regarding the presence of phenols or other antioxidant compounds in cactus pear fruits. In fact, the purple cultivar has the highest concentration of total phenols, at approximately 660 mg/l juice (Stintzing et al., 2005). Other studies have identified the presence of flavonoids as flavonol glycosides, amongst which significant amounts of isorhamnetin-3- rutinoside, rutin and kaempferol-3-rutinoside were found in a blend of yellow and red cultivars (Galati et al., 2003). Besides, according to Wallace (1986), flavonoids content in *Opuntia* fruits vary with variety, tissue type, and maturation. Subsequently, previous studies have shown that plant polyphenolics, antioxidant and antimicrobial activities depend significantly on biological factors (genotype, organ and ontogeny), as well as, edaphic and environmental (temperature, salinity, water stress and light intensity) ones (Lisiewska et al., 2006).

The objectives of this study were to investigate antioxidant activities using different tests, to estimate the antimicrobial capacities against human pathogen strains

that may be from food poisoning microorganisms, and to quantify and identify the fatty acid present in *O. ficus-indica* fruits.

MATERIALS AND METHODS

Chemical and reagents

Folin-Ciocalteu reagent, butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), vanillin, nicotinamid-adenin-dinucleotid (NADH), 1,1- diphenyl-2-picrylhydrazyl (DPPH), sodium bicarbonate (NaCO₃), and nitrite sodium (NaNO₂) were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). Authentic standards of phenolic compounds (gallic acid and catechin) were purchased from Sigma and Fluka (Buchs, Switzerland).

Plant sampling

Three *O. ficus-indica* cultivars of different colours (Rossa, Gialla, Bianca) were used in this study (Photo 1). Fruits were harvested in August [Rossa Fruit of August (RFA), Gialla Fruit of August (GFA) and Bianca Fruit of August (BFA)], and in November [(Rossa Fruit of November (RFN), Gialla Fruit of November (GFN) and Bianca Fruit of November (BFN)] in the same year (2009) when "scozzolatura" is applied at full ripening in Kasserine locality (286 km center-west Tunis; semi arid bioclimatic stage; mean annual rainfall: 335 mm/year). The samples were rinsed with distilled water then freeze-dried and ground to a fine powder and stored at - 20°C until analysis began.

Extraction of total lipid because soxhlet method is more efficient

To determine de oil content the ISO 659-1988 (E) procedure was used. Soxhlet extractions were performed using 40 g of cactus. The amount was transferred in a 33x100 cellulose thimble and placed in the extraction chamber of a 200 ml capacity Soxhlet apparatus. The cellulose thimble was plugged with cotton in order to avoid transfer of sample particles in the distillation flask fitted with a condenser, placed on a 500 ml distillation flask containing 300 ml of solvent and 3 boiling glass regulator. Samples were thus, extracted under reflux with *n*-hexane during 4 h (18 to 22 cycles/h). Thereafter, the cellulose cartridge was cooled to room temperature in a desiccators and its content was then milled before being transferred again in the thimble. The described procedure was thus repeated within 2 h until a total extraction of 8 h (4 + 2 + 2 h). After the extraction, the major solvent was eliminated in a vacuum rotary evaporator. The content was then transferred in a smaller tarred flask and concentrated to dryness with a vacuum rotary evaporator during 1 h at 80°C before cooling again in desiccators for 1 h. The flask was then weighed and the operation repeated during 30 min until difference between two consecutive weights was less than 10% (m/m). Extractions were performed at least three times and the mean values were reported. Results obtained were expressed as described hereinafter:

$$\% \text{ Oil content} = \frac{\text{Weight of oil obtained after extraction}}{\text{Weight of dry sample}} \times 100$$

Analysis of fatty acid composition

Fatty acid methyl esters (FAMES) of the studied oil samples were

prepared based on a method described by Parish and Felker (1997). Briefly, 0.1 g of oil was weighed in a 20 ml test tube (with screw cap). The sample was dissolved in 3 ml of hexane, and 0.5 ml of potassium hydroxide (2N) in methanol was added. The test tube was capped and vortexed for 30 s. The mixture was centrifuged at $402 \times g$ for 10 min, and the extracts were transferred to a 2 ml auto sampler vial, and analyzed using gas chromatography.

Gas chromatography (GC) condition

Fatty acid composition of oil samples were analyzed using a GC system (Agilent 6890, Wilmington, Delaware, USA) equipped with a split-splitless injector. Hewlett-Packard EL-980 flame ionization detection (FID) system was used to separate and quantify each FAMEs component. FAMEs were separated using DB-23 column (30 m \times 0.25 mm, i.d. 0.25 μ m polyethylene glycol film (Muskegon, Michigan, USA). Chromatography data was recorded and integrated using Chemstation software (version 6.0, Hewlett-Packard, Waldbronn, Germany). Oven temperature was held at 50°C for 1 min, then increased to 175°C/min and increased to 230°C, held for 5 min at 230°C. The temperatures of injector and detector were set at 250 and 280°C. Oil sample (1 μ L) was injected with split ratio of 1:50 at column temperature of 110°C. Helium (1 ml/min) was used as carrier gas controlled at 103.4 kPa, while hydrogen and air were used for FID and was held at 275.6 kPa. FAME standards were used to determine each type of fatty acid. Identification of fatty acids of the samples was carried out by comparing the retention times of reference standards and was analyzed under the same operating conditions as those employed for FAME of the standards and was expressed in percentage.

Determination of antioxidant activities and polyphenolic contents

Extraction of phenolic compounds

Sample extracts were obtained by magnetic stirring of 2.5 g of dry fruits powder with 25 ml of pure methanol for 30 min (Mau et al., 2005). All extracts were kept for 24 h at 4°C, filtered through a Whatman No. 4 filter paper, and evaporated under vacuum to dryness. They were stored at 4°C until analysis began.

DPPH radical-scavenging activity

The DPPH quenching ability of plant extracts was measured according to Hanato et al. (1988). One ml of the extract at different concentrations was added to 0.25 ml of a DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect AA (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where, A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in three replications. A higher percentage value corresponds to a higher antiradical activity of plant extract.

Iron reducing power

The capacity of plant extracts to reduce Fe^{3+} was assessed by the method of Oyaizu (1986). Methanol extract (1 ml) was mixed with

2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. After that, 2.5 ml of 10% trichloroacetic acid were added, and the mixture was centrifuged at $650 \times g$ for 10 min. The upper layer fraction (2.5 ml) was mixed with 2.5 ml of deionised water and 0.5 ml of ferric chloride and thoroughly mixed. The absorbance was measured at 700 nm and ascorbic acid was used as a positive control. A higher absorbance indicates a higher reducing power. The results are expressed in $\mu\text{g/ml}$ values.

β - Carotene bleaching test (BCBT)

A modification of the method described by Koleva et al. (2002) was employed. β - Carotene (2 mg) was dissolved in 20 ml chloroform. To 4 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 ml of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. An aliquot (150 μ L) of the β -carotene: linoleic acid emulsion was distributed in each of the wells of 96-well micro titre plates and fruits methanolic extracts (10 μ L) were added. Three replicates were prepared for each of the samples. The micro titre plates were incubated at 50°C for 120 min, and the absorbance was measured using a model EAR 400 micro titre reader (Labsystems Multiskan MS) at 470 nm. Readings of all samples were performed immediately ($t = 0$ min) and after 120 min of incubation. The antioxidant activity (AA) of the extracts was evaluated in term of β -carotene bleaching using the following formula:

$$\text{AA (\%)} = [(A_1 - A_0)/(A_0 - A_1)] \times 100 \quad (2)$$

Where, A_0 is the absorbance of the sample at 0 min, and A_1 is the absorbance of the sample at 120 min. The results are expressed in percentage.

Screening for antimicrobial activity

The antibacterial activity of fruit extracts was assessed by the agar disk diffusion assay (Mann, 2004) against four human pathogenic bacteria: Gram-positive cocci including *Staphylococcus aureus* (ATCC 25923) and Gram-negative bacteria including *Escherichia coli* (ATCC 35218), *Enterococcus faecium*, and *Salmonella typhi*. The bacterial strains were first grown on Muller Hinton medium at 37°C for 24 h prior to seeding onto the nutrient agar. One or several colonies of the indicator bacteria were transferred into API suspension medium (Bio Mérieux) and adjusted to the 0.5 McFarland turbidity standard with a Dens mat (Bio Mérieux). A sterile filter disc with 6 mm in diameter (What man paper no. 3) was placed on the infusion agar seeded with bacteria, and 10 μ L of several extract concentrations were dropped onto each paper disc, representing. The treated Petri dishes were kept at 4°C for 1 h, and incubated at 37°C for 24 h. The antibacterial activity was assessed by measuring the zone of growth inhibition surrounding the discs. Standard discs of gentamycin (10 UI) served as positive antibiotic controls according to CASFM 2005 guidelines.

Discs with 10 μ L of pure methanol were used as negative controls. For the antifungal activity, the agar-disc diffusion method was used as previously described (Cox et al., 2000). One *Candida* strains (*Candida albicans*) was first grown on Sabouraud chloramphenicol agar plate at 30°C for 18 to 24 h. Several colonies were transferred into API suspension medium and adjusted to two McFarland turbidity standards. The inoculate of the respective yeasts were streaked onto Sabouraud chloramphenicol agar plates at 30°C using a sterile swab and then dried. A sterile filter disc, diameter 6 mm (What man paper no. 3) was placed in the plate. Ten micro litres of extract concentration were dropped on each paper disc. The treated Petri dishes were placed at 4°C for 1 to 2 h

Table 1. Levels and fatty acid compositions of total lipids (TL) in *Opuntia ficus-indica* fruit.

Fatty acid	Relative content (%) ^a				
	RFA	BFA	GFA	GFN	RFN
C12:0	0.01±0.00	0.01±0.00	0.07±0.07	0.08±0.00	0.1±0.00
C14:0	0.12±0.00	0.11±0.01	0.15±0.08	11.47±0.24	0.09±0.01
C16:0	12.48±0.05	12.15±0.5	12.04±0.98	1.24±0.50	11.44±0.24
C16:1	1.36±0.00	1.25±0.07	1.05±0.79	1.82±0.23	0.69±0.1
C17:0	8.32±0.00	NI	5.18±5.75	NI	NI
C18:0	1.73±0.00	1.76±0.04	2.55±0.21	19.32±0.28	1.96±0.04
C18:1	18.88±0.10	24.59±0.08	16.90±1.29	63.57±0.19	18.46±0.19
C18:2	55.40±0.05	58.41±0.27	59.05±5.39	0.94±0.02	64.60±0.12
C18:3	0.88±0.00	0.54±0.00	1.69±0.97	0.22±0.03	1.34±0.00
C20:0	0.16±0.00	0.27±0.00	0.28±0.06	0.33±0.00	0.24±0.00
C20:1	0.23±0.00	0.38±0.01	0.44±0.09	0.20±0.02	0.44±0.1
C22:0	NI	0.19±0.01	0.37±0.07	0.28±0.03	0.30±0.08
C22:1	NI	0.31±0.01	0.16±0.08	0.48±0.01	0.38±0.03
U/S ^b	3.35±0.05	5.89±0.47	3.83±6.68	2.05±0.51	6.11±0.14

^aResults are given as the average of triplicate determinations standard deviation, ^bRatio of unsaturated to saturated fatty acids. NI, Not identified. **RFA**, Rossa Fruit of August; **GFA**, Gialla Fruit of August; **BFA**, Bianca Fruit of August; **RFN**, Rossa Fruit of November; **GFN**, Gialla Fruit of November; **BFN**, Bianca Fruit of November.

and then incubated at 37°C for 18 to 24 h. As for the antibacterial activity, the antifungal one was evaluated by measuring the diameter of the growth inhibition zone around the discs. The susceptibility of the standard was determined using a disc paper containing 20 µg of amphoterecin B. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

Colorimetric quantification of phenolics

Determination of total polyphenol content

An aliquot of 125 µL of diluted extract were added to 500 µL of distilled water and 125 µL of the Folin-Ciocalteu reagent. The mixture was shaken, before adding 1250 µL Na₂CO₃ (7%), adjusting with distilled water to a final volume of 3 mL, and mixed thoroughly. After incubation for 90 min at 23°C in the dark, the absorbance versus prepared blank was read at 760 nm (Dewanto et al., 2002). Total phenol content of fruits was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid, ranging from 0 to 400 µg/mL. All samples were analysed in triplicate.

Estimation of total flavonoid content

Total flavonoids were measured by a colorimetric assay (Dewanto et al., 2002). An aliquot of diluted sample or standard solution of (+)-catechin was added to a 75 µL of NaNO₂ solution (5%), and mixed for 6 min before adding 0.15 ml AlCl₃ (10%). After 5 min, 0.5 ml of NaOH was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the blank where the sample was omitted. Total flavonoid content was expressed as mg catechin per gram of DW (mg CE/g DW), through the calibration curve of (+)-catechin, ranging from 0 to 400 µg/mL. All samples were analysed in triplicate.

Quantification of total condensed tannins

To 50 µL of properly diluted sample, 3 ml of 4% vanillin solution in methanol and 1.5 ml of concentrated hydrochloric acid were added (Sun et al., 1998). The mixture was allowed to stand for 15 min, and the absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin/g DW. The calibration curve range of catechin was established between 0 and 400 µg/ml. All samples were analysed in triplicate.

Statistical analysis

For all cultivars parameters, three replicates were used. To determine their relative variability on phenol content, fatty acid composition, antioxidant and antibacterial activities. Analysis of variance (ANOVA) was achieved for whole data, using the XLSTAT statistical program. Means were compared using the Newman-Keuls (SNK) test at the *P*<0.05 level, when significant differences were found.

RESULTS

Fatty acid compositions

In our study, the fatty acid composition differed greatly between *O. ficus-indica* fruit depending on pulp colour and ripening time. Typical CpG chromatogram of fruit extracts is presented in Table 1. Thirteen (13) fatty acids were identified in fruit (pulp and seed) extracts. Major fatty acids were significantly different when comparing the ecotypes. Wherein the analysis of FAME exhibited that the major fatty acids in summer crop are linoleic C18:2 (Ω6) (55.40; 58.41 and 59.05 %) followed by oleic C18:1 (18.88; 24.59 and 16.90%) and palmitic acids C16:0

(12.48; 12.15 and 12.0 4%) for RFA; BFA and GFA, respectively. The fatty acid composition of RFN presented nearly the similar trends to that of summer crop with linoleic C18:2 (Ω_6) (64.60%) followed by oleic C18:1 (18.46%) and palmitic C16:0 (11.44%) acids. Whereas the major fatty acids in GFN are oleic C18:1 (63.57%) followed by stearic C18:0 (19.32%) and myristic C14:0 (11.44%) acids. Results analysis illustrate that "scozolatturae" technique probably had an effect on linoleic and myristic acids composition of Gialla fruit collected in November as compared to those harvested in August.

Antioxidant activities

Antiradical activity

DPPH is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction capability of DPPH radical was determined by the decrease in absorbance induced by plant antioxidants. BHT was the reagent used as standard. The scavenging effect of methanol extracts and standard on the DPPH radical expressed in percentage was in the following order: Bianca fruit collected in August (96.14%) > Gialla fruit collected in August (93.15%) > Rossa fruit collected in August (93.15%) > Rossa fruit collected in November (85.51%) > Bianca fruit collected in November (39.65%) > Gialla fruit collected in November (32.37%) (Figure 1A). The experimental data of this species reveals that ecotype extracts have a stronger effect of scavenging free radical than positive control BHT (79.75%).

Iron reducing power

Another reaction pathway in electron donation is the reduction of an oxidized antioxidant molecule to regenerate the "active" reduced antioxidant. Iron reducing power values of all tested samples were ranged from 0.04 to 0.55 $\mu\text{g mL}^{-1}$ and arranged in the following increasing efficiency order: ascorbic acid (1.32 $\mu\text{g mL}^{-1}$) > Bianca fruit (August) (0.55 $\mu\text{g mL}^{-1}$) > Gialla fruit (August) (0.45 $\mu\text{g mL}^{-1}$) > Rossa fruit (August) (0.16 $\mu\text{g mL}^{-1}$) > Rossa fruit (November) (0.10 $\mu\text{g mL}^{-1}$) > Bianca fruit (November) (0.05 $\mu\text{g mL}^{-1}$) > Gialla fruit (November) (0.04 $\mu\text{g mL}^{-1}$). As shown in (Figure 1B), the reducing power of fruits collected in August extracts was clearly more important than fruits collected in November extracts. Nonetheless, the ascorbic acid (positive control) concentration required to reduce the ferric iron was higher (1.32 $\mu\text{g/ ml}$) than all extracts, indicating probably a low activity.

Antioxidant assay using β -carotene linoleate system

In this model, β -carotene undergoes rapid discoloration in the absence of an antioxidant. The presence of an antioxi-

dant such as phenolics can hinder the extent of β -carotene destruction by "neutralizing" the linoleate free radical and any other free radicals formed within the system (Kamath and Rajini, 2007). Figure 1C depicts the inhibition of β -carotene bleaching by the fruit extracts of *O. ficus-indica*, and by the two positive controls [butylated hydroxyanisole (BHA) and BHT].

In terms of β -carotene bleaching effect, those samples exhibited the following order: BHT (79.75 %) > BHA (61.69 %) > GFN (26.16 %) > BFN (18.33 %) > RFN (15.36 %) > GFA (8.37 %) > BFA (7.57 %) > RFA (3.39 %). This method showed significant differences between the different ecotypes. Indeed, fruits collected in November were statistically as efficient as those collected in August.

Antimicrobial activity

Table 2 shows the antibacterial activities of *O. ficus-indica* fruits measured by the agar diffusion method against selected pathogenic bacteria. The mean inhibition zone for all bacteria treated with Rossa fruit extracts varied from 9.33 to 12 mm and from 7.33 to 11 mm at 50 mg/l treated with Bianca fruit varied from 8.33 to 10.33 mm and from 7.33 to 10 mm and treated with Gialla fruit varied from 6.33 to 8mm and from 5.33 to 7 mm, respectively for ecotype collected in November and ecotype collected in August.

The strongest activity was recorded against *Enterococcus faecium* for the two ecotypes with an inhibition zone equal to 12 and 11 mm, respectively for RFN and RFA. Pertaining to antifungal tests, fruit extracts concentration (50 mg/ml) inhibit *Candida albicans* growth with an inhibition zone equal to 9.33 and 7.33 mm, respectively for RFN and RFA. These results suggest in one hand that "scozolatturae technique" had an impact on antibacterial activities of *O. ficus-indica* fruit, in the other hand that methanolic extracts of fruit were more efficient to inhibit bacterial growth than fungal one for the two ecotypes, probably in relation to their active molecules.

Analysis and quantification of phenolic compounds

Total phenolics contents in *Opuntia ficus-indica* fruit

Results of total phenolic, flavonoid, and condensed tannin quantifications are represented in Figure 2. Total phenolic content measured on *O. ficus-indica* fruit varied significantly depending on ripening-time and on ecotypes and ranged from 2.61 to 15.48 mg GAE g^{-1} DW. Among ecotypes, RFA was the richest in phenolics.

They were significantly decreased in the order: RFA (15.48 mg GAE g^{-1} DW) > GFA (7.31 mg GAE g^{-1} DW) > GFN (3.89 mg GAE g^{-1} DW) > BFA (3.13 mg GAE g^{-1} DW) > BFN (2.90 mg GAE g^{-1} DW) > RFN (2.61 mg GAE g^{-1} DW).

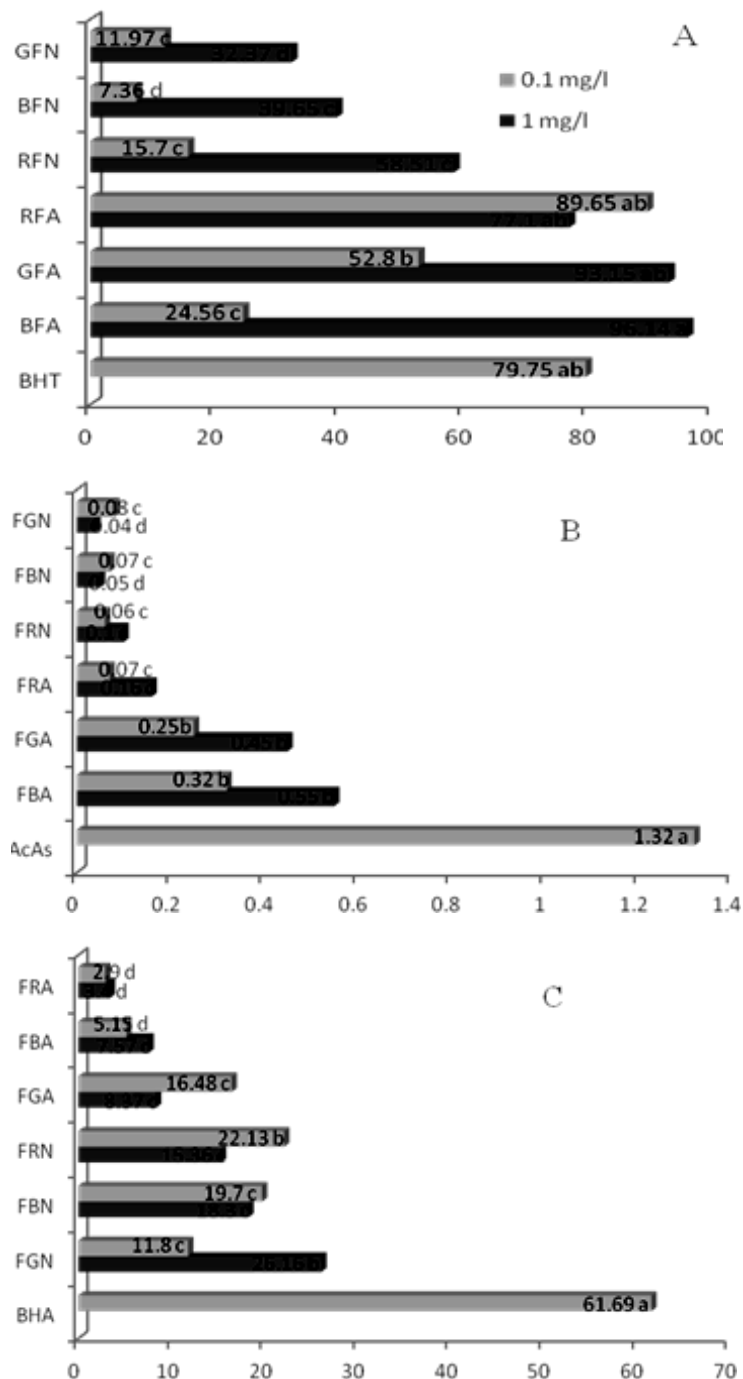


Figure 1. 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (A), reducing power (B) and β -carotene bleaching inhibition capacity (C) in the *Opuntia ficus-indica* fruit and authentic standards (BHT, ascorbic acid and BHA). Means of three replicates followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls post-hoc test.

Total flavonoid content of *Opuntia ficus-indica* extracts

Variability of total flavonoid contents presented similar

trends to that of total polyphenols. Thus, Fruits collected in August had the highest total flavonoids. Total flavonoid values were significantly decreased in the order: GFA ($5.43 \text{ mg CE g}^{-1} \text{ DW}$) > RFA ($3.79 \text{ mg CE g}^{-1} \text{ DW}$) > BFA

Table 2. Antimicrobial activity of *Opuntia ficus-indica* fruit extracts against five human pathogen bacteria, compared to that of positive standard (gentamycin) at 50 mg/ml. Inhibition zone calculated in diameter around the disc (mm \pm SD).

Bacterial strain	Diameter of growth inhibition (mm \pm SD) ^a						
	Gentamycin	RFN	RFA	BFN	BFA	GFN	GFA
<i>Echerchia coli</i> ATCC 35218	22.23 \pm 0.67	11.66 \pm 1.06	7.66 \pm 1.40	11.66 \pm 1.06	10.33 \pm 2.06	6.33 \pm 1.06	6.66 \pm 2.06
<i>Staphylococcus aureus</i> ATCC 25923	18.3 \pm 0.6	9.66 \pm 2.32	10 \pm 1.84	11.66 \pm 1.06	10.33 \pm 1.06	8.00 \pm 1.66	7.33 \pm 2.46
<i>Salmonella thyphi</i>	24.7 \pm 0.60	10 \pm 0.92	9 \pm 0.92	11.66 \pm 1.06	9.66 \pm 1.86	7.33 \pm 1.96	5.33 \pm 1.96
<i>Enterococcus facium</i>	22.95 \pm 0.66	12 \pm 0.92	11 \pm 0.92	11.66 \pm 1.06	8.33 \pm 1.46	7.66 \pm 1.86	7.00 \pm 1.99
<i>Candida albicans</i>	19.85 \pm 0.66	9.33 \pm 2.32	7.33 \pm 1.40	11.66 \pm 1.06	9.66 \pm 1.06	6.66 \pm 1.06	6.66 \pm 1.89

^aData are reported as means \pm SD of three measurement. The diameter of disc was 6 mm. SD, Standard deviation.

RFA, Rossa Fruit of August; **GFA**, Gialla Fruit of August; **BFA**, Bianca Fruit of August; **RFN**, Rossa Fruit of November; **GFN**, Gialla Fruit of November; **BFN**, Bianca Fruit of November.

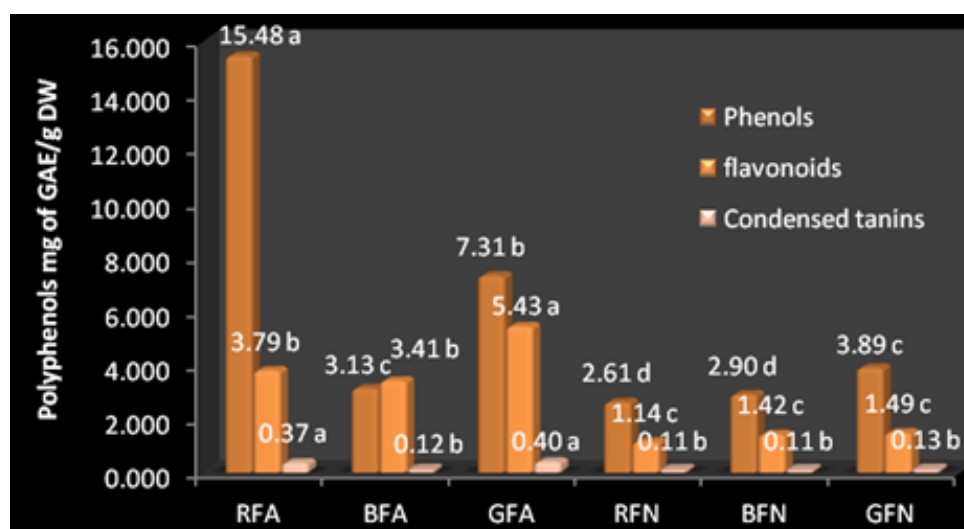


Figure 2. Total polyphenol, flavonoid, and condensed tannin of *Opuntia ficus-indica* cultivars. Means (three replicates) followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls post-hoc test. **RFA**, Rossa Fruit of August; **GFA**, Gialla Fruit of August; **BFA**, Bianca Fruit of August; **RFN**, Rossa Fruit of November; **GFN**, Gialla Fruit of November; **BFN**, Bianca Fruit of November.

(3.41 mg CE g⁻¹ DW) > GFN (1.49 mg CE g⁻¹ DW) > BFN (1.42 mg CE g⁻¹ DW) > RFN (1.14 mg CE g⁻¹ DW).

compared to Bianca fruit collected in August (0.11 mg CE g⁻¹ DW).

Condensed tannins in methanolic extract

Condensed tannin content measured varied significantly, depending on ripening-time and on ecotypes and ranged from 0.40 to 0.11 mg CE g⁻¹ DW. Hence, Gialla and Rossa fruit collected in August displayed the highest values (0.40 and 0.37 mg CE g⁻¹ DW, respectively) as

DISCUSSION

For a plant to be suitable for oil production, it must meet the following two criteria:

1. The oil content must reach the minimum for commercially viable exploitation, and

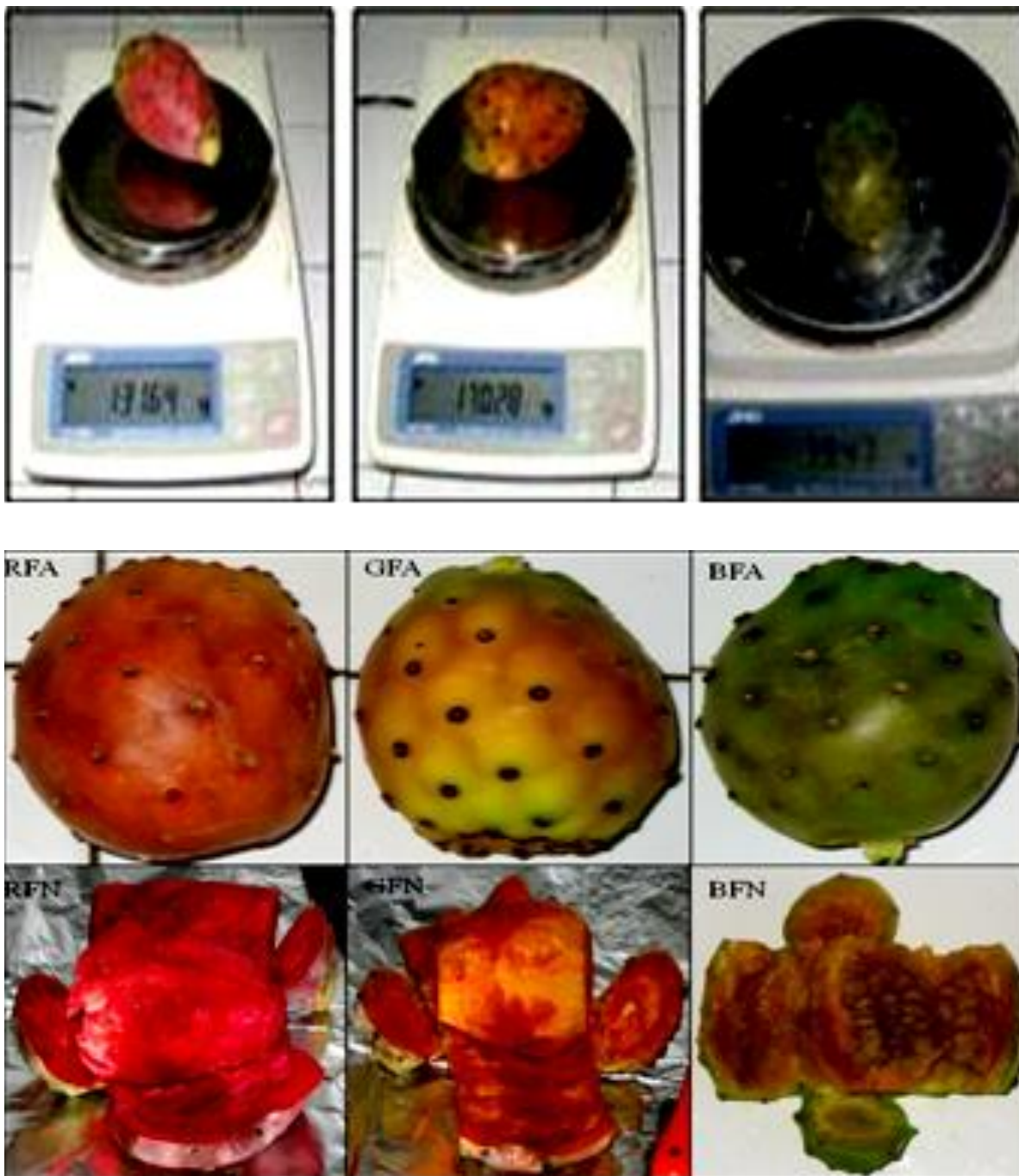


Photo 1. *Opuntia Ficus-Indica* fruits. **RFA**, Rossa Fruit of August; **GFA**, Gialla Fruit of August; **BFA**, Bianca Fruit of August; **RFN**, Rossa Fruit of November; **GFN**, Gialla Fruit of November; **BFN**, Bianca Fruit of November.

2. The plant must be suitable for high acreage cultivation.

The only exceptions are plants that contain oils or fats unique in their composition or with properties that cannot be found elsewhere (Bockisch, 1998). Cactus pear pulp which resembles the edible part of the fruit can be divided into seeds (15%) and strained pulp (85%), the latter being the basis for fruit and juice products. It is found that

seeds contain the maximum amount of oil (98.8 g/kg dry weight) while total lipid, recovered from lyophilised strained pulp, accounted for 8.70 g/kg. It is well known that the mesocarp, or pulp, of fruits generally contains very low levels of lipid materials (0.1-1.0%) and, as such, does not constitute an important source of edible or industrial oils (Kamel and Kakuda, 2000). Amounts of oil recovered from seeds which represent a potential source

of oil, are in agreement with literature data (Pimienta-Barrios, 1994; Sawaya and Kahn, 1982). The levels of total lipids, however, may depend on fruit cultivar, degree of ripeness and fruit processing or storage conditions. Previous data on cactus pear seed oil show rather, a similar pattern in that linoleic acid is the fatty acid marker (Sawaya and Kahn, 1982). Fatty acid profile of seed oil evinces the lipids as a good source of the nutritionally essential linoleic acid and unsaturated oleic acid, wherein the ratio of linoleic acid to oleic acid was about 3:1. In both seed and pulp oils, linoleic acid is the dominating fatty acid, followed by palmitic and oleic acids, respectively (Ramdan, 2003). Based on our results, high percentage of palmitic acid (C16:0) in Rossa fruit oil can be exploited as new source of vegetable oil. Additionally, the high palmitic and linoleic acids content will be advantageous in hypercholesterolemic conditions (Kuti, 1992; 2004).

Additionally, our findings show that *Opuntia* oil may possess comparable nutritional content, as the palm oil, which has been extensively accepted and used as cooking oil in various food applications; due to its superior frying quality and oxidative stability owing to high content of monounsaturated and saturated fatty acids (Saenz et al., 1993). As recommended by the National Cholesterol Education Program/American Heart Association, C16:0 and C18:0 are best saturated fatty acid (SFA) from natural source (Odoux and Dominguez, 1996). It is found that antioxidant molecules such as ascorbic acid, tocopherol, flavonoids, and tannins reduce and decolorize DPPH due to their hydrogen donating ability (Kumaran and Karunakaran, 2007). Phenolic compounds of the *O. ficus-indica* fruit extracts were probably involved in their antiradical activity. Several studies attributed the inhibitory effect of plant extracts against bacterial pathogens to their phenolic composition (Kamath and Rajini, 2007; Espinet et al., 2007). The inhibitory effect of these phenolics could be explained by adsorption to cell membranes, interaction with enzymes, substrate and metal ion deprivation (Nacz and Shahidi, 2006).

Our results suggest that the antibacterial capacity needed, as compared to antioxidant activity, which has a good efficiency with crude extracts of *O. ficus-indica* fruit, more concentration and even purification of phenolic compounds. In fact, numerous studies evocate the analgesic and anti-inflammatory actions of the genus *Opuntia* by using the phytosterols from fruit and stem extract (Parck et al., 1998). Gastric lesions in rat animal studies were reduced both by stem and fruit powder (Lee et al., 2001; 2002). Besides, the ethanol extract of *O. ficus-indica* fruits inhibited the writhing syndrome induced by acetic acid, indicating that, they contain analgesic effect (Parck et al., 1998). Hence, our findings shows that ripening time, climatic conditions and cultivars had significantly and differently affect phenol content of *O. ficus-indica* fruits compared with those harvested in

August in Morocco by Maataoui et al. 2006 (0.2 and 0.28 mg/100 ml for Gialla and purple, respectively). Moreover, total phenolic content of RFA was higher than that reported by Stintzing et al. (2005) of 660 mg/l, by Morales (2007) of 777.4 mg/l and by (Maataoui et al., 2006) of 0.2 and 0.28 mg/100 ml for Gialla and Rossa fruit, respectively. Results depicted also, that summer crop displayed much higher polyphenol content than those collected in November. Probably, this can be attributed to the "scozolattura" technique, which had a positive impact on fruit size other than seems to exert a negative effect on phenol content.

Accordingly, it could be a positive relationship between the intensity of solar radiation and the quantity of phenolics produced by plants (Niknam and Ebrahimzadeh, 2002). A rise in total phenolics was generally found in plants grown in sunny situations relative to shady ones, and such relationship could also be seen at the intra-individual level by comparing plant parts exposed to different amounts of light. It seems that rainfall scarcity and long light exposure may be involved in the activation of phenol biosynthesis (Nacz and Shahidi, 2006). Actually, plant species have inherent physiological differences, as a result of interactions with their environment (Taulavuori et al., 2010). These differences may be reflected by the presence of various chemical compounds that provide information regarding the ecotype conditions (Taulavuori et al., 2010). Moreover, BFA displayed the highest polyphenol content and the lowest antiradical activity; which may probably depend in one hand on phenolic nature rather than quantity and in other hand, on fatty acid composition. Previous results in cactus pear, Galati et al. (2003) analyzed *O. ficus-indica* (L.) Mill. juices (95% yellow and 5% red cultivars) and they identified the presence of major flavonoids, isoharmnetin triglycoside, rutin and kaempferol, as the main water-soluble constituents. Total flavonoid content corresponds to 652.5 µg/ml and ascorbic acid concentration is 26.9 mg/100 ml of juice. Many types of flavonoids have been reported in *Opuntia* sp. and an important role has been proposed for their presence as the main responsible of the antioxidant power of its extracts (Lee and Lim, 2000). Their chemical structure and concentration are very variable and depend on the variety, ripening stages and the kind of tissue of the plants (Wallace, 1986) 3-O-glycosilated flavonols, dihydroflavonols, flavonones and flavonols have been found in Cactaceae plants and fruits (Kuti, 2000).

Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits, such as, red grape (Negro et al., 2003), vegetables (Luo et al., 2002) and medicinal plants (Bourgou et al., 2008) and shows that plant flavonoid levels can be influenced by environmental factors such as light, temperature, and mineral nutrition (Jaakola et al., 2002). Accordingly, flavonoid contents were higher in plants growing in more stressful climatic

conditions (Tunisia) than those coming from more appropriate environment (Texas). In this way, Rodrigues et al. (2011) suggested that the high levels of red onion flavonols are probably related to the high radiation and low rainfall during growing season. Moreover, Germ et al. (2010) showed that the subjection of *Hypericum perforatum* to increasing doses of UV radiation significantly increased its foliar flavonoids (from 6.5 to 9 g 100 g⁻¹ DW). Several authors have reported a positive and significant relationship between the antioxidant components including phenols, polyphenols and tannins, respectively with the reducing power and DPPH radical scavenging capacity (Connan et al., 2006; Huang et al., 2005). It is extremely important to point out that, there was a positive correlation between antioxidant potential and phenolic content estimated by the Folin-Ciocalteu method (Ksouri et al., 2007) in other species. Nevertheless, these antioxidant and antimicrobial activities depend on phenolic nature, structure and synergistic interactions (Djeridane et al., 2006). In fact, phenolics were able to reduce damages induced in the photosynthetic systems by absorbing UV-radiation and they were produced to protect plants from stresses such as, long exposure to dryness and/or solar radiation (Macheix et al., 2005; Wahid and Ghazanfar, 2006). In literature, phenolic compounds are assumed to directly contribute to antioxidative action since their level is strongly correlated with in vitro-measured antioxidant activities (Duh and Yen, 1999; Falleh et al., 2008).

Overall, the literature describes that antioxidant capacities are more variable in plants of different species (inter-specific) than within the same species (intra-specific). Accordingly, previous studies had shown that the purple-skinned cactus pear varieties of *Opuntia lindheimeri* (from Texas) had the highest total flavonoids (93.5 ± 12.4 mg/g of fruit), followed by the green-skinned of *O. ficus-indica* (69.5 ± 3.8 mg/g of fruit), the red-skinned of *Opuntia streptacantha* (54.8 ± 5.1 mg/g of fruit) and the yellows skinned of *Opuntia stricta* var. *stricta* (9.8 ± 3.0 mg/g of fruit), respectively (Kuti, 2004).

Conclusion

The data obtained will be important as an indication of the potentially nutraceutical and economic utility of cactus pear as a new source of fruit oils and functional foods. Moreover, these results provide useful information for the industrial application of *O. ficus-indica* fruits. Thirteen fatty acids were identified in fruit extracts oleic, linoleic and palmitic acids were the major fatty acids. Furthermore, this species displayed a high antiradical and antibacterial activity; and possessed a high phenolic content as compared to other medicinal plants and fruits (e.g. grape or apple) or beverages (tea) well known for their richness in polyphenols. This make *O. ficus-indica* a novel natural source of antioxidants with numerous health benefits. Besides, purified components from fruits may be

used as natural antimicrobials in food systems. This need to be confirmed in future studies.

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Full Length Research Paper

Experimental design applied to the optimization and partial characterization of pectin-methyl-esterase from a newly isolated *Penicillium brasilianum*

Jamile Zeni, Jonáina Gomes, Éllin Ambroszini, Geciane Toniazzo, Débora de Oliveira* and Eunice Valduga

Department of Food Engineering - URI - Campus de Erechim Av. Sete de Setembro,
1621 - Erechim - RS, 99700-000, Brazil.

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The objective of this work was to optimize the medium composition for maximum pectin-methyl-esterase (PME) production from a newly isolated strain of *Penicillium brasilianum* by submerged fermentation. A Plackett-Burman design was first used for the screening of most important factors, followed by a 2³ full experimental design, to maximize the enzyme production. The maximum pectin-methyl-esterase activity was 4.0 U/mL at 24 h of bioproduction using a pectin concentration of 32.0 g/L, yeast extract of 30.0 g/L, potassium phosphate of 8.0 g/L, iron (II) sulfate of 0.02 g/L, at 30°C, stirring rate of 180 rpm and initial pH of 5.5. The kinetic evaluation showed that after 27 h of fermentation, a consumption of 15% of total organic carbon and 10% of nitrogen was observed. The crude enzymatic extract kept about 80% of its initial activity after 1848 h under low temperatures. An increase of PME activity was observed after incubation at high temperatures. The residual activity of the extracts after 1728 h of incubation was about 95% for all tested pH values (5, 7, 9 and 11). The application of 0.5% (v/v) of PME crude extract for clarification of peach juice showed a reduction on the viscosity (7.20%) and turbidity (14.11%).

Key words: *Penicillium brasilianum*, pectin-methyl-esterase, experimental design, pectin-methyl-esterase (PME).

INTRODUCTION

Pectin is a major plant cell wall polysaccharide contributing to tissue integrity and rigidity. The main structural element of pectin, governing most of its functional properties, is a linear homopolymer of α -(1-4) linked D-galacturonic acids, which can be methyl-esterified (Ridley et al., 2001). Changes in the pectin structure take place during fruit ripening, processing and storage, and can be both chemical (for example, alkaline demethoxylation, β -eliminative depolymerization) and enzymatic (Bordenave, 1996; Rexova-Benkova and Markovic, 1976).

Many of the structural polysaccharides in the plant-cell wall undergo enzymatic hydrolysis, transglycosylation reactions, solubilization and depolymerization, which are thought to contribute to wall loosening (Brummell, 2006; Fischer and Bernnett, 1991; Wakabayashi et al., 2001). The disassembly of the cell wall structure occurs through the action of hydrolases including pectinases like polygalacturonase (PG), pectate lyase and pectin methylesterase (PME) (Brummell and Haspster, 2001).

Pectin methylesterase (PME, EC 3.1.1.11) is an enzyme of either plant or microbial origin that catalyses

the demethoxylation of pectin, thus altering the degree and pattern of methyl-esterification and releasing methanol (Voragem et al 2001). PME's are thought to play an important role in determining the extent to which demethylated polygalacturonans are accessible to degradation by PGs (5), by releasing galacturonic acid (exo-PG) or oligogalacturonate (endo-PG). Their action also influences the availability of homogalacturonan carboxylic groups for Ca^{2+} binding. Increasing the number of Ca^{2+} bridges between homogalacturonan chains leads to a decrease in their accessibility to cell wall hydrolases (Micheli, 2001).

In spite of its industrial importance, just a few works are presented in the current literature about the microbial PME's. Acidic microbial PME's (*Aspergillus japonicus*, *Aspergillus niger*, *Aspergillus foetidus*) catalyze a random cleavage of esterified carboxyl groups (Jayani et al., 2005; Ishii et al., 1979) (multiple chain mechanism). Alkaline PME's from higher plants (tomato, orange, alfalfa) and from fungal origin (*Trichoderma reesei*) produce blocks of free carboxyls groups, that is, they catalyze demethoxylation of pectin linearly along the chain (single chain mechanism). Pectinases synthesized by filamentous fungi have some advantages, since the microorganisms have the ability of adaptation to a wide type of substrates, being excellent decomposers of organic material. Moreover, the fungi pectinases present important characteristics for application in bioprocesses, as stability to pH and temperature (Martins et al., 2004).

In this context, the objective of this study was to evaluate the PME production by a newly isolated strain of *Penicillium brasilianum* using submerged fermentation of a conventional media. After the media optimization, a kinetic evaluation was carried out and the enzymatic extract was partially characterized and tested for juice clarification.

MATERIALS AND METHODS

Microorganism

The microorganism used in the present study was previously isolated from tea. Newly isolated microorganism was identified by molecular biology technique, following the methodology described here. Firstly, after incubation in potato dextrose (PD) medium at 28°C for three to four days, we used a protocol for extraction of yeast genomic DNA using liquid nitrogen for cell disruption (Fernandes-Salomão et al., 1996) following DNA quantification using a spectrophotometer model NanoDrop, ND-1000 (NanoDrop Technologies). The newly isolated microorganism was identified as *P. brasilianum*.

The propagation of this culture was done on potato dextrose agar (PDA) slant medium containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L) and agar (20 g/L) and incubated at 30°C until sporulation (1 week). Stock cultures of these strains were prepared with 20 weight% glycerol water and stored at -80°C (MDF - U3086S - Sanyo). The harvesting of the spores from the slants was done using 5 ml of Tween 80-water (0.02%). For spores counting, 1 ml of the suspension, aseptically withdraw, was diluted 10 to 10^3 times in sterile aqueous solution of Tween 80 (0.1 % v/v).

The resulting suspension was transferred to Neubauer chamber for spores counting. To determine the best spore concentration (spores/mL) for the production of PME, a previous study was carried out (data not shown) using different spores concentration: 5.10^3 , 5.10^4 , 5.10^5 , 5.10^6 , 5.10^7 and 5.10^8 . In 100 ml of bioproduction medium (10 g/L pectin, 10 g/L yeast extract, pH 5.5) 1 ml of spores was added, in the desired concentration. The cultivation conditions were 30°C, 180 rpm for 24, 48, 72 and 96 h. Higher PME activity (3.0 U/mL in 24 h) was obtained using a spore concentration of 5.10^6 spores/mL.

Pectin-methyl-esterase bioproduction

A Plackett-Burman experimental design was employed to evaluate the effects of the culture medium and the operational conditions on the bioproduction of pectin-methyl-esterase (PME). The independent variables were: concentrations of pectin (2 to 22 g/L), L-asparagine (0 to 4 g/L), yeast extract (0 to 20 g/L) and magnesium sulfate (0 to 1 g/L). The variables temperature (30°C), agitation (180 rpm), pH (5.5) and cell concentration (5.10^6 spores/mL) were kept fixed in all experiments.

A full 2^3 experimental design was carried out, taking into account the results of the first planning, evaluating the effects of concentrations of pectin (22 to 32 g/L), yeast extract (20 to 30 g/L), potassium phosphate (4 to 8 g/L), keeping fixed the temperature (30°C), agitation (180 rpm), pH (5.5), fermentation time (24 h) and iron sulfate (0.02 g/L).

Kinetic evaluation

The knowledge of the kinetics of a fermentative process is of extreme importance when transposing a laboratory experiment to an industrial scale, as well as when a quantitative comparison between different culture conditions is required.

The kinetics of substrate consumption (total nitrogen, potassium, magnesium and total organic carbon - TOC), cell mass, pH evolution and PME production was evaluated in the experimental condition previously maximized in the experimental design step (32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate and 0.02 g/L iron sulfate, at 180 rpm, 30°C, pH 5.5 and initial cell concentration of 5.10^6 spores/mL) by periodic sampling of the fermentation medium.

Effect of salts and pectin concentration on the measurement of the pectin-methyl-esterase (PME) activity

From the maximized experimental condition defined previously, the effect of NaCl and CaCl_2 concentrations on the measurement of PME activity was evaluated. The salts concentration varied from 0.02 to 1 M, keeping fixed the citric pectin concentration at 1% (wt/v). Keeping the CaCl_2 concentration fixed at 0.5 M, the effect of pectin concentration on the enzyme activity was studied in the range from 0.5 to 2% (wt/v).

Partial characterization of the crude enzymatic extract

The partial characterization of enzymatic extracts is of fundamental importance for establishing the conditions of application. Such characteristics are optima temperature and pH and stability of the extract to the temperature and pH.

To determine the optimum values of temperature and pH in terms of enzyme activity for the PME from *P. brasilianum*, a central composite rotatable design (CCRD) 2^2 was accomplished using the enzymatic extract. The studied range for pH was from 5 to 11 and

the temperature varied from 30 to 80°C. The temperature of stability of the enzymatic extract was determined by enzyme incubation at a fixed pH (5.5) and different temperatures: 30 to 80°C. The stability pH was determined by incubation of the extract obtained by *P. brasiliense* at 40°C at the pH values ranging from 5.0 to 11.0. The samples were withdrawn from the reaction medium at regular time intervals. The stability of the crude enzymatic extract to low temperature was evaluated by the storage of the extract at 4, -20 and -80°C and determining the enzyme activity periodically.

Application of the crude enzymatic extract for the clarification of peach juice

A commercial peach juice (Sarandi, Brazil) was treated by the crude enzymatic extract obtained at the maximized conditions of bioproduction, defined previously. Enzyme concentrations of 0.01, 0.05, 0.1 and 0.5% (v/v) at 40°C, 100 rpm and 60 min were tested by evaluation of the viscosity, turbidity and percent of clarification of the juice.

Analytical determinations

Pectin-methyl-esterase activity

For all determination of PME activity, the bioproduction medium was filtered to separate the biomass. The filtrate was denominated by crude enzymatic extract: the activity of the pectin-methyl-esterase (PME) was determined following the methodology described by Hultin et al. (1966), with some modifications. 1 ml of enzymatic extract was added to 30 ml of citric pectin and 1ml of NaCl 0.2 M. The pH of the solution was adjusted to 7.0 for 10 min using NaOH 0.01 N. One unit of PME was defined as the amount of enzyme able to catalyze the demethylation of pectin corresponding to the consumption of 1 μ mol of NaOH/min/mL, under the assay conditions.

pH

The pH was monitored using a potentiometer (DMPH-2, Digimed), after calibration with standard solutions pH 4.0 and 7.0.

Total organic carbon (TOC)

The total organic carbon (TOC) content was determined by the method of oxidation by catalytic combustion at 680°C and detection by infrared, in equipment Shimadzu model TOC-VCSH (AOAC, 1995).

Mineral compounds

Macro magnesium (Mg) and manganese (Mn) and micronutrients iron (Fe) and potassium (K) were determined by flame atomic absorption spectrometry - FAAS (Varian Spectra AA-55), following the methodology described by Association of Analytical Communities (AOAC, 1995). Hollow cathode lamps of Mg and Fe were used as radiation source. The elements were measured in optimized operation conditions by FAAS in flame of air/acetylene nitrous oxide/acetylene. The readings of Mg, Mn, Fe and K were performed by FAAS, in absorption mode. To eliminate possible interferences in the determination of Mg content, lanthanum chloride was added to the samples and to the standard solutions at a proportion of 1% (wt/v). For the determination of the minerals contents on the samples, calibration curves of standard solutions

were used. The nitrogen content was determined by the Kjeldahl method (VELP DK-20 e UDK-126D), following the methodology of AOAC (1995).

Viscosity

The reduction of the viscosity of the peach juice after the enzymatic treatment using the crude extract was evaluated in viscometer "Falling Ball" (Ustok et al., 2007).

Clarification

The clarification of the peach juice after enzymatic treatment with the crude extract was determined based on the color intensity (CI), by readings in spectrophotometer at 440 nm and 520 nm, with optical path of 1 mm (Silva et al., 2005), expressed as % of clarification, calculated taking into account the color intensity of control juice (without enzymatic treatment) and the enzyme-treated juice.

Turbidity

The reduction of turbidity of the juice after enzymatic treatment using the crude extract was calculated based on the absorbance, measured at wave length of 540 nm, using optical path of 1 mm against water, in spectrophotometer (Silva et al., 2005), expressed as % of reduction of turbidity, calculated based on the absorbance of the control juice.

Statistical analysis

The statistical analysis related to the estimated effects of each variable and process optimization was performed using the global error and the relative standard deviation between the experimental and predicted data. The other results were treated by analysis of variance followed by Tukey's test. All analysis was performed using the software Statistica version 6.0 (Statsoft Inc, USA).

RESULTS AND DISCUSSION

Pectin-methyl-esterase bioproduction

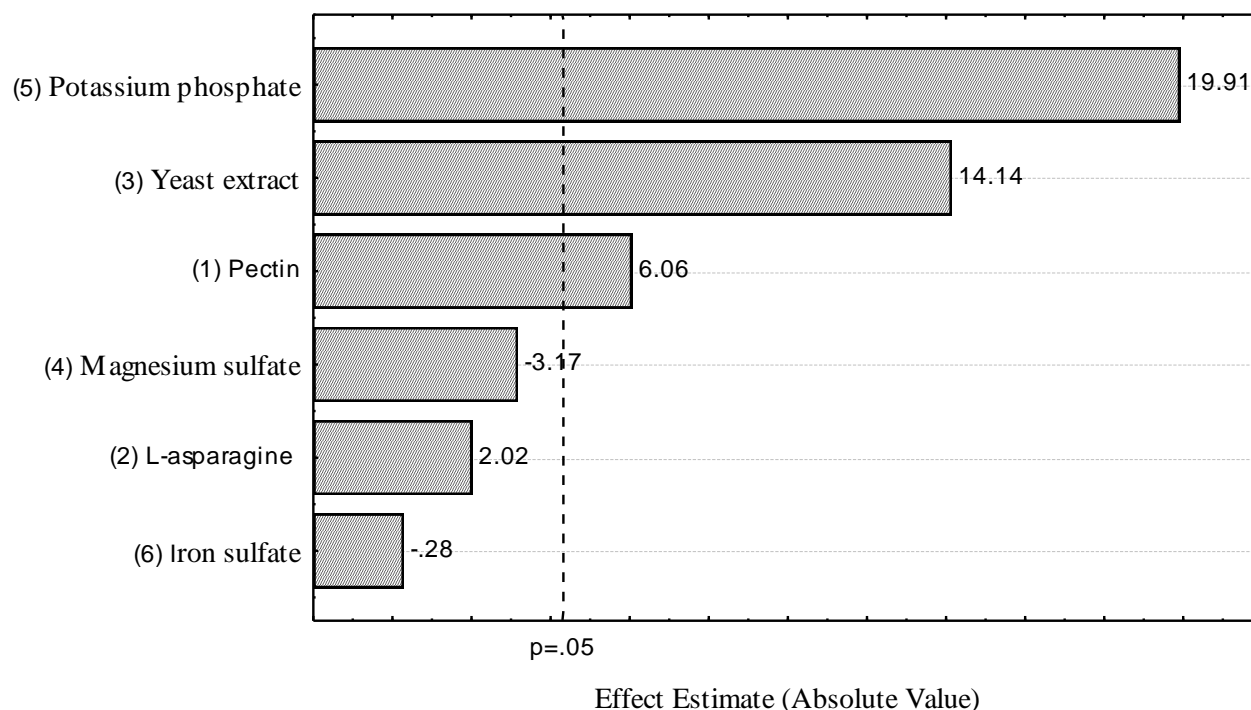
Table 1 presents the matrix of the Plackett-Burman experimental design with the real and coded values of independent variables and the response in terms of pectin-methyl-esterase (PME) activity. From this table, one can verify that the maximum PME activity (2.50 U/mL) was obtained in the experiment 6, corresponding to the highest levels of pectin (22 g/L), yeast extract (20 g/L), L-asparagine (4 g/L), potassium phosphate (4 g/L) and iron II sulfate (0.02 g/L) concentrations.

The data presented in Table 1 were statistically treated and Figure 1 presents the Pareto chart with the estimated values of independent variables. The variables potassium phosphate, yeast extract and pectin concentrations presented a positive significant effect ($p < 0.05$), demonstrating that the displacement of these variables to upper levels could lead to an increase of enzyme activity. On the other side, the variables magnesium sulfate, L-

Table 1. Matrix of the Plackett-Burman experimental design with the coded and real values of independent variables and the response as pectin-methyl-esterase (PME) activity and pH.

Assay	Independent variable						Response	
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	PME (U/mL)	pH
1	1 (22)	-1 (0)	1 (20)	-1 (0)	-1 (0)	-1 (0)	1.00	5.15
2	1 (22)	1 (4)	-1 (0)	1 (1)	-1 (0)	-1 (0)	0.40	4.67
3	-1 (2)	1 (4)	1 (20)	-1 (0)	1 (4)	-1 (0)	2.00	5.65
4	1 (22)	-1 (0)	1 (20)	1 (1)	-1 (0)	1 (0.02)	1.00	5.38
5	1 (22)	1 (4)	-1 (0)	1 (1)	1 (4)	-1 (0)	1.30	4.56
6	1 (22)	1 (4)	1 (20)	-1 (0)	1 (4)	1 (0.02)	2.50	5.68
7	-1 (2)	1 (4)	1 (20)	1 (1)	-1 (0)	1 (0.02)	0.60	5.37
8	-1 (2)	-1 (0)	1 (20)	1 (1)	1 (4)	-1 (0)	2.00	5.50
9	-1 (2)	-1 (0)	-1 (0)	1 (1)	1 (4)	1 (0.02)	0.80	5.20
10	1 (22)	-1 (0)	-1 (0)	-1 (0)	1 (4)	1 (0.02)	1.50	4.44
11	-1 (2)	1 (4)	-1 (0)	-1 (0)	-1 (0)	1 (0.02)	0.20	4.56
12	-1 (2)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	0.00	4.92
13	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,20	5.14
14	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,10	5.17
15	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,00	5.11

X₁= Pectin (g/L); X₂= L-asparagine (g/L); X₃= yeast extract (g/L); X₄= magnesium sulfate (g/L); X₅= potassium sulfate (g/L). X₆ = iron II sulfate (g/L); Fixed variables: 180 rpm, 30°C, 24 h, pH_{initial} 5.5 and initial cell concentration of 5.10⁶ spores/mL.

**Figure 1.** Pareto chart with the estimated effect (absolute value) of the studied variables on the Plackett-Burman experimental design for pectin-methyl-esterase (PME) production.

asparagine and iron (II) sulfate concentrations did not influence significantly ($p < 0.05$) the enzyme production,

within the studied ranges. Based on this information, magnesium sulfate and L-asparagine were not added to

Table 2. Matrix of the 2³ factorial design and response as pectin-methyl-esterase (PME) activity and pH.

Assay	Independent variable			Response	
	X ₁	X ₃	X ₅	PME (U/mL)	pH
1	-1 (22)	-1 (20)	-1 (4)	1.70	5.20
2	1 (32)	-1 (20)	-1 (4)	2.10	4.92
3	-1 (22)	1 (30)	-1 (4)	2.50	5.16
4	1 (32)	1 (30)	-1 (4)	2.40	5.02
5	-1 (22)	-1 (20)	1 (8)	3.10	5.07
6	1 (32)	-1 (20)	1 (8)	3.50	4.96
7	-1 (22)	1 (30)	1 (8)	3.50	5.14
8	1 (32)	1 (30)	1 (8)	4.00	5.00
9	0 (27)	0 (25)	0 (6)	3.20	5.01
10	0 (27)	0 (25)	0 (6)	3.00	5.05
11	0 (27)	0 (25)	0 (6)	3.20	5.10

*X₁= Pectin (g/L), X₃= yeast extract (g/L), X₅= potassium phosphate (g/L). Fixed: iron (II) sulfate 0.02 g/L, 180rpm, 30°C, 24 h and pH_{initial}=5.5.

the production medium in the second experimental design. The iron (II) sulfate was fixed at 0.02 g/L, based on the highest PME activity achieved in experiment 6 of Table 1. A 2³ full experimental design was carried out in order to maximize the enzyme production. Results obtained in this step are presented in Table 2.

From Table 2, one can verify that the maximum PME activity (4.0 U/mL) was obtained in experiment 8 (32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate, 0.02 g/L iron (II) sulfate) at 180 rpm, 30°C, 24 h of bioproduction, initial pH of 5.5 and initial cell concentration of 5.10⁶ spores/ml. The variables potassium phosphate and yeast extract presented a positive significant effect ($p < 0.05$) on the enzyme production and the pectin concentration influenced the pH reduction.

Equation 1 presents the first order coded model as a function of pectin, yeast extract and potassium phosphate concentrations. The model was validated by analysis of variance, with a correlation coefficient of 0.95 and a calculated F 7.48 times higher than the tabled one. The non significant parameters were added to the lack of fit for the analysis of variance (ANOVA) test.

$$\text{PME} = 2.93 + 0.25 X_3 + 0.67 X_5 \quad (1)$$

Where, PME = pectin-methyl-esterase activity (U/mL); X₃ = yeast extract (g/L); X₅= potassium phosphate (g/L).

Kinetic evaluation

The kinetic evaluation of the PME bioproduction, substrate consumption, biomass and pH was carried out taking into account the experimental conditions maximized (32 g/L pectin, 30 g/L yeast extract, 8 g/L potas-

sium phosphate, 0.02 g/L iron (II) sulfate, 5.10⁶ spores/mL, 180 rpm and initial pH 5.5) in the experimental design step performed in flasks (Figure 2).

From Figure 2a, one can observe that the maximum activity (4.80 U/mL) was reached after 27 h of bioproduction. The highest PME activity can be related to the beginning of the exponential phase for microorganism growth. The pH value was stable (approximately 5.5) during the first 36 h of bioproduction, with posterior decrease, reaching, after 48 h, values of about 4.5.

The analysis of the substrates assimilation permit us to observe a similar behavior compared to the nitrogen (Figure 2a), iron and potassium (Figure 2b), showing a slow and gradual decrease during the bioproduction, with a consumption of 13, 13 and 11%, respectively. From the mineral sources, the more expressive assimilation was observed for manganese and magnesium, corresponding to 26 and 82%, respectively.

The concentration of total organic carbon was practically constant until 24 and at 27 h period of maximum PME bioproduction (4.80 U/mL); it had the highest consumption of the carbon source (15%). After this period, the carbon concentration was constant (222 to 210 mg/L).

Studies about the production of PME by microorganisms are scarce in the current literature. The most cited works are referred to the enzymatic extract from papaya (Chatterjee et al., 2004; Lourenço and Catutani, 1984), apple (Johnston et al., 2002), peach (Oliveira et al., 2005), raspberry (Lannetta et al., 1999), carambola (Chin et al., 1999), pear (Brummell and Haspster, 2004), tomato (Hobson, 1963; Resende et al., 2004) banana (Hiltin and Levine, 1966; Sales et al., 2004) and mango (Prasanna et al., 2003). Here, it is important to mention that the PME activity obtained by

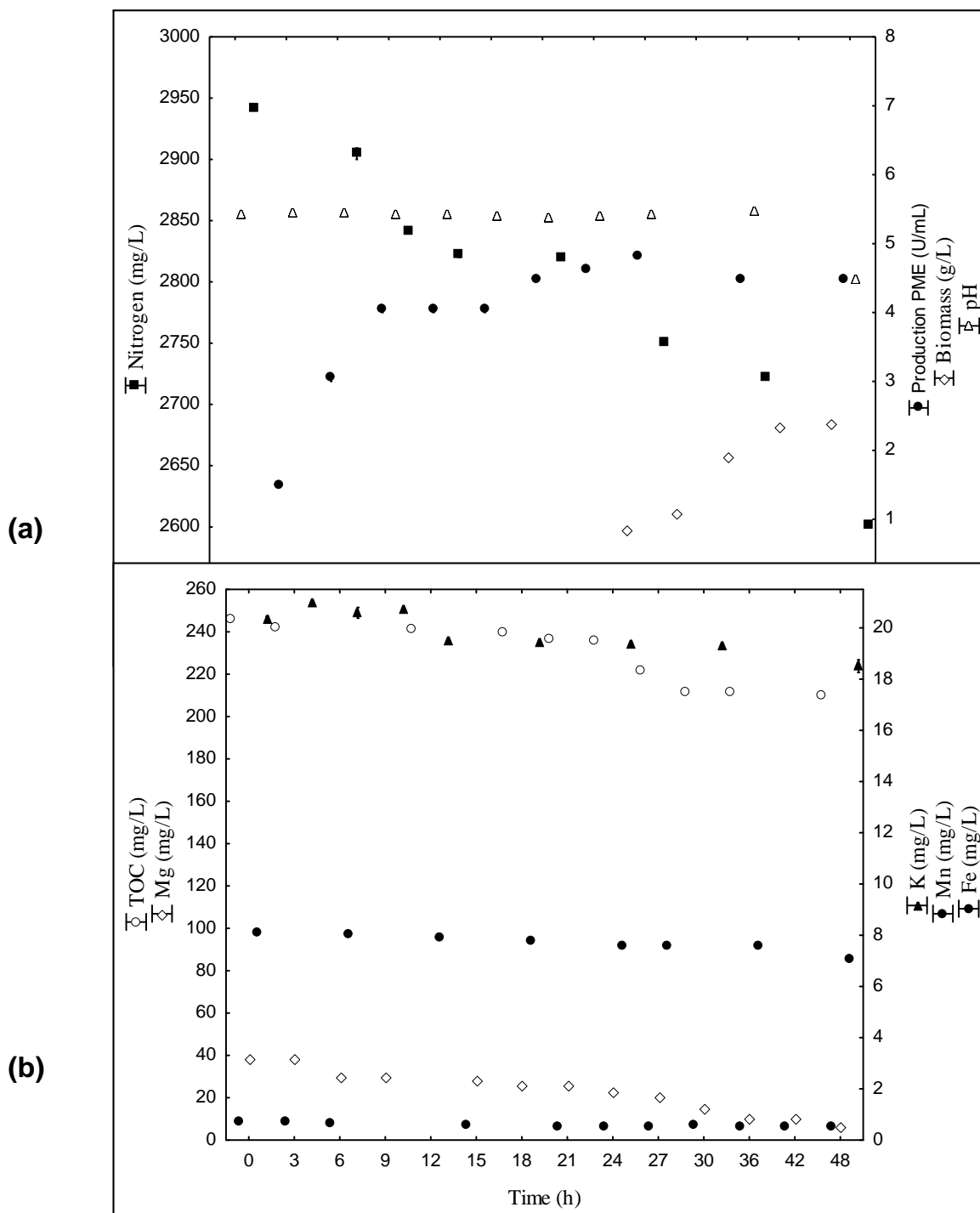


Figure 2. Kinetic curves for bioproduction of pectin-methyl-esterase (PME) as a function of enzyme activity (U/mL), biomass production (g/L), nitrogen and pH (a) and carbon consumption and minerals content (b).

the filamentous fungi *P. brasillianum* (4.80U/mL) was similar to that cited by Mantovani et al. (2005) for a commercial PME extract (Pectinex AR), which presented activity of 3.58 U/mL and higher than that obtained from industrial residues of pineapple pump (*Ananas comosus*

L. Merr) (1.47 U/mL) (Kapoor et al., 2001).

Table 3 presents the PME activity (U/mL) in different concentrations of NaCl, CaCl₂ and mixture of NaCl/CaCl₂ for the measurement of the enzyme activity. From this table, one can observe that when CaCl₂ and 1.0% of citric

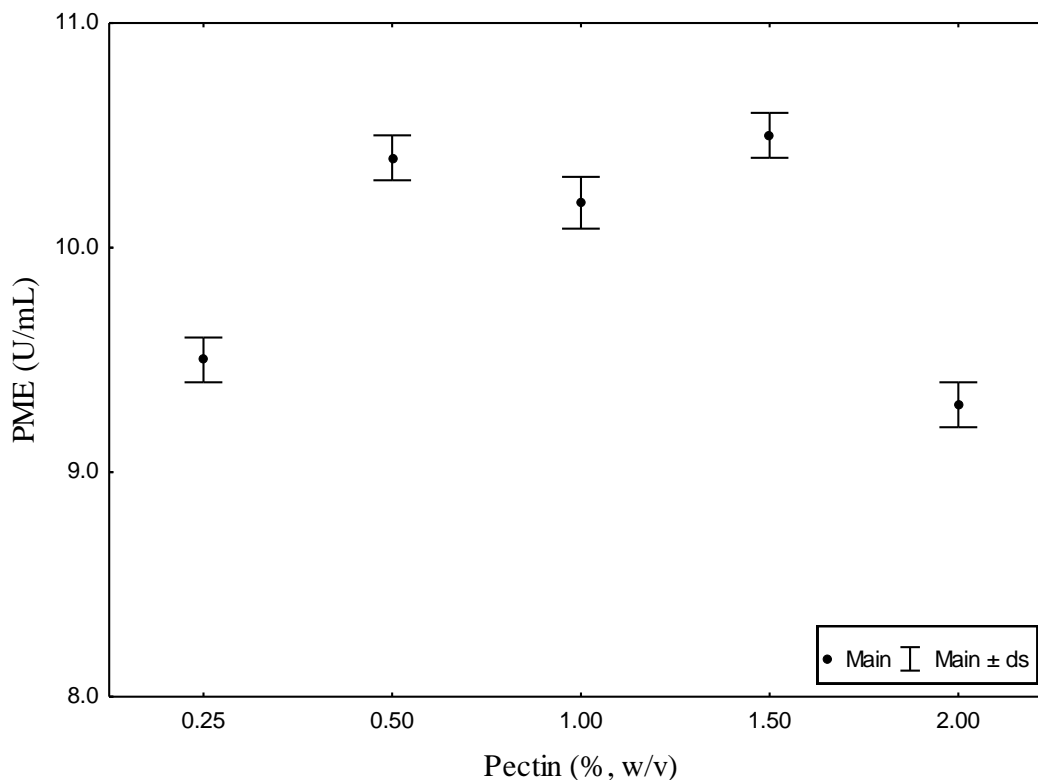


Figure 3. Pectin-methyl-esterase (PME) activity (U/mL) using different concentrations of citric pectin.

Table 3. Pectin-methyl-esterase (PME) activity (U/mL) in different concentrations of NaCl, CaCl₂ and mixture of NaCl/CaCl₂.

Salt concentration (M)	PME activity (U/mL)		
	NaCl	CaCl ₂	NaCl/CaCl ₂
0.02	5.10 ^{aA} (± 0.14)	4.87 ^{eA} (± 0.25)	3.30 ^{iB} (± 0.20)
0.04	5.25 ^{Aa} (± 0.16)	5.43 ^{eA} (± 0.12)	5.00 ^{hA} (± 0.28)
0.06	5.55 ^{aB} (± 0.29)	6.63 ^{dAB} (± 0.32)	7.27 ^{fA} (± 0.29)
0.08	5.90 ^{aB} (± 0.15)	6.70 ^{dA} (± 0.20)	6.9 ^{fA} (± 0.10)
0.1	5.05 ^{aC} (± 0.07)	7.27 ^{cdB} (± 0.23)	8.15 ^{eA} (± 0.07)
0.15	5.70 ^{aB} (± 0.30)	8.47 ^{cA} (± 0.25)	8.55 ^{deA} (± 0.35)
0.2	5.27 ^{aB} (± 0.35)	8.63 ^{bA} (± 0.23)	8.80 ^{dA} (± 0.10)
0.25	5.75 ^{aB} (± 0.35)	8.33 ^{cA} (± 0.21)	6.93 ^{fB} (± 0.32)
0.3	5.25 ^{aC} (± 0.07)	7.80 ^{cA} (± 0.30)	7.17 ^{fB} (± 0.31)
0.4	4.73 ^{aC} (± 0.15)	9.00 ^{bB} (± 0.15)	9.97 ^{bA} (± 0.15)
0.5	5.20 ^{aC} (± 0.12)	10.17 ± 0.25 ^{aA}	9.33 ± 0.15 ^{cB}
0.75	5.27 ± 0.35 ^{aB}	10.13 ± 0.38 ^{aA}	10.70 ± 0.10 ^{abA}
1	5.57 ± 0.45 ^{aB}	11.23 ± 0.23 ^{aA}	11.20 ± 0.20 ^{aA}

*PME activity (U/mL) ± standard deviation followed by equal lowercase/uppercase letters on the columns/lines indicates no significant difference at a confidence level of 5%.

pectin was used, the highest PME activities were obtained: 10.17, 10.13 and 11.23 U/mL, at salt concentration of 0.50, 0.75 and 1 M, respectively; these did not

differ statistically ($p < 0.05$) from each other. This study was performed because the ions Na⁺ and Ca⁺² on the reaction medium can interact with the substrate (pectin)

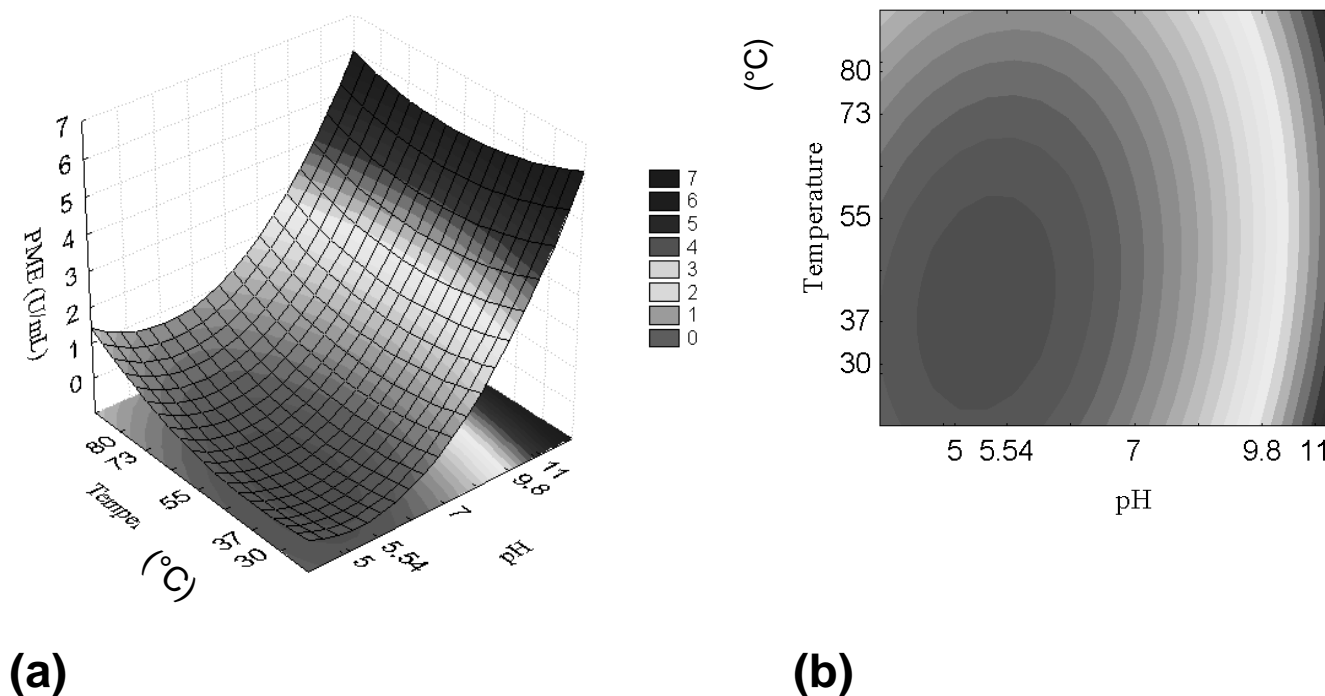


Figure 4. Response surface (a) and contour curve (b) for pectin-methyl-esterase (PME) activity (U/mL) as a function of temperature (°C) and pH.

catalyzing the removal of methoxyl groups. In this case, the PME activity is higher when the pectin is methylated.

Figure 3 presents the PME activity using different concentrations of citric pectin. From this figure, it can be observed that the pectin concentrations of 0.5, 1.0 and 1.5% did not present significant difference (confidence level of 95%) among them, but differed from those that used 0.25 and 2% of pectin for the measurement of enzyme activity.

Cardello and Lourenço (1992) evaluated the use of NaCl and CaCl₂ and 0.125% of pectin in the reaction medium and verified that the best results were obtained using NaCl and CaCl₂ concentrations of 0.15 and 0.04 M, respectively, reaching activities of about 6 U/ml for both salts. When the same authors evaluated the effect of salts association (NaCl and CaCl₂) and 0.5% of citric pectin, varying the molarity, they verified that the activity was about 9U/mL, for all tested molarities (0.04 to 0.5 M).

Partial characterization of pectin-methyl-esterase (PME) crude extract

Optimum temperature and pH

Table 4 presents the matrix of the 2² full experimental design with the response as PME activity as a function of temperature and pH. The highest PME activity (40 U/mL) was obtained in experiment 6, at 55°C and pH 11. Similar

results were observed in experiments 2 and 4, showing a range of optimum temperature (37 to 73°C) and pH (9 to 11).

Equation 2 presents the second order coded model for the PME activity as a function of temperature and pH, within the evaluated range. It is possible to observe that the variables influenced the enzyme activity. The model was validated by analysis of variance, with a correlation coefficient of 0.98 and a calculated F 4.9 times higher than the tabled one, making possible to build the response surface and contour curve, presented in Figure 4. The non significant factors were added to the lack of fit by analysis of variance (ANOVA).

$$\text{PME (U/mL)} = 11.744 + 13.754.\text{pH} + 6.314.(\text{pH})^2 + 1.537.T + 1.761.(T)^2 - 1.150.(\text{pH}).(T) \quad (2)$$

Where, PME = pectin-methyl-esterase activity (U/mL); T = temperature (°C).

From Figure 4, it is possible to verify a wide range of temperature (30 to 80°C) and pH (9 to 11) that lead to higher PME activities. The literature points out the influence of pH and temperature on the measurement of the PME activity, mainly for pectinases extracted from fruits. Delgado et al., (1992) determined the optimal conditions for measurement of the activity of commercial PME (Pectinex 100 L Plus, Panzym Univers and Panzym

Table 4. Matrix of the full 2² experimental design with real and coded values and the response as pectin-methyl-esterase (PME) activity as a function of temperature and pH.

Assay	*Independent variable		PME activity (U/mL)
	pH	Temperature (°C)	
1	-1 (5.54)	-1 (37)	5.00
2	1 (9.8)	-1 (37)	36.00
3	-1 (5.54)	1 (73)	11.60
4	1 (9.8)	1 (73)	38.00
5	-1.41 (5)	0 (55)	2.90
6	1.41 (11)	0 (55)	40.00
7	0 (7)	-1.41 (30)	11.10
8	0 (7)	1.41 (80)	13.70
9	0 (7)	0 (55)	11.60
10	0 (7)	0 (55)	11.80
11	0 (7)	0 (55)	11.90

Clears) and observed that higher activities were obtained in pH values from 4.0 to 4.5 and temperature of 45°C. Assis et al. (2002) related higher activities for PME from acerola (*Malpighia glabra* L.) at pH of 9.0. Researches using other fruits as PME source presented higher activities at lower values of pH, as 8.0 for pectin-methyl-esterases from orange juice (Amaral et al., 2005), or 7.5 (Termote and Ilnik, 1977). Ly-Nguyen et al. (2002) found the optimal pH as 7.0 using crude PME extracts from strawberries. Targano et al. (1994), evaluating the activity of pectin-methyl-esterase (PME) and polygalacturonase (PG) of peaches, verified that the PG showed maximum activity at pH values around 5.0, and the PME showed higher activities at pH 7.0. The optimal temperature for both enzymes was 25°C.

Arbaisah et al. (1997) studied the purification of PME extracted from pulp of graviola (*Anona muricata*) and determined that the optimal range of pH was 7.0 to 8.0. Amaral et al. (2005) evaluated the effect of temperature on the activity of purified PME from orange and determined the optimal temperature as 50°C.

Thermal stability

The crude PME extract was stored at -80, -20 and 4°C and the enzyme activity was measured periodically. The extract kept 80% of its initial activity after storage at all three temperatures for, at least, 1848 h.

The stability of PME extract at high temperatures showed a differenced behavior. An increase of 15, 25, 10, 10 and 50% was observed for PME activity at 30, 40, 50, 60 and 70°C, after 1848 h, respectively. The PME activity at 100°C presented a decrease of 25% compared to its original value.

Results obtained here are similar to those of Kapoor et al., (2001) which evaluated the thermal stability of concentrated extract of PME from pineapple and verified

an increase on the activity of 45.9% at 25°C and 100% after incubation at 40°C, both during 60 min. Leite et al. (2006) evaluated the thermal stability of concentrated PME extract from guava cv. Paluma and verified that samples submitted to 75°C presented an enhancement on enzyme activity of 64.77% compared to the initial activity.

Stability to pH

The stability of the crude PME was evaluated by incubation of the extract at different pH values (5, 7, 9 and 11) and monitoring the activity with time. The residual activity of the extracts after 1728 h of incubation was about 95%, for all tested pH values.

Cardello and Lourenço (1992) evaluated the stability of the partially purified PME extract from eggplant by incubation during 60 min and verified that the extract was stable in pH values in the range of 5 from 8.

Preliminary application of crude pectin-methyl-esterase (PME) extract to the clarification of peach juice

Table 5 presents the reduction of viscosity, turbidity, pH and soluble solids of the peach juice treated with pectin-methyl-esterase. From this table one can observe that the maximum reduction was 7.20 and 14.11% on the viscosity and turbidity, respectively, using an enzyme concentration of 0.5% (v/v) at 40°C, 100 rpm during 60 min. The pH of the juice was 3.69.

Results obtained here can be explained since pectinases hydrolyze pectin, leading to the flocculation of the complex pectin-protein (Lee et al., 2006) resulting in juice with reduced content of macromolecules and low turbidity (Sandri et al., 2011). The enzymatic treatment

Table 5. Reduction of viscosity, turbidity, pH and soluble solids of the peach juice treated with pectin-methyl-esterase.

Parameter	Enzymatic treatment**			
	0.01 % (v/v)	0.05 % (v/v)	0.1 % (v/v)	0.5 % (v/v)
Reduction of viscosity (%)	5.65 ^c (± 0.74)	4.42 ^d (± 0.20)	5.93 ^b (± 0.01)	7.20 ^a (± 0.20)
Reduction of turbidity (%)	0.08 ^d (± 0.14)	4.85 ^c (± 0.53)	11.96 ^b (± 0.08)	14.11 ^a (± 0.28)
pH				
Control*	3.67 ^a (± 0.02)	3.67 ^a (± 0.02)	3.69 ^a (± 0.01)	3.68 ^a (± 0.02)
Treated	3.66 ^a (± 0.03)	3.68 ^a (± 0.01)	3.68 ^a (± 0.01)	3.69 ^a (± 0.01)
Soluble solids (°Brix)				
Control*	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)
Treated	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)	13.00 ^a (± 0.01)

*juice without enzymatic treatment; ** Mean ±standard deviation followed by equal letters in the lines indicates no significant difference at a confidence level of 5%.

for degradation of pectin could cause reduction on the ability to retain water, reducing the viscosity of the juice by the liberation of water to the system (Sandri et al., 2011). The pectin, in aqueous solution, can make the environment more viscous, even at low concentrations, depending on the degree of methoxylation (Lofgren and Hermansson, 2007).

The low efficacy of the PME extract on the reduction of turbidity and viscosity can be associated to the fact that the extract also presents pectin-lyase activity (2.96 U/mL) and possibly other non-measured activities. Clemente and Pastore, (1998) and Vámos-Vigyázó (1981) used a commercial enzyme (Pectinex), with PME and cellulase activities, and verified that the use of this enzyme improved performance of clarification process for peach juice, compared to the employment of isolated enzymes.

The enzymatic treatment and the time of processing (60 minutes) did not influence the values of pH and soluble solids. Similar values compared to the *in natura* juice were observed. This behavior was also verified by Ushikubo et al. (2007), Cao et al. (1992) and Barros et al. (2003), in the clarification process of different fruit juices. Santin et al. (2008) evaluated the viscosity of peach juice using a commercial pectinase, Pectinex AFPL-3 (Novozymes), at 45°C, 30 min of processing and enzyme concentration of 200 ppm. A reduction on viscosity of about 29% was observed.

Taking into account the results obtained for the clarification of peach juice evaluated here and also the partial characterization of crude PME extract, one can suggest other promising industrial applications for the extract, especially those that occur at high temperatures and pH near to the neutrality, such as production of processed fruit and/or lettuce food (purees, pulps, ketchup and pet food). Kashyap et al. (2001) showed that peels, seed residues and pulp ejected by the citrus fruit extractor can be used to make dried animal feed and the mixture is lime treated to increase the pH to 8.0 to take advantage of the citrus PME. Papaya puree is prepared

by conversion of papaya-fresh into a semi-liquid product. As the puree is viscous even if the fruit contains endogenous PME and PG, it is necessary to depectinize it before concentration.

Conclusions

The experimental condition that conducted to the maximal production of PME (4.8 U/mL) in shaken flasks was: 32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate and 0.02 g/L iron sulfate, 5.10⁶ spores/mL, 30°C, 27 h of fermentation, initial pH 5.5 and 180 rpm. The addition of CaCl₂ (0.5 M) and citric pectin (0.5%) led to an increase of 2.3 times in the PME activity.

The partial characterization of the crude PME extract indicated that higher activities (36 to 40 U/mL) were obtained at pH range from 9 to 11 in a wide interval of temperature (30 to 80°C). The extract kept about 80% of its activity after storage at 4°C during 1848 h. An increase on the PME activity was observed at high temperatures. The extract was stable during 1728 h at different pH values (5, 7, 9 and 11). The application of 0.5% (v/v) of PME crude extract for clarification of peach juice showed a reduction on the viscosity of 7.20% and turbidity of 14.11%.

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Full Length Research Paper

Purification and characterization of protease from *Bacillus cereus* SU12 isolated from oyster *Saccostrea cucullata*

S. Umayaparvathi*, S. Meenakshi, M. Arumugam and T. Balasubramanian

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai - 608 502, Tamil Nadu, India.

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Protease-producing bacterium *Bacillus cereus* SU12 was isolated from oyster *Saccostrea cucullata*. Fifteen strains of bacteria were isolated from oyster *S. cucullata* and screened for secretion of protease on casein agar plates. Among them, SU12 isolate was selected due to its high enzyme production capacity and was identified as *B. cereus* SU12 on the basis of its morphological, biochemical and 16S rDNA properties. Media and cultivation conditions were studied to optimize bacterial growth and protease production which includes different carbon and nitrogen sources, in addition to different factors such as incubation time, pH, temperature, NaCl concentrations. At pH 7, temperature 40°C and 2.5% NaCl concentration, carbon source such as starch and beef extract as nitrogen source, the protease activity was maximum. Extracellular protease was isolated, purified to 8.73 fold by diethyl aminoethyl (DEAE) ion-exchange chromatography and its specific activity was determined to be 886.56 U/mg and the sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) showed a single band for the purified enzyme, with an apparent molecular weight of 66 kDa. These findings suggest that the scope for the use of *B. cereus* SU12 strain as suited organism for the industrial production of the extracellular protease enzyme.

Key words: Protease, *Bacillus cereus* SU12, Oyster, diethyl aminoethyl (DEAE) ion-exchange chromatography, optimization.

INTRODUCTION

With recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts have been directed towards the selection of microorganisms producing enzymes with new physiological properties and tolerance to extreme conditions used in industrial processes such as temperature, salts, pH among others. More than 75% of industrial enzymes are observed as hydrolases (Leiola

et al., 2001). Proteases constitute one of the most important groups of hydrolytic enzymes which act upon native proteins to disintegrate them into small peptides and amino acids (Nascimento and Martins, 2004).

Marine microorganisms have recently emerged as rich sources for the isolation of industrial enzymes (Adinarayana and Ellaiah, 2002). Researchers throughout the world target marine niches for enzymes as they

*Corresponding author. E-mail: shanuma1987@gmail.com. Tel: 8903673229.

Abbreviations: PCR, Polymerase chain reaction; BLAST, Basic Local Alignment Search Tool; NCBI, National Centre for Biotechnological Information; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

display novel characteristics such as tolerance to extreme pH, temperature, salinity among others. In marine habitats, there are relatively high proportions of proteolytic bacteria as compared to freshwater or soil habitats (Atlas and Bartha, 1981). Marine microbes are now being looked upon as a potential source of various compounds: pharmaceutical, nutritional supplements, agrochemicals, cosmetics and enzymes (Vignesh et al., 2011; Baharum et al., 2010). The basic characteristics of the enzymes derived from the marine sources differ from the terrestrial sources due to their natural habitat. Marine microbial enzymes are reported to be more stable and active than those originating from plant and animal sources since they possess almost all characteristics desired for their biotechnological applications (Bull et al., 2000).

Proteases are the most important industrial enzymes accounting for about 50% of the total industrial enzymes (Rao et al., 1998). They are the class of enzymes which occupy a pivotal position due to their wide-spread application in detergent, pharmaceutical, photography, leather, laundry, food and agricultural industries. These enzymes are also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, and bioremediation process and as treatment against inflammation and virulent wounds (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Microbial enzymes find a broad spectrum of application due to their broad biochemical diversity, ease of mass culture that is, rapid growth in limited space and genetic manipulation (Adsul et al., 2007; Das and Prasad, 2010). Among the various proteases, bacterial proteases are most significant compared to animal and fungal proteases. Amongst bacteria, *Bacillus* species are specialized producers of extracellular proteases. Most of the commercial alkaline proteases were isolated from *Bacillus* species (Priest, 1977).

Bacillus cereus is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of protease enzyme. Bioactive potential of bivalve species was rarely studied. Oysters are edible sea food. It is abundant resource in marine environment. However, there is no study on isolation of microbial enzyme from oyster. Therefore, the present study was focused on the isolation, identification and characterization of protease producing bacteria from oyster *Saccostrea cucullata*. We also report here the purification and characterization of protease from *B. cereus* SU12 isolated from gut of oyster *S. cucullata*.

MATERIALS AND METHODS

Sample collection

Oysters (*S. cucullata*) were collected from the Mandapam coast (Lat. 09° 17.417'N; Long. 079° 08.558'E). Then the oysters were transported to the laboratory in an ice box within 2-3 h after collection. Further, the animals were cleaned, opened aseptically

and shucked as followed by Hunt et al. (1984).

Isolation and characterization of protease producing bacteria

Gut is important tissue for isolation of potential microbes in many marine species. Oyster gut tissue was weighed in a sterile beaker, and ground in mortar and pestle. Then the sample was serially diluted (10^{-2} to 10^{-9}) and spread onto nutrient agar media. Plates were incubated at 28°C for 48 h.

The bacteria were sub-cultured on casein agar and the isolates, which produced clear zone on casein agar after 24 h incubation were maintained on nutrient agar plates. The potential isolate showed clear zone of inhibition than other strains on casein agar was retained for this study. The isolated bacterial strain was identified on the basis of their morphological, cultural and biochemical characteristics. The obtained data were compared with standard description provided in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994).

16S rDNA sequencing and analysis

Genomic DNA was extracted from the cells of an 18 h culture using Wizard® genomic DNA Purification kit (Promega). The 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 27f (5'-AGAGTTTGATCCTGTGCTCAG-3') and 1492r (5'-GACTTACCAGGGTATCTAATCC-3'). PCR products were electrophoresed on 1% agarose gel and documented (Kumaran et al., 2010). The PCR product was purified by using PCR purification kit (Genei, Bangalore, India). The nucleotide sequences of the PCR product was determined by using the automated DNA sequencer with forward and reverse primers (Bio-serve Bio Technologies Pvt. Limited Hyderabad, India). Sequence comparison with the databases was performed using Basic Alignment Search Tool (BLAST) through the National Centre for Biotechnological Information (NCBI), USA, server. The isolate was identified as *B. cereus*.

Nucleotide accession number

The obtained nucleotide sequence of *B. cereus* strain was submitted in GenBank database and the assigned accession number is JX080200.

Phylogenetic analysis

Sequence similarity search was made for the 16S rDNA sequence of the identified strain by applying their sequence to BLAST in NCBI (USA). Phylogenetic analysis was performed using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Kumar and Tamura, 2001) after multiple alignment of data by CLUSTAL X (Thompson et al., 1997) and a phylogenetic tree was reconstructed by using the neighbor-joining method of Saitou and Nei (1987).

Production of protease

Production of protease from *B. cereus* SU12 was carried out in a medium containing 0.5% casein (w/v), 0.2% starch (w/v), 0.2% KH_2PO_4 (w/v), 0.2% KNO_3 (w/v), 0.5% NaCl (w/v), 0.005% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v), 0.002% CaCO_3 (w/v), 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v) with pH adjusted to 7.4. The fermentation medium was maintained at 37°C for 36 h at 200 rpm in a shaking incubator. At the end of the fermentation period, the broth was centrifuged at 10,000 rpm for 15

min at 4°C. The clear supernatant was recovered as crude enzyme preparation and subjected to purification for further studies.

Optimization of culture conditions

The strain SU12 was subjected to different culture conditions to derive the optimum conditions for protease production. Protease production was estimated at various temperatures, pH, sodium chloride, carbon sources and nitrogen sources. Experiment was conducted in triplicate at various temperatures viz. 35, 40, 45, 50, 55 and 60°C to study their effect on protease production. Average of triplicate values has been plotted. Different pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) of the casein broth was tested to study their effect on protease production. All the experiments were carried out in triplicate and average values has been plotted.

The effect of various concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) on protease production was studied by changing the ratio of volume of sodium chloride solution to the casein broth and the flasks were incubated in triplicate and the average values were plotted. Casein broth was used for studying the effect of various carbon compounds viz. starch, mannitol, glucose, dextrose, xylose, and arabinose. The broth was distributed into various flasks and 1% of each carbon source was added before inoculating of the strain (SU12) and was incubated at the optimum pH, temperature and NaCl. The experiments were conducted in triplicate and the average values were plotted.

Casein broth was used for studying the effect of various nitrogen compounds viz. beef extract, peptone, yeast extract, potassium nitrate (KNO₃) and ammonium sulfate. The broth was distributed into various flasks and 1% of each nitrogen source was added before strain inoculation and was incubated at standardized parameters. The experiments were conducted in triplicate and the average values were plotted.

Incubation period

The effect of incubation periods on protease production was studied. For this, 50 ml of casein broth containing optimized carbon and nitrogen sources was taken in each 100 ml conical flask. After autoclaving, the flasks were inoculated with equal quantity of inoculums and incubated at optimal conditions (temperature, pH) for 6, 12, 18, 24, 30, 36 h. The culture filtrates were collected and used for enzyme activity. The experiments were conducted in triplicate and the average values were plotted.

Enzyme purification

The clear supernatant (crude protease) was precipitated with different concentrations of ammonium sulphate that is, from 10 - 80% saturation. The precipitated protein was dissolved in 20 mM potassium phosphate buffer and dialyzed against same buffer. Further purification was carried out in ion exchange chromatography (DEAE-Cellulose). The dialyzed protein was applied to a DEAE-cellulose column (2.5 × 70 cm), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 volume of equilibration buffer, bound proteins were eluted stepwise by using phosphate buffers in increasing molarity and decreasing pH values at room temperature. The flow rate was adjusted to 0.5 ml/min and fractions (1 ml each) were collected. The fractions showing high enzyme activity were pooled and lyophilized.

Determination of protein

Protein concentration was measured following Lowry et al. (1951)

method. Bovine serum albumin (BSA) was used as the standard.

Protease assay

Protease activity was determined following the method of Kembhavi et al. (1993) and 2.0% casein was used as substrate. One milliliter (1 ml) diluted enzyme solution was mixed with 1 ml of 2.0% casein in 50 mM Tris-HCl (pH 8.5) and incubated at 30°C for 10 min. After incubation, the reaction was stopped by the addition of 2 ml of 0.4 M trichloroacetic acid. Then, the precipitate was removed by centrifugation at 10,000 rpm for 10 min, and 1 ml of supernatant was neutralized with 5 ml of 0.4 M sodium carbonate and incubated with 1 ml of 1 N Folin Ciocalteu's reagent solution at 40°C for 20 min. Subsequently the absorbance at 660 nm was measured. The enzyme activity was calculated from standard curve of L-tyrosine. One unit of activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per milliliter of reaction mixture per minute.

Molecular weight determination of protease in SDS-PAGE

The molecular weight of the protease was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli (1970) using 4% stacking gel and 12% resolving gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. The molecular weight of the protease was determined by comparison with the migration distances of standard marker proteins consisting of phosphorylase B (97.4 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.3 kDa).

RESULTS AND DISCUSSION

Protease is an industrially important enzyme having wider applications in pharmaceutical, leather, laundry, food and waste processing industries. Industrial enzyme production would be effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes. Primary screening for protease producing bacterial strains was done in casein agar medium based on zone formation. The bacterial isolates which formed zone around the colonies were considered to be protease positive strain. The clear zone formation may be attributed to the hydrolysis of casein. Fifteen (15) bacterial colonies (SU1 to SU15) secreting protease were isolated. The isolates were purified through repeated streaking onto casein agar plates. Among the isolates, the best (SU12) was selected based on highest zone formation (casein hydrolysis) on casein agar (Figure 1). The potential strain was maintained on agar slants and stored at 4°C.

Identification of the protease producing strain

Microscopic observation of isolate SU12 showed motile, Gram positive rod shaped bacterium; the bacterium grew aerobically and formed white colonies. The morphological and biochemical characteristics are presented in Table 1.



Figure 1. Proteolytic activity of *B. cereus* SU12.

Table 1. Characteristics of *Bacillus cereus* SU12.

Morphological and biochemical tests	<i>Bacillus cereus</i> SU12
Morphological identification	Small, white, dried colonies
Gram's staining	Gram positive, rod
Indole production	-
Methyl red test	-
Voges - Proskauer test	+
Citrate utilization test	+
Starch hydrolysis	+
Casein hydrolysis	+
Urease test	-
Motility	+
Carbohydrate fermentation test	
a) D-glucose	+
b) Mannitol	-
c) Lactose	-
d) Sucrose	+

+, Positive results; -, negative results.

Based on Bergey's manual of determinative bacteriology the phenotypic characteristics of isolate (SU12), belongs to the genus *Bacillus*.

Genomic DNA was isolated from the strain SU12 and its quality was checked by loading in 1% agarose gel with the DNA marker which showed the intact DNA. 16S rDNA of the strain SU12 was amplified through PCR which shows the molecular weight of 1.472 kb corresponding to that of the DNA ladder in 1% agarose gel (Figure 2). The amplified product was sequenced (Figure 3). The obtained 16S rDNA gene sequence (1472 bp) of the strain SU12 was preliminary compared with previously obtained sequen-

ces of *Bacillus* sp deposited in GenBank (NCBI) and it indicated that this organism is phylogenetically related to the members of the genus *Bacillus*. The phylogenetic tree of 16S rDNA sequences was constructed by using the three valid representative species of the genus *Bacillus* to know the relationship of the strain SU12 and the BLAST result showed that they appeared close match in neighboring tree. The sequence of *B. cereus* (HM771668.1) served as outside reference of operational taxonomic unit (Figure 4). The species *B. cereus* (AB682146.1), *Bacillus thuringiensis* (EU429660.1), *Bacillus* sp (GQ407198.1) has the closest sequence similarity of 99%.

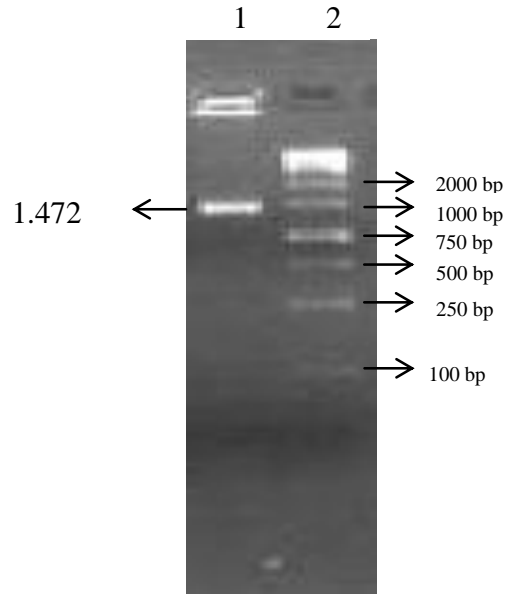


Figure 2. Amplified product of 16S rDNA of strain SU12. Lane 1, Strain SU12; Lane 2, DNA marker.

ORIGIN

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1 accggaggcg cgccccgctt aaaacatggc aagtcggagc gaatggatta agagcttgct
61 cttatgaagt tagcggcgga cgggtgagta acacgtgggt aacctgcccc taagactggg
121 ataactccgg gaaaccgggg ctaataccgg ataacatttt gaaccgcatg gttcgaattt
181 gaaaggcggc ttcggctgtc acttatggat ggaccgcgct cgcattagct agttggtagg
241 gtaacggctc accaaggcaa cgatgcgtag ccgacctgag agggatgatc gccacactgg
301 gactgagaca cggcccagac tcctacggga ggcagcagta gggaatcttc cgcaatggac
361 gaaagtctga cggagcaacg ccgcgtgagt gatgaaggct ttcgggtcgt aaaactctgt
421 tgtaggggaa gaacaagtgc tagtgaata agctggcacc ttgacggtac ctaaccagaa
481 agccacggct aactacgtgc cagcagccgc ggtaatacgt aggtggcaag cgttatccgg
541 aattattggg cgtaaagcgc gcgcaggtgg tttcttaagt ctgatgtgaa agcccacggc
601 tcaaccgtgg agggtcattg gaaactggga gacttgagtg cagaagagga aagtggattt
661 ccatgtgtag cggtgaaatg cgtagagata tggaggaaca ccagtggcga aggcgacttt
721 ctggtctgta actgacactg aggcgcgaaa gcgtggggag caaacaggat tagataccct
781 ggtagtccac gccgtaaacg atgagtgcta agtggttagag ggtttccgcc ctttagtgct
841 gaagttaacg cattaagcac tccgcctggg gagtacggcc gcaaggctga aactcaaagg
901 aattgacagg ggcccgcaca agcgggtggag catgtggttt aattcgaagc aacgcgaaga
961 accttaccag tcttgacatc ctctgacaac cctagagata gggcttctcc ttcgggagca
1021 gagtgacagg tgggtgcatg ttgtcgtcag ctcgtgtcgt gagatggttg gttaagtccc
1081 gcaacgagcg caacccttga tcttagttgc catcatttag ttgggcactc taagggtgact
1141 gccggtgaca aaccggagga aggtggggat gacgtcaaat catcatgccc cttatgacct
1201 gggctacaca cgtgctacaa tggacgggtac aaagagctgc aagaccgcca ggtggagcta
1261 atctcataaa accgtttctc gttcggattg taggctgcaa ctcgcctaca tgaagctgga
1321 atcgctagta atcgcggatc agcatgccgc ggtgaatac tccccgggcc ttgtacacac
1381 cgcccgctac accacgagag tttgtaacac ccgaagtcgg tggggtaacc tttttggagc
1441 cagcccgccc taaatggggg aacaagaatt tt

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Figure 3. 16S rDNA sequence of the strain SU12.

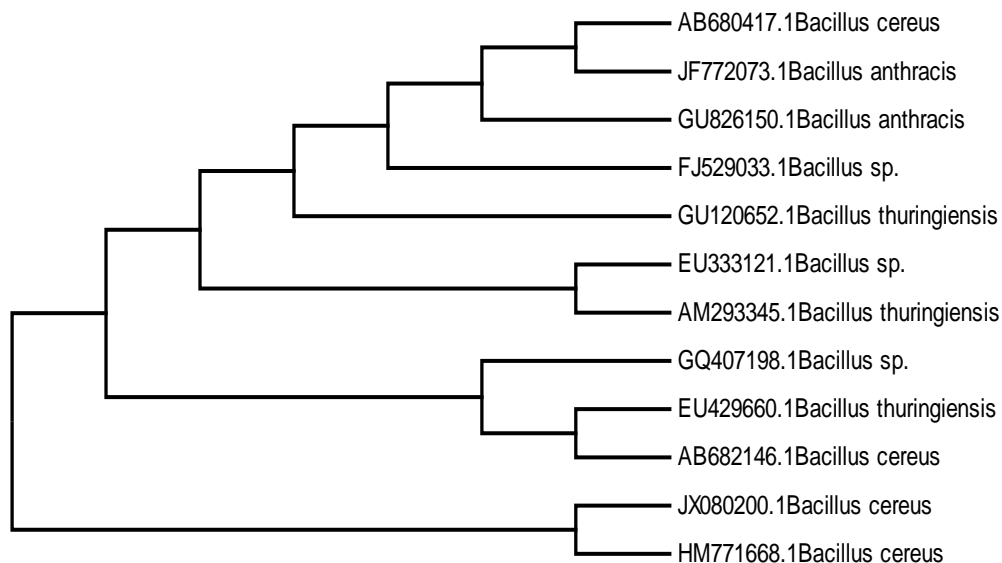


Figure 4. Neighbor-joining tree based on 16S rDNA sequences, showing relationship between the strain SU12 (1472 nucleotides) and 11 bacterial species.

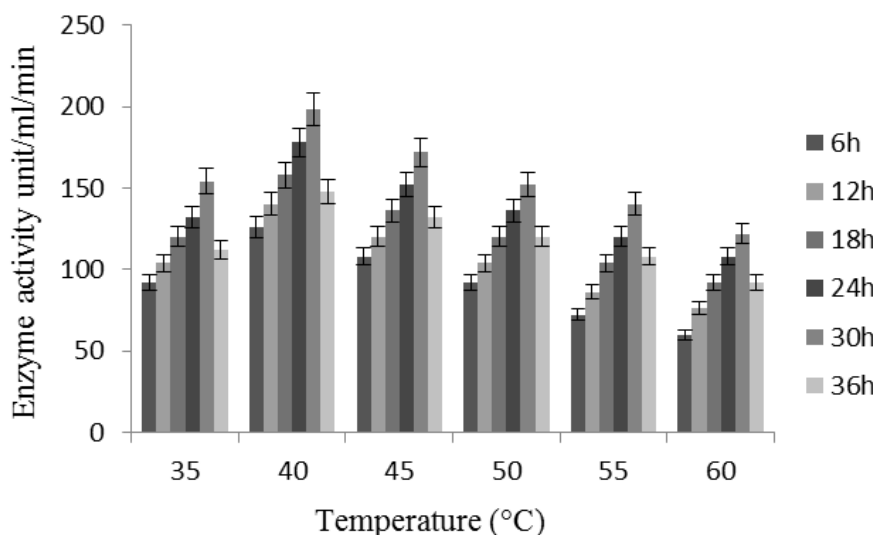


Figure 5. Effects of temperature on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

Optimization of culture conditions

Factors such as temperature, pH, sodium chloride concentration, different carbon sources and nitrogen sources which may influence the secretion of protease enzyme were optimized for maximum protease production and activity by *B. cereus* SU12. Standardization of culture condition was carried out in one parameter at one time and the standardized values were used for subsequent experiments.

Effects of temperature on protease activity

The activity of protease at different temperatures was determined by incubating the reaction mixture at temperatures ranging from 35 to 60°C for different time intervals before determining protease activity. Experiments were carried out in triplicate and the average values were reported. Maximum enzyme production (198 \pm 0.3 unit/ml) was observed at 40°C (Figure 5), and relatively minimum level (122 \pm 0.2 unit/ml) was observed

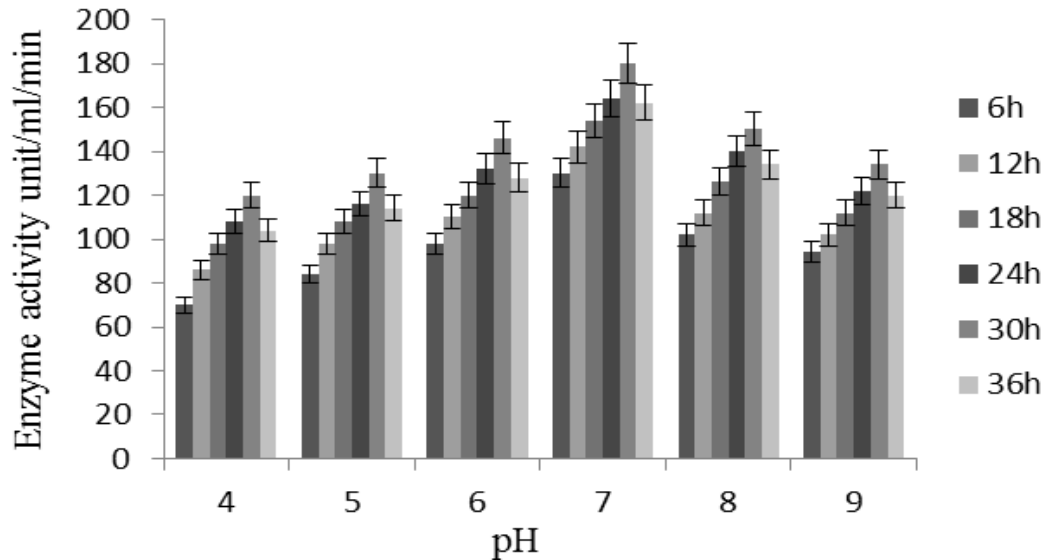


Figure 6. Effects of pH on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

at 60°C for 30 h of incubation. Similar results were reported for other *Bacillus* proteases. For example, the optimum temperature for protease from *Bacillus amovivorus* (Sharmin et al., 2005), *Bacillus fastidiosus* (Shumi et al., 2004) and *Pseudomonas fluorescens* CM1₁₂ (Al-Saleh and Zahran, 1997) was 37°C. The present study recorded 40°C as optimal, which is in agreement with earlier findings of VijayAnand et al. (2010) and Fulzele et al. (2011), optimum temperature for protease was 40°C. El-Safey and Abdul-Raouf (2004) reported the same findings in production, purification and characterization of protease enzyme from *Bacillus subtilis*. Related studies also reported that protease production by *Bacillus laterosporus* was best at 40°C (Usharani and Muthuraj, 2010).

Effects of pH on protease activity

The effect of pH on protease activity was determined in different pH values ranging from 4.0 to 9.0. Reaction mixtures were incubated at different time intervals and the activity of the protease was measured. The result showed that the enzyme activity was maximum (180 \pm 0.06 unit/ml) at pH 7 and minimum (120 \pm 0.02 unit/ml) at pH 4 (Figure 6). Similar results were obtained for the optimum pH for enzymatic activity of other *Bacillus* sp: pH 7.5 for *B. subtilis* ITBCCB 148 (Yandri et al., 2008), *Bacillus* sp. HS08 (Huang et al., 2006) and *Bacillus* sp. S17110 (Jung et al., 2007). In another study, Maal et al. (2009) reported, pH 7 is optimal for the maximum production of alkaline protease from *B. cereus* and *Bacillus polymixa*. Several other findings also described pH 7 is optimal for the maximum protease

production (Gouda, 2006; Fulzele et al., 2011; Vidyasagar et al., 2006).

Sodium chloride

As the organism was isolated from marine environment, the use of varying percentage of NaCl in the production medium was of interest. The results confirmed that the enzyme activity was at its best (190 \pm 0.04 unit/ml) when the concentration of NaCl was 2.5% (Figure 7). Similarly, Manivasagan et al. (2010) observed the maximum protease activity at 2% sodium chloride concentration by the actinobacteria isolated from sediment samples. Vonothini et al. (2008) also reported maximum protease activity of actinomycete Strain, PS-18A at 3% sodium chloride concentration.

Carbon compounds

Different carbon sources were used in the production medium for determining the highest yield of enzyme production. The highest enzyme activity (184 \pm 0.5 unit/ml) was obtained when starch was used as the carbon source (Figure 8), while the activity was minimum (116 \pm 0.4 unit/ml) with arabinose. This is in agreement with the previous reports which showed that starch and glucose caused high level of enzyme expression in *Bacillus* sp. and *B. cereus* strain 146, respectively (Mahmood et al., 2000; Shafee et al., 2005). Similarly, some research groups have shown an increase in protease production by *Bacillus* sp. in the presence of starch (Ferrero et al., 1996; Gusek et al., 1988; Hubner et

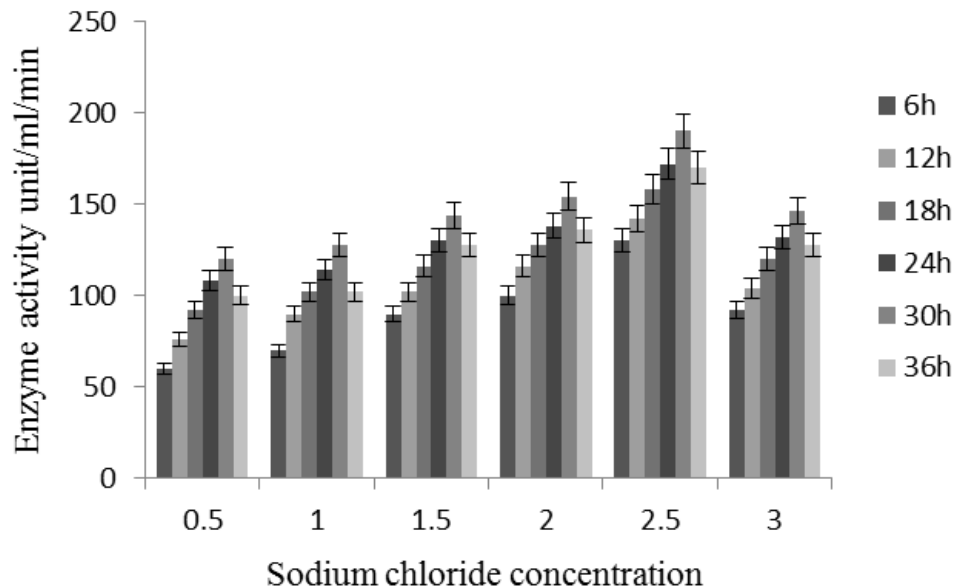


Figure 7. Effects of sodium chloride on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

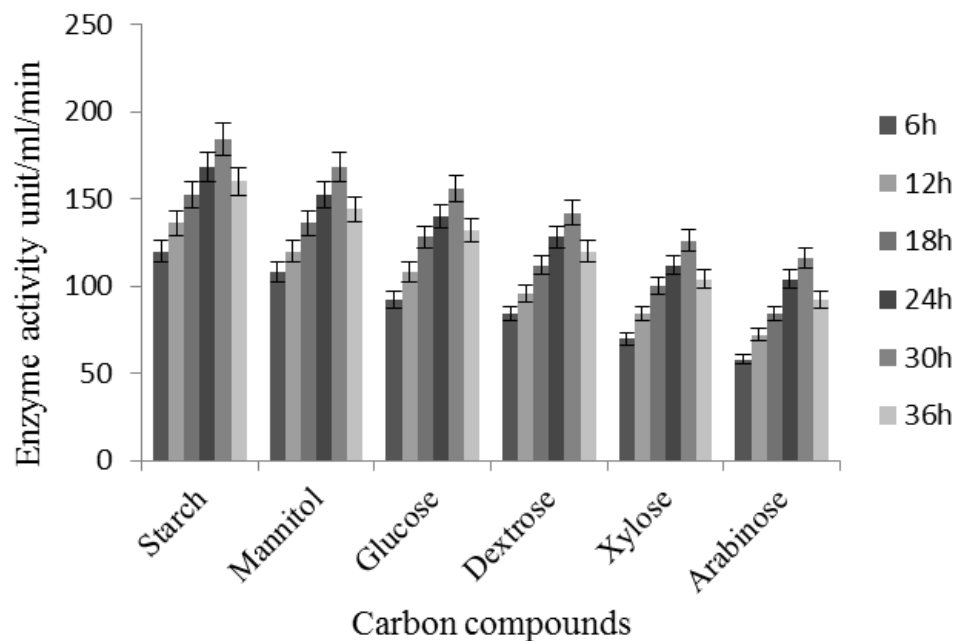


Figure 8. Effects of carbon compounds on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

al., 1993; Puri et al., 2002). Studies on alkaline proteases reported that the addition of starch to the culture medium induced enzymes synthesis (Chauhan and Gupta, 2004; Fang et al., 2001). However, other works reported better protease synthesis in the presence of glucose as carbon source (Ferrero et al., 1996; Mehrotra et al., 1999).

Nitrogen compounds

Culture broth was used for studying the effect of various nitrogen compounds viz. beef extract, peptone, yeast extract, potassium nitrate (KNO₃) and ammonium sulfate

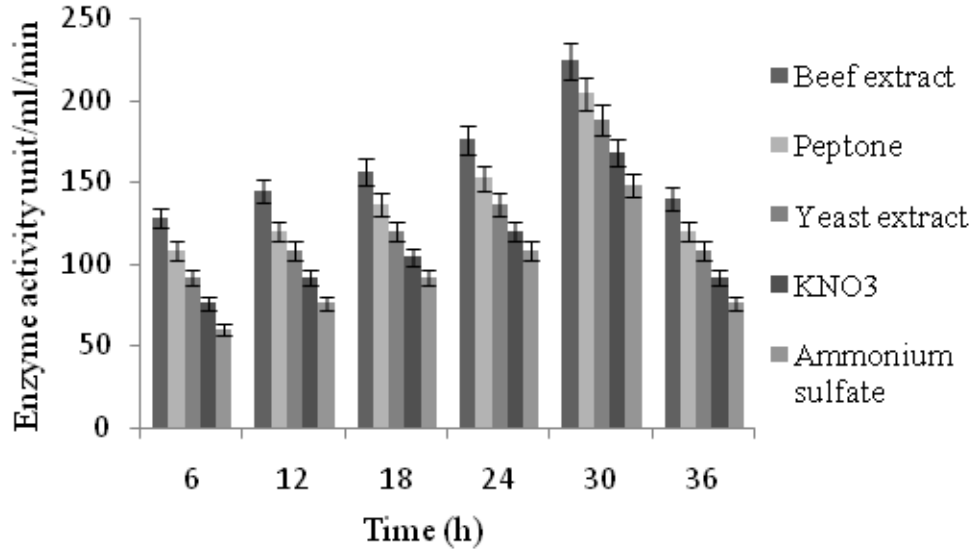


Figure 9. Effects of nitrogen compounds on protease activity of *B. cereus* SU12. Each value is the mean±SD of triplicate measurements.

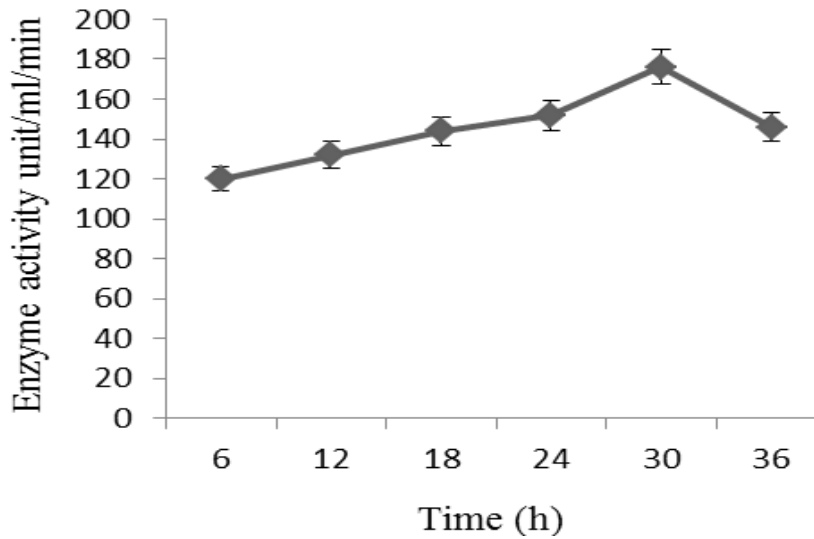


Figure 10. Effects of incubation period on protease activity of *B. cereus* SU12. Each value is the mean±SD of triplicate measurements.

on enzyme activity. Of the different nitrogen sources, the test strain showed maximum enzyme activity (224±0.05 unit/ml) in the presence of beef extract (Figure 9). Similarly Vonothini et al. (2008) and Manivasagan et al. (2010) also observed maximum enzyme activity in the beef extract as nitrogen source in actinobacteria. Researchers have reported soybean meal as the best nitrogen source for protease production from *Bacillus* sp (Masse and Tilburg, 1983; Bhunia et al., 2010; Hubner et al., 1993; Puri et al., 2002). Likewise, other reports demonstrated peptone as the better nitrogen source for

protease production from *Bacillus* sp (Das and Prasad, 2010; Adinarayana and Ellaiah, 2002).

Effects of incubation period on protease activity

Since microorganisms show considerable variation at different incubation period, it is very essential to detect the optimum incubation time at which an organism shows the highest enzyme activity. Figure 10 shows maximum level of protease production (176±0.05 unit/ml) at 30 h

Table 2. Effects of incubation period on the biomass yield of *B. cereus* SU12.

Incubation periods (h)	Biomass yield (absorbance at 600 nm)
6	0.65
12	0.752
18	0.856
24	0.915
30	0.974
36	1.143

Table 3. Optimal levels of growth parameters and sources for protease production by the strain SU12.

S/N	Parameter	Optimal value/sources
1	Incubation time	30 h
2	Temperature (°C)	40
3	pH	7.0
4	NaCl concentration	2.5%
5	Carbon source	Starch
6	Nitrogen source	Beef extract

Table 4. Summary of the protease purification from *B. cereus* SU12.

Purification step	Volume (ml)	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	100	4579.45	137.8	33.23	1	100
Ammonium sulfate precipitation (80% saturation and dialysis)	25	2975.32	29.3	101.55	3.05	64.97
DEAE-cellulose chromatography	5	1125.93	1.27	886.56	8.73	37.84

and minimum level of enzyme production (120 ± 0.03 unit/ml) at 6 h of incubation period. Maximum production of proteases with 48 h of incubation by bacteria was reported by Hoshino et al. (1997) and Shumi et al. (2004). In the present study, 30 h of incubation is suitable time for maximum production of proteases. The highest biomass yield was recorded at 36 h of incubation period (Table 2). Optimal levels of the growth parameters observed in the present study for the production of protease with respect to the potential bacterial strain SU12 are shown in Table 3.

Purification of protease enzyme

The protease from *B. cereus* SU12 was purified with ammonium sulfate fractionation, followed by DEAE-ion exchange chromatography. Results of protease purification are summarized in Table 4. The specific activity after the

80% saturation with ammonium sulfate was 101.55 U/mg protein and the purification was 3.05 fold. The purified enzyme of strain SU12 after DEAE cellulose chromatography exhibited a specific activity of 886.56 U/mg protein with 8.73 fold purification.

Molecular weight determination in SDS-PAGE

Molecular weight of the protease enzyme was determined by SDS-PAGE (Figure 11). Single protein band was observed when stained with Coomassie Brilliant Blue and it clearly indicated the purity of the protein. The molecular weight of the purified enzyme was 66 kDa. A variety of molecular mass for proteases from other *Bacillus* species had been reported: 30.9 kDa thermophilic *Bacillus* strain HS08 (Huang et al., 2006); 27.0 kDa *Bacillus megaterium* (Reungsang et al., 2006); 75.0 kDa *Bacillus sp.* S17110 (Jung et al., 2007); 34.0 kDa *B. thuringiensis* (Kunitate et

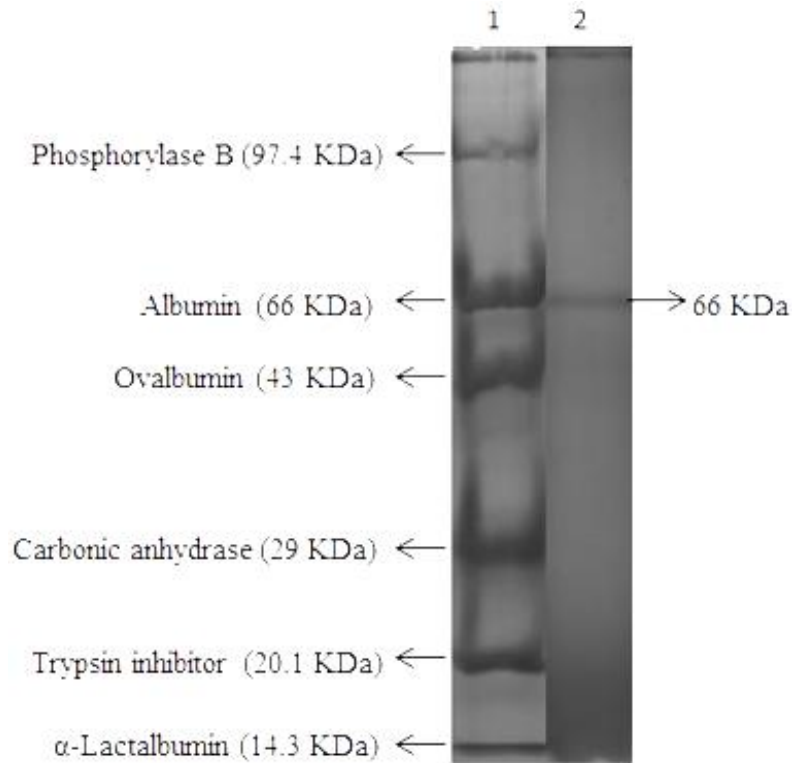


Figure 11. SDS-PAGE of the purified enzyme: Lane1: Molecular weight markers; Lane2: Protease enzyme.

al., 1989); 38.0 kDa *B. cereus* KCTC 3674 (Kim et al., 2001); 15.0 kDa *B. subtilis* PE-11 (Adinarayana et al., 2003); 34.0 kDa *B. cereus* BG1 (Ghorbel-Frikha et al., 2005); 66.2 kDa, 31.0 kDa and 20.1 kDa *Bacillus licheniformis* strains BLP1, BLP2 and BLP3, respectively (Cheng et al., 2006).

Conclusions

From the present study, it is concluded that the identified species, *B. cereus* SU12 isolated from the *S. cucullata* possesses good protease activity. The study has also standardized the growth parameters of bacteria for the maximum enzyme production, which can be effectively used in the large scale production of protease for commercial purposes. The optimum temperature and pH were determined as 40°C and 7.0 and best carbon and nitrogen sources were starch and beef extract. This information has enabled the formulation of media composition for maximum protease production by this organism. The protease was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. The molecular weight of protease was found to be 66 kDa. The purified protease will be used for the various purposes in detergent industries, food industries and pharmaceutical industries.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Ginger-supplemented diet ameliorates ammonium nitrate-induced oxidative stress in rats

Amira Messaadia^{1*}, Saad Saka¹, Meriem Krim¹, Imen Maida¹, Ouassila Aouacheri¹ and Rachid Djafer²

¹Applied Biochemistry and Microbiology Laboratory, Department of Biochemistry, Faculty of Sciences, Badji Mokhtar University, Annaba – Algeria.

²Toxicological Laboratory, CHU Ibn Sina, Annaba – Algeria.

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The present study was designed to evaluate the capacity of ginger to repair the oxidative stress induced by ammonium nitrate. 50 male rats were divided into 5 groups; they underwent an oral treatment of ammonium nitrate and/or ginger (N mg/kg body weight + G% in diet) during 30 days. Group I served as control (C); group II (G) received a diet with 2% of ginger; group III (N) received a toxic dose of ammonium nitrate and normal diet; group IV (NG) received a toxic dose of ammonium nitrate and a diet containing 2% ginger and group V (N⁺G) received a highly toxic dose of ammonium nitrate and an experimental diet containing 2% ginger. The treatment by ammonium nitrate was found to elicit a rise in blood biochemical parameters, a disorder in hematological parameters and significant decrease in the tissue glutathione level. Feeding ginger supplemented diets to ammonium nitrate treated rats restored all the parameters studied compared to the controls. These findings suggest that ginger treatment exerts a protective effect on metabolic disorders by decreasing oxidative stress.

Key words: Ammonium nitrate, toxicity, ginger, oxidative stress, rats.

INTRODUCTION

The massive use of fertilizers lead to an increase in agricultural outputs, but caused a greater pollution of continental waters and farmlands (Koller, 2009). Ammonium nitrate is one of the most commercially important ammonium compounds in terms of usage. It finds extensive use in the area of nitrogen fertilizers (Testud, 2004), explosives (Presles et al., 2009) and in the manufacturing of meat products (Honikel, 2008). It is a crystalline, hygroscopic and odorless substance which tends to coagulate in lumps, and very soluble in water. Excessive nitrate concentrations in drinking water may have serious

implications for public health; it can enhance the proliferation of phytoplankton, contributing to the phenomenon of eutrophication in aquatic ecosystems (Camargo and Álvaro, 2006). Once in the organism, the conversion of nitrate in the mouth is particularly important. The dorsal surface of the tongue symbiotically harbors a specialized flora of anaerobic nitrate reducing bacteria, which can rapidly reduce nitrate to nitrite (Duncan et al., 1995). Nitrite will be converted to varieties of nitrogen compounds in the stomach. The main products are NO and N-nitroso compounds (Mitsui and Kondo, 2002).

*Corresponding author. E-mail: missmayra2007@yahoo.fr. Tel/Fax + 213 30 82 07 36.

Abbreviations: GPx, Glutathione peroxidases; SOD, superoxide dismutases; CAT, catalase; EDTA, ethylenediaminetetra acetate; CBC, complete blood count; ALP, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase, LDH, lactate dehydrogenase; GSH, glutathione; metHb, methemoglobin; MCV, mean corpuscular volume; IDDM, insulin-dependent diabetes mellitus.

Recent research indicates that there is a close correlation between NO generation and nitrite content in plasma; and thereby the nitrite is suggested to be the storage form of NO (Bryan, 2006). Nitric oxide (NO) has been shown to be involved in many important biological events. However, the presence of the unpaired electron gives NO paramagnetic properties, prevents its dimerization and increases its reactivity with a variety of atoms and free radicals leading to oxidative stress. One important reaction of NO is its interaction with oxyhemoglobin to form methemoglobin (metHb) (Mansouri and Lurie, 1993); it can also react with secondary amines to produce N-nitroso compounds susceptible to be carcinogenic (Volkmer et al., 2005). Moreover, NO interact with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$); which is a potent oxidant which can nitrosate proteins and nucleic acids, and can cause lipid peroxidation (Squadrito and Pryor, 1998). Additionally, the protonated form of $ONOO^-$ may be decomposed to form highly reactive (OH^\bullet) and nitrogen dioxide (Beckman et al., 1990).

Organisms have several mechanisms to counteract damage by free radicals (Koechlin-Ramonatxo, 2006). One important line of defence is an enzyme system, including glutathione peroxidases (GPx), superoxide dismutases (SOD) and catalase (CAT), which decrease concentrations of the harmful oxidants in the tissues. The second line of defence against free radical damage is the presence of antioxidants, which are stable molecules enough to donate an electron to scavenging free radical and neutralize it. Some antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the cells (Yu, 1994). Other antioxidants are found in the diet, the best known are vitamin E, vitamin C and carotenoids. Many natural substances, such as phenolic or polyphenolic compounds, display antioxidant properties, and thus, important for health. Spices are recognized as sources of natural antioxidants which can protect against the oxidative stress and play an important role in the prevention of numerous pathologies (Gião et al., 2010).

Ginger (*Zingiber officinale*, Roscoe, Zingiberaceae) is one of the most commonly used spices around the world, especially in the South-Eastern Asian countries (Chrubasik et al., 2005). The constituents of ginger are numerous and vary depending on the place of origin and whether the rhizomes are fresh or dry. Many chemical investigations on this plant led to the identification of a large number of compounds, like the gingerols, shogaols, and the gingerdiones (Schwertner and Rios, 2007). Ginger has been demonstrated to have various pharmacological activities (Ali et al., 2008) such as antiemetic, antiulcer, anti-inflammatory, antioxidant, glucose and lipid lowering, cardiovascular as well as anti-cancer activities. Therefore, the present study was undertaken to investigate the potential role of ginger in reducing the toxic effect and oxidative stress induced by ammonium nitrate in experimental rats.

MATERIALS AND METHODS

Preparation of ammonium nitrate solution

Pure ammonium nitrate (Fluka Macedonia) was dissolved in mineral water, and induced by oral voice (*per os*); the volume of each dose was adjusted to deliver 400 and 600 mg/kg of rat body weight.

Preparation of ginger powder

The rhizomes of fresh ginger was purchased from the local market (imported from China), peeled, washed, coarsely minced, air dried and pulverized with a blender to fine powder. It was preserved in airtight containers at room temperature until the formulation of experimental diets. Ginger powder was added (w/w) to already pulverized feed and thoroughly mixed so as to give a diet containing 2% ginger.

Animals, diet and treatment

Fifty (50) male rats with an average body weight of 200 g were provided by the Algiers Pasteur Institute, Algeria. The animals were housed in clean polypropylene cages, and kept under standard laboratory conditions of light/dark cycle (12/12 h) and controlled temperature room ($25 \pm 2^\circ\text{C}$). The rats were given a nutritionally adequate diet which was prepared according to Upreti et al. (1989) and water *ad libitum* throughout the experimental period. After acclimatization, the rats were randomly allocated into five groups of ten animals each and the groups were treated as follows: the 1st group (C) served as control and received mineral water and normal diet (0 + 0); the 2nd group (G) feed with 2% ginger diet (0 + 2%); the 3rd group (N) received by gavage 400 mg/kg of ammonium nitrate (400 mg/kg + 0); the 4th group (NG) received 400 mg/kg of ammonium nitrate and feed with 2% ginger diet (400 mg/kg + 2%); and the 5th group (N⁺G) received 600 mg/kg of ammonium nitrate and feed with 2% ginger diet (600 mg/kg + 2%).

Experimental methods

After 30 days of treatment, animals were sacrificed by decapitation and blood samples were collected into two tubes: one contained ethylenediaminetetra acetate (EDTA) as anticoagulant for metHb and complete blood count (CBC) test, while the other was kept dry and centrifuged at 5000 rpm for 15 min to obtain serum, which is used for various biochemical investigations. Some organs were retrieved, weighed and preserved at -20°C for the determination of reduced glutathione levels.

Analytical methods

Methemoglobin assay was carried out according to Evelyne and Malloy (1938) method. Hemathological parameters were measured by an automated ERMA INC Analyzer (full automatic blood cell counter model PCE-210N). The serum glucose concentration was measured by portable glucometer (Accu-Chek Active). Uric acid, urea, creatinine, cholesterol, triglycerides, total lipids, alkaline phosphatase (ALP), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), lactate dehydrogenase (LDH), total and direct bilirubin were measured spectrophotometrically in serum with kits purchased from Spinreact (Spain). The concentration of tissue reduced glutathione (GSH) was measured, as described by Weckbecker and Cory (1988). The proportioning of proteins was carried out by the method of Bradford (1976).

Statistical analysis

All data were represented as means \pm SD of 10 rats. Data were analyzed by Minitab software (version 13.31) using the Student's t-test to assess differences compared to the control group.

RESULTS

Physiological study

Exposure of rats to ammonium nitrate did not produce any overt sign of toxicity/mortality. We find that hepatosomatic index in (N) group increased significantly ($p < 0.05$) indicating hepatomegaly, as well as the spleenosomatic index in (N⁺G) group was significantly decreased (Figure 1).

Hematological study

Table 1 shows the result of hematological parameters in control and ginger-diet supplemented rats. These results reveal that the treatment by ammonium nitrate (N) caused significant changes ($p < 0.05$) in hematocrit, red and white blood cells number; hemoglobin (Hb), methHb level and mean corpuscular volume (MCV). However, ginger supplementation in treated rats prevented alterations in these parameters except hematocrit and methHb.

Renal study

Significant increase ($p < 0.01$) in serum urea, creatinine and uric acid were observed in the ammonium nitrate treated rats compared with control group. Feeding of rats with ginger-supplemented diet maintains these parameters in normal values, which reflect a re-establishment of the renal function (Table 2).

Biochemical study

As presented in Table 3, oral administration of 400 mg/kg of ammonium nitrate to rats caused a significant increase in the concentration of glucose, total and direct bilirubin, cholesterol, triglycerides, total lipids, ALAT, ASAT, LDH and ALP in comparison with control group. Meanwhile, ginger supplementation in NG group restored these biochemical parameters to near control levels. The N⁺G group, which was given 600 mg/kg ammonium nitrate and 2% of ginger, showed a very significant alteration in all biochemical markers.

Toxicological study

High nitrate intake caused an impressive oxidative stress which occurs mainly through a significant reduction of GSH levels in all removed organs. On the other hand, ginger supplementation showed a remarkable antioxidant activity. We recorded an increase of GSH levels in all organs and became equivalent to those of the control rats

(Figure 2).

DISCUSSION

A large number of xenobiotics have been identified to have potential to generate free radicals in biological systems (Kehrer, 1993). Free radicals have become an attractive means to explain the toxicity of numerous xenobiotics. It is now known that both nitrates and nitrites are the precursor of NO leading to ONOO⁻ production in particular if oxidative stress is present in biological areas. ONOO⁻ reacts with and damages many important biological molecules including thiols, lipids, proteins, and nucleic acids by a number of mechanisms (Raat et al., 2009). However, it is clear that dietary intake of naturally occurring antioxidants may be an effective means to develop prevention strategies in biochemical alterations and diseases risk factors associated with free radicals formation.

The most important effect of nitrate in the cellular elements of the blood is its hemolytic action inducing microcytosis (reduction of MCV) (Lukyanenko et al., 2004). Increased formation of MetHb is the most emphasized adverse effect caused by nitrate pollution after its subsequent reduction to nitrite which is ten times more toxic because of its oxidative properties (Sadeq, 2008; Rodriguez-Estival, 2010).

Nitrites are absorbed into the blood stream, from where they can reach other tissues. It leads to the oxidation of the ferrous iron (Fe²⁺) to the ferric (Fe³⁺) valence state, converting the hemoglobin to methemoglobin with a resultant inability to deliver oxygen to tissues, causing hypoxia and cyanosis (Jaffe, 1981). Nitrites can also, be a source of nitric oxide (NO) and other reactive oxygen as well as nitrogen species such as hydrogen peroxide (H₂O₂), peroxyxynitrite (ONOO⁻) and superoxide anion (O₂⁻), which disturb the balance between pro-oxidants and antioxidants in favor of the former, resulting in oxidative stress (Halliwell and Gutteridge, 1984). Inducing a peroxidation of the unsaturated fatty acids of phospholipids. Thus, it appears like an osmotic brittleness of the erythrocyte membrane as well as a disturbance of membrane transport which leads to the hemolysis (Ozturk et al., 2003).

Therefore, ginger is considered to be effective in reducing the rate of methemoglobin and restoring the values of hematological parameters in combination groups, demonstrating its antioxidant properties. Polyphenols, the largest compound family in ginger roots, possess ideal structure for free radical scavenging activity; and some of them have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate (Bolkhina et al., 2010). Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), as well as their ability to chelate transition metal

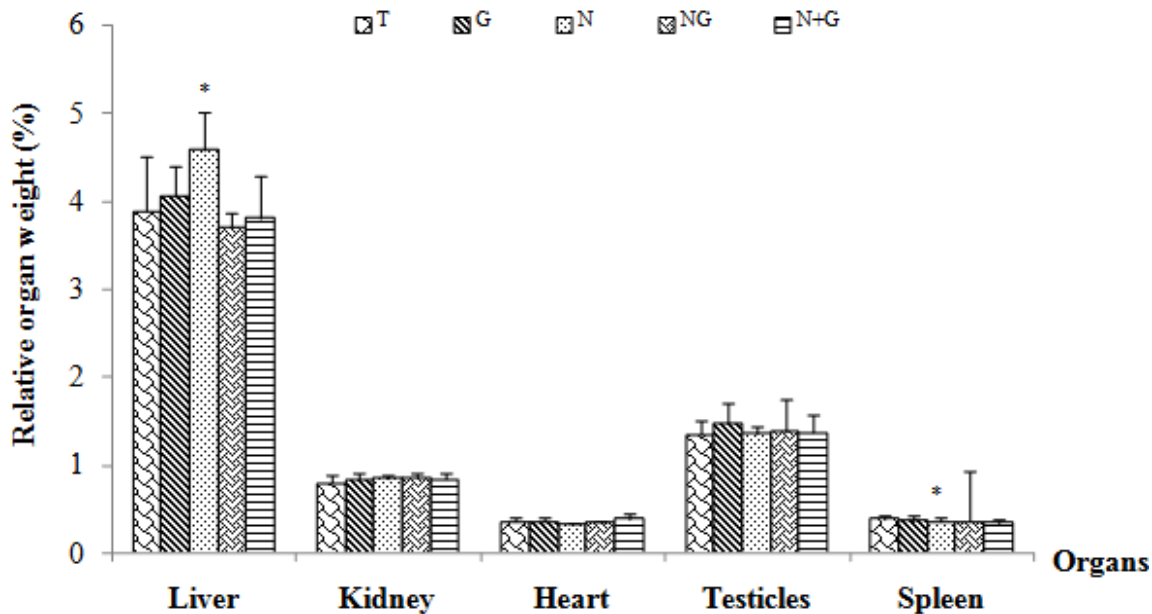


Figure 1. Organosomatic indexes (relative organ weight) in control (C) and treated rats with ammonium nitrate and ginger (G, N, NG and N⁺G) after 30 days treatment. Each value represents the mean \pm SD of 10 rats. *, Significantly different from control at $P < 0.05$.

Table 1. Concentrations of hematological parameters in control (C) and treated rats with ammonium nitrate and ginger (G, N, NG and N⁺G) after 30 days treatment, (each value represents the mean \pm SD of 10 rats).

Parameter	Experimental batch				
	C (0 + 0)	G (0 + 2%)	N (400 mg/kg + 0)	NG (400 mg/kg + 2%)	N ⁺ G (600 mg/kg + 2%)
WBC ($\times 10^3$ /ul)	9.07 \pm 1.26	8.54 \pm 0.80	7.68 \pm 1.22*	8.96 \pm 1.41	9.66 \pm 1.26
LY ($\times 10^3$ /ul)	5.98 \pm 0.84	5.72 \pm 0.90	5.32 \pm 0.74	6.08 \pm 0.61	6.54 \pm 0.92
MO ($\times 10^3$ /ul)	1.21 \pm 0.27	1.22 \pm 0.28	1.05 \pm 0.16	1.14 \pm 0.17	1.24 \pm 0.24
RBC ($\times 10^6$ /ul)	8.92 \pm 0.67	7.97 \pm 1.34	7.79 \pm 0.57*	8.89 \pm 0.55	8.55 \pm 0.63
Hgb (g/dl)	13.54 \pm 0.85	12.52 \pm 1.29	12.12 \pm 1.47*	13.80 \pm 1.03	12.88 \pm 1.27
HCT (%)	51.21 \pm 3.37	47.54 \pm 5.69	39.66 \pm 4.89***	45.02 \pm 4.77**	42.11 \pm 3.12***
MCV (fl)	54 \pm 3.79	50.26 \pm 4.46	49.53 \pm 2.39*	50.49 \pm 2.68	50.30 \pm 3.20
MCHC (g/dl)	32.33 \pm 2.90	33.03 \pm 3.47	31.80 \pm 2.65	32.17 \pm 2.56	31.89 \pm 3.22
PLT ($\times 10^3$ /ul)	303.6 \pm 80.1	248.4 \pm 37.2	235.2 \pm 90.7	290.6 \pm 67.8	282.1 \pm 55
MetHb (%)	1.69 \pm 0.82	1.75 \pm 0.60	10.51 \pm 1.50***	3.65 \pm 1.11**	4.62 \pm 1.02***

WBC, White blood cells; LY, lymphocytes; MO, monocytes; RBC, red blood cells; Hgb, hemoglobin; HCT, hematocrit; MVC, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; MetHb, methemoglobin; *, **, and ***, significantly different from control at $P < 0.05$; $P < 0.01$; $P < 0.001$, respectively.

Table 2. Concentrations of renal parameters in control (C) and treated rats with ammonium nitrate and ginger (G, N, NG and N⁺G) after 30 days treatment, (each value represents the mean \pm SD of 10 rats).

Parameter	Experimental batch				
	C (0 + 0)	G (0 + 2%)	N (400 mg/kg + 0)	NG (400 mg/kg + 2%)	N ⁺ G (600 mg/kg + 2%)
Uric acid (mg/dl)	4.90 \pm 0.85	3.70 \pm 0.87*	6.80 \pm 1.23**	5.63 \pm 1.07	6.36 \pm 1.25*
Urea (mg/dl)	27.08 \pm 4.51	26.71 \pm 3.69	46.41 \pm 11.20**	35.19 \pm 9.94	39.28 \pm 10.50**
Creatinine (mg/dl)	1.02 \pm 0.25	1.29 \pm 0.29	2.06 \pm 0.58**	1.34 \pm 0.35	1.67 \pm 0.39**

* and **, Significantly different from control at $P < 0.05$; $P < 0.01$, respectively.

Table 3. Concentrations of biochemical parameters in control (C) and treated rats with ammonium nitrate and ginger (G, N, NG and N⁺G) after 30 days treatment.

Parameter	Experimental batch				
	C (0 + 0)	G (0 + 2%)	N (400 mg/kg + 0)	NG (400 mg/kg + 2%)	N ⁺ G (600 mg/kg + 2%)
Glucose (g/l)	1.15 ± 0.15	1.10 ± 0.14	1.45 ± 0.27*	1.17 ± 0.13	1.19 ± 0.10
Cholesterol (mg/dl)	96.69 ± 14.40	76.86 ± 15.40*	150.71 ± 27.20***	121.70 ± 25.40*	130.56 ± 25.50**
Triglyceride (mg/dl)	132.25 ± 22.20	87.83 ± 16.20**	199.81 ± 33***	160.63 ± 24.90*	169.36 ± 25.40**
Total lipids (mg/dl)	326.95 ± 84.50	281.94 ± 80.50	720.85 ± 98.60***	426.79 ± 90.60*	506.56 ± 92.80**
ASAT (UI/l)	30.07 ± 9.32	37.26 ± 9.55	60.50 ± 10.30***	42.33 ± 9.82*	46.39 ± 10.10**
ALAT (UI/l)	26.43 ± 6.45	32.89 ± 6.54	45.47 ± 8.20***	35.58 ± 6.09*	41.05 ± 7.38**
PAL (UI/l)	130.90 ± 14.60	145.25 ± 13.20	243.83 ± 23.70***	159.63 ± 20.60*	169.75 ± 20.90**
LDH (UI/l)	165.08 ± 32.80	177.93 ± 31.90	296.18 ± 36.20***	229.60 ± 34.50**	274.76 ± 35.40***
Total bilirubin (mg/dl)	1.13 ± 0.28	0.92 ± 0.27	1.74 ± 0.31**	1.25 ± 0.30	1.44 ± 0.31*
Direct bilirubin (mg/dl)	0.56 ± 0.20	0.61 ± 0.22	1.01 ± 0.24**	0.76 ± 0.21	0.86 ± 0.22***

* and **, Significantly different from control at $P < 0.05$; $P < 0.01$, respectively. Each value represents the mean ± SD of 10 rats.

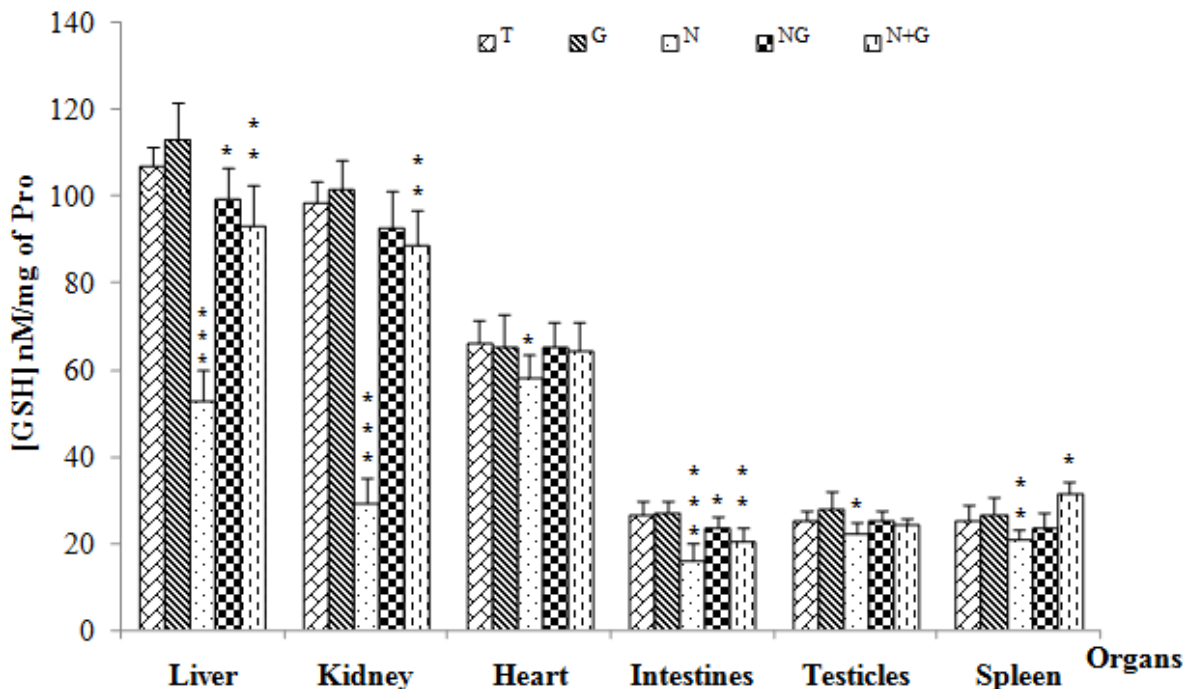


Figure 2. Glutathione levels in control (C) and treated rats with ammonium nitrate and ginger (G, N, NG and N⁺G) after 30 days treatment (each value represents the mean ± SD of 10 rats); *, **, and ***, significantly different from control at $P < 0.05$; $P < 0.01$; $P < 0.001$, respectively.

ions (termination of the Fenton reaction) (Rice-Evans et al., 1997). Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes. These changes could sterically hinder diffusion of free radicals and restrict lipids peroxidative reactions (Arora et al., 2000).

The results obtained in the present study showed an increase in blood glucose levels in ammonium nitrate

treated rats (N). Nitrate has been suspected to have a diabetogenic effect in children. Thus, several studies conducted in Europe have attempted to determine if a relationship exists between drinking contaminated water with nitrate and childhood-onset Type 1 insulin-dependent diabetes mellitus (IDDM) (Parslow et al., 1997; Moltchanova et al., 2004). Peroxynitrite acts as a terminal mediator of cellular injury in various pathophysiologic conditions. Typical cytotoxic reaction pathways triggered by peroxynitrite include lipid peroxidation, DNA breakage

and base modification, activation of the nuclear enzyme poly (ADP-ribose) polymerase, as well as tyrosine nitration (Beckman et al., 1990). Tyrosine nitration has been demonstrated in a variety of pathophysiologic conditions, including diabetes mellitus. In the pancreatic islets of spontaneous autoimmune diabetic mice, a significant increase in tyrosine nitration was found and the degree of beta-cell destruction showed a good correlation with the frequency of nitrotyrosine-positive beta cells. It was therefore proposed that the intra-islet formation of peroxynitrite plays an active pathogenic role in the pathogenesis of diabetes mellitus (Ischiropoulos et al., 1992). However, the administration of ginger to ammonium nitrate treated rats reduced blood glucose levels, in accordance with earlier reports (Al-Amin et al., 2006; Shahidul-Islam and Choi, 2008). *Zingiber officinale* may have beneficial effects on diabetes that hold the hope of a new generation of antidiabetic drugs.

Significant increase was obtained in serum cholesterol, triglyceride and total lipids levels in rats treated by ammonium nitrate. This hyperlipidemia finds several explanations. The hyperglycemia, dysthyroidism and renal failure observed in these rats can cause dyslipidemia, which can be deteriorated according to the intensity of the imbalance (Krauss and Siri, 2004; Pearce, 2004; Baumelou et al., 2005). Furthermore, possible relation between nitrate intake and effects on the thyroid have also been reported. Experimental study has shown that inorganic nitrate is a goitrogenic agent at short term inducing a hypertrophy of the epithelial cells of the thyroid gland follicles (Gatseva and Argirova, 2008). On the other hand, ginger diet supplemented rats witnessed reduced levels of cholesterol, triglyceride and total lipids. These data were consistent with the previous study (Schwertner and Rios, 2007). The lipid lowering effect of ginger could have possibly resulted from several phenomena, such as attenuation of cellular cholesterol biosynthesis, which was associated with increased activity of the LDL receptor leading to the enhancement of the removal of LDL from plasma (Ness et al., 1996), inhibition of intestinal absorption of dietary fat by inhibiting its hydrolysis (Han et al., 2005), reducing lipid peroxidation (Liu et al., 2003), increasing pancreatic lipase and amylase activity (Patel and Srinivasan, 2000), increasing intestinal peristalsis (Hashimoto et al., 2002), and/or increasing cholesterol conversion to bile acids (Srinivasan and Sambaiah, 1991).

Creatinine, urea and uric acid are wastes produced by protein metabolism and eliminated by the kidneys, generally used as indicators of renal function. When kidneys failure occurs, rates of these parameters increase; this was observed in rats of the N group proving the nephrotoxic effect of ammonium nitrate (Boukerche et al., 2007). In the present study, the levels of creatinine, uric acid and urea were decreased significantly in group with ginger supplementation. The results agree with the earlier published data (Al-Qattan et al., 2008). Also, Ajith et al. (2007) reported that ginger ameliorated cisplatin-induced

nephrotoxicity and this protection is mediated either by preventing the cisplatin-induced decline of renal antioxidant defense system or by direct free radical scavenging activity of ginger.

Oxidative stress generated by external toxic agents may have a negative impact on many tissues, including liver. The obtained result of hepatosomatic index in this study shows that a higher intake of ammonium nitrate can cause hepatomegaly and in order to assess the extent of the injury, determination of some biochemical parameters were performed. The activities of ASAT, ALAT, PAL and LDH were significantly elevated in N group compared to control group. Also, the increased levels of bilirubin in the serum indicate impaired excretory and synthetic functions of the liver (Ogur et al., 2005; Rodriguez-Estival et al., 2010). The obtained results in this study show the effectiveness of ginger in the prevention of ammonium nitrate-induced hepatotoxicity as indicated by normalization of the hepatic enzymes and bilirubin (El-sharaky et al., 2009).

Glutathione serves as a sensitive marker of oxidative stress and it plays an important role in maintaining the integrity of the cell. GSH is involved in several detoxification-reactions in the organism and it is one of the most prominent non-enzymatic antioxidants (Meister and Anderson, 1983). It was therefore interesting to study the GSH level in the liver, kidneys, testes, intestines, spleen and heart. In this study, the GSH level decreased in all organs of ammonium nitrate treated-group. Significant depletion in tissue GSH levels enhanced cellular damage caused by oxidative stress and suggests its increased utilisation against reactive oxygen species (Tachi et al., 2001). However, ginger treatment in combination groups reversed the GSH to normal levels; which also implies that ginger has an antioxidant property (Ahmed et al., 2008; Shanmugam et al., 2011). Gingerol was found to exert inhibitory effect on xanthine oxidase responsible for generation of ROS, such as superoxide anion (Chang et al., 1994). Other studies revealed that [6]-gingerol, the major pungent constituent of ginger, inhibits nitric oxide production and prevents oxidation and nitration reactions induced by peroxynitrite (Shimodo et al. 2010).

Conclusion

In conclusion, our study shows that ginger exerts a protective effect against oxidative stress induced by ammonium nitrate via increasing the antioxidant defence (GSH) and restoring the levels of biochemical and hematological parameters to their normal values. Further study on antioxidant enzyme activities and MDA levels in tissues is necessary to get a better idea on the antioxidant properties of ginger.

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Full Length Research Paper

Acute and sub-chronic pre-clinical toxicological study of *Averrhoa carambola* L. (Oxalidaceae)

Débora L. R. Pessoa, Maria S. S. Cartágenes, Sonia M.F. Freire, Marilene O. R. Borges and Antonio C. R. Borges*

Federal University of Maranhão, Physiological Science Department, Pharmacology Research and Post-Graduate Laboratory. Av. dos Portugueses. S/N, Bacanga, São Luís – Maranhão-Brazil, CEP 65085-582.

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Averrhoa carambola L., a species belonging to the Oxalidaceae family, is associated with neurological symptoms in individuals with renal diseases. The objective of this work was to accomplish a pre-clinical toxicological study of the hydroalcoholic extract (HE) from *A. carambola* leaves. Wistar rats and Swiss mice, both male and female, were used in these experiments. The rats were used in the acute toxicity assessment, with the extract administered at doses of 0.1 to 8.0 g/kg (oral route), and 0.5 to 3.0 g/kg (via intraperitoneal route). The mice received the extract in doses of 0.5 to 5.0 g/kg (via oral and intraperitoneal routes) and were observed for 14 days. Rats were also used in the sub-chronic toxicity evaluation, and divided into three groups (n=10): control group, HE 0.125 g/kg and HE 0.25 g/kg. These animals received HE for a 60 day period, at the end of which a macroscopic analysis of selected organs was performed with biochemical analysis of the blood. The acute toxicity assessment revealed that the HE of *A. carambola* L. presented low toxicity in the mice and rats. Furthermore, no signs of toxicity were present in the sub-chronic assessment.

Key words: *Averrhoa carambola* L., Oxalidaceae, acute toxicity, sub-chronic toxicity.

INTRODUCTION

The use of medicinal plants is common in popular culture, where it represents the result of centuries of accumulation of empirical knowledge on the action of plants by diverse ethnic groups. Some of the most valuable and most widely used medicines were developed from the accumulation of this knowledge (Simoes, 1989). The species *Averrhoa carambola* L., popularly known in Brazil as "carambola" (starfruit), belongs to the Oxalidaceae family. This family is comprised of eight genera distributed predominantly in the southern hemisphere, in the tropical and subtropical zones, and made up of trees cultivated for ornamental purposes because of their fruit (Carreira and Schatzmayr, 1982; Joly, 1979). Two species

are present in this genus: *A. carambola* L. and *Averrhoa bilimbi* L. The assessment of the physicochemical characteristics of the fruits of *A. bilimbi* L. showed the presence of oxalic acid and ascorbic acid (Lima et al., 2001). Hypolipidemic and hypoglycemic activities were observed using this plant species in Sprague-Dawley rats with induced diabetes (Tan et al., 2005) and the methanol extract from the leaves was shown to contain antioxidant activity (Abas et al., 2006).

Popularly, *A. carambola* L. is used as an anti-thermal, against the bites of poisonous animals, and as an anti-dysentery and anti-scurvy treatment. Chemical and phytochemical analyses performed on the plant revealed

*Corresponding author. E-mail: romao@ufma.br. Tel: +55 98 33018538.

Abbreviations: MPO, Myeloperoxidase; HE, hydroalcoholic extract; CT, control animals; HDL, high density lipoprotein; ALT, alanine aminotransferase; AST, aspartate amino transferase.

the presence of oxalic acid, vitamin C and tannins (Prance, 1975), moderate amounts of sugar, fiber, and calcium, as well as a low phosphorus and iron content (Oliveira et al., 1989), isoforms of the galactoside enzymes (Balasubramanian et al., 2005), volatile compounds (Macleod and Ames, 1990) and carotenoids (Gross et al., 1983). Several studies have reported that the ingestion of *A. carambola* L. fruit is related to the appearance of neurological symptoms in individuals affected by renal insufficiency, with symptoms including intractable hiccups, vomiting, paresthesias, paresis of the upper and lower limbs and several different disturbances related to consciousness present in varying degrees (Moysés et al., 1998; Tse et al., 2003; Moysés and Mep, 2004; Lo et al., 2001; Wu et al., 2002; Yap et al., 2002; Chan et al., 2002). Provasi et al. (2001) investigated the acute toxicity of *A. carambola* L. in doses of 3.5 g/kg in mice and 1.14 g/kg in rats by intra-gastric route and did not observe any deaths among treated animals in the 4 h following the administration of the extract. On the other hand, Signate et al. (2009) reported the cases of two patients with undiagnosed chronic renal insufficiency who developed severe encephalopathy after ingestion of star fruit. The two patients developed intractable hiccups, vomiting, impaired consciousness and status epilepticus. Diffusion-weighted magnetic resonance imaging showed cortical and thalamic hyperintense lesions related to epileptic status.

The use of insoluble fibers of the *A. carambola* L. fruit had beneficial effects on intestinal activity, leading to a decrease in the pH in the cecum, together with a decrease in the cecal and fecal ammonia levels (Chau and Chen, 2006). In addition to their effects on digestive system function, the insoluble fibers present in the fruit, when evaluated against glycemia *in vitro* demonstrated potential hypoglycemic activity, besides inhibiting the enzyme alpha amylase (Chau et al., 2004). Provasi et al. (2001) observed that a 30 mg/kg dose of extract from the leaves of *A. carambola* L. had anti-hypoglycemic activity in rats and when the authors examined the effect of the crude hydroalcoholic extract and isolated fractions of leaves on the glucose metabolism in Wistar rats treated for two weeks with a 20 mg/kg dose, they found a reduction in glycemia. However, when the extract's activities were investigated in isolated muscle, there was no stimulation of the production of glycogen and lactate (Provasi et al., 2005). In a study on the effect of the hydroalcoholic extract of the leaves on glycemia during fasting in rats treated orally (20 mg/kg), we found that the *A. carambola* L. extract lowered glycemia in comparison with the control group, while this reduction was not caused by inhibition of hepatic gluconeogenesis nor by an increase in glucose uptake by muscle (Ferreira et al., 2008).

Anti-inflammatory activity was observed in the skin of mice treated with the crude ethanolic extract of *A. carambola* leaves, as well as its hexane, ethyl acetate, and butanol fractions and two isolated flavonoids. The

activity was measured using a croton oil-induced ear edema model of inflammation, and topically applied ethanolic extract reduced edema in a dose-dependent manner, with a maximum inhibition of 73±3% and an ID₅₀ value of 0.05 (range: 0.02–0.13) mg/ear. Myeloperoxidase (MPO) activity was also inhibited by the extract, with a maximum inhibition of 60±6% (0.6 mg/ear). All of the fractions tested inhibited edema formation as well as MPO activity, although treatment with the ethyl acetate fraction was the most effective, resulting in inhibition of 75±5 and 54±8% for edema formation and MPO activity, respectively. However, treatment of mice with isolated compounds [apigenin-6-Cb- L-fucopyranoside and apigenin-6-C-(200-O-a-L-rhamnopyranosyl)-b-L-fucopyranoside] did not yield notable results, with the latter compound causing only a mild reduction in edema formation (28± 11%) (Cabrin et al., 2011).

In another work, Khoo et al. (2010) examined the toxic effect of *A. carambola* juice, kept under different storage conditions, in Sprague Dawley (SD) rats, observing no deaths and consequently being unable to determine the LD₅₀ for the juice. The authors did report an increase in alanine aminotransferase (ALT) levels ($P < 0.05$) in those rats treated with *A. carambola* juice stored for 3 h.

Studies that examine the potential toxicity of *A. carambola* L. remain scarce, which explains the importance of assessing its toxic effects in experimental studies. The objective of this work was to assess the acute and sub-chronic toxicity of the hydroalcoholic extract of *A. carambola* L. leaves in rats and mice.

MATERIALS AND METHODS

Plant material and extract preparation

The *A. carambola* L. leaves were collected from the municipal area of Paço do Lumiar, in the state of Maranhão, Brazil in February 2006, and identified at the "Atico Seabra" herbarium (Federal University of Maranhão), where a voucher specimen was registered under number 0561 SLS/MA. The hydroalcoholic extract (HE) was obtained from the dehydrated and ground leaves by maceration in 70% ethanol for 72 h. Next, the extract was concentrated in a rotary evaporator at a reduced pressure and at a temperature of 50°C. Finally, the dry weight of the extract was determined.

Animals

The experiments were carried out in Wistar rats (*Rattus norvegicus*), between 60 and 90 days of age, weighing between 220 and 360 g (male) and between 135 and 260 g (female), and Swiss mice (*Mus musculus*), between 60 and 90 days of age, weighing between 26 and 32 g (male) and between 21 and 28 g (female), supplied by the Central Animal Facility of the Federal University of Maranhão. These animals were divided into groups of 6 to 10, including both males and females, according to the experiment performed. The control animals (CT, rats and mice respectively) received water at 0.1 ml/100 g or 0.1ml /10g body weight and the remaining animals received HE at varying doses. The results obtained with the HE are compared to the control animals. Animals were handled based on the guidelines of the animal ethics committee of the Universidade Estadual do Maranhão (UEMA), who approved this research (license number 002/ 2008).

Assessment of acute toxicity

The experimental protocols for the assessment of acute toxicity were mainly based on the Specific Resolution (SR 90/2004) of the National Sanitary Vigilance Agency (ANVISA; Brazil, 2004). Rats and mice were divided into five or six experimental groups with ten animals each (five male and five female), and increasing doses of extract were administered. The rats received the following via intraperitoneal route (i.p.): water (CT group), *A. carambola* L. hydroalcoholic extract at doses of 0.5 g/kg (group HE 0.5), 1.0 g/kg (group HE 1.0), 2.0 g/kg (group HE 2.0) and 3.0 g/kg (group HE 3.0). By oral route (v.o.), they were given: water (CT group), *A. carambola* L. HE at doses of 1.0 g/kg (group HE 1.0), 2.0 g/kg (group HE 2.0), 5.0 g/kg (group HE 5.0) and 8.0 g/kg (group HE 8.0). After administration of the HE, the animals remained under observation for a 14-day period to monitor signs of toxicity, according to criteria established by Malone (1997). The animals were weighed weekly by group (sex/dose), with the mean value for each group being recorded. At the end of this period, the surviving animals were sacrificed, and had the kidneys, heart, liver and spleen weighed and subjected to a macroscopic analysis.

Assessment of sub-chronic toxicity

The rats (*R. norvegicus*) were divided into three groups of six animals each (three male and three female). The groups were treated (v.o) with *A. carambola* L. HE at doses of 0.125 g/kg (HE 0.125) or 0.25 g/kg (HE 0.25), or water, for 60 days. During this interval, the body weight and consumption of food were determined three times a week, and the average weekly values of each group (sex/dose) were recorded. The behavioral parameters were also measured daily. At the end of the treatment period, the animals were subjected to a 12 h fast for subsequent blood collection and analysis of the following biochemical parameters: Glucose, urea, creatinine, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, albumin, total proteins, aspartame amino transferase, alanine amino transferase and alkaline phosphatase. Afterwards, the animals were sacrificed and selected organs (kidneys, lungs, heart, liver, spleen and pancreas) were analyzed macroscopically for the detection of possible alterations caused by the *A. carambola* L. extract.

Statistical analysis

The results of the experiments were expressed as mean \pm standard error of the mean and submitted to analysis of variance (ANOVA), followed by Newman Keuls test, considering significant differences between the groups when $p \leq 0.05$. The analyses were done with the aid of the GraphPad Prism 3.0 program (GraphPad Prism, 1999).

RESULTS

Preparation of hydroalcoholic extract (HE)

The HE prepared from the *A. carambola* L. leaves presented a dark green color, with a residue that was easily soluble in water and a yield of 17.6%.

Assessment of acute toxicity

Mice

Among the animals treated by oral route who received the lowest doses (0.5 and 1.0 g/kg), no significant changes

were observed, similar to the animals in the control group. In relation to the animals treated with the doses of 2.0 and 5.0 g/kg, in the first few hours following treatment, some signs of toxicity were observed including convulsions and tachycardia (Table 1). From the second day, the animals were found to be sedated, although the intensity of this effect was not proportional to the dose employed. These effects occurred in both sexes, although the only animals that died were both female, one at each dose of HE over the course of the 14 days of observation (Table 1). No change in body weight was seen in the animals at any of the doses employed (Table 2). As a result of this profile of mortality, the mean lethal dose (LD₅₀) could not be determined in these animals. Among the mice treated with the HE by intraperitoneal route that received the lowest doses (0.5 and 1.0 g/kg), no significant changes were noted; however, in the animals treated with the doses of 2.0 and 5.0 g/kg, some signs of toxicity such as abdominal pain, grouping, convulsions and tachycardia were observed in the first few hours after treatment, and these can be seen in Table 1. From the second day onwards, the animals exhibited sedation and death proportional to the dose employed. These effects occurred in both sexes, although mortality only occurred in females at a dose of 2.0 g/kg, and in males and females at a dose of 5.0 g/kg, as shown in Table 1. Once again, as a result of the profile of mortality in these animals, the mean lethal dose (LD₅₀) could not be determined. Significant weight loss was not seen in the animals (Table 2). Macroscopic examination of the kidneys, heart, liver and spleen of the surviving animals did not reveal any relevant changes. Furthermore, there were no changes in the weights of organs as shown in Table 3.

Rats

The rats that received higher doses of HE by the intraperitoneal route (2.0 and 3.0 g/kg) presented a mortality index proportional to the dose, as can be seen from Table 4. The LD₅₀ of the HE of *A. carambola* L. in rats treated by this route was 1.49 g/kg. Among the treated animals that were given the lowest doses (0.5 and 1.0 g/kg), no significant behavioral changes were observed, which was a similar result to that seen in the control group. In relation to the rats treated with the doses of 2.0 and 3.0 g/kg, in the first few hours after treatment, some signs of toxicity were observed which are presented in Table 4; these were piloerection, writhing, sedation and depression. With this treatment the animals died within a few hours of the administration of the extract. Among the rats treated by oral route no behavioral changes were noted and none of the animals died (Table 4). The acute toxicity in rats was not associated with any detectable macroscopic changes in the organs analyzed (kidneys, liver, spleen, heart and pancreas) after the 14 days of observation. Small superficial inclusions of extract were found in the liver of the only surviving animal treated by

Table 1. Effect of single oral and intraperitoneal dose of *Averrhoa carambola* L. extract in mice.

Route – dose (g/kg)	Gender	D/T	Latency (h)	Symptom
Oral route				
Control	M	0/5	-	None
	FM	0/5	-	None
0.5	M	0/5	-	None
	FM	0/5	-	None
1.0	M	0/5	-	None
	FM	0/5	-	None
2.0	M	0/5	4-48h	Tachycardia, convulsions and sedation
	FM	1/5	4-48h	Tachycardia, convulsions and sedation
5.0	M	0/5	4-48h	Tachycardia, convulsions and sedation
	FM	1/5	4-48h	Tachycardia, convulsions and sedation
LD ₅₀ = -				
Intraperitoneal route				
Control	M	0/5	-	None
	F	0/5	-	None
0.5	M	0/5	-	None
	F	0/5	-	None
1.0	M	0/5	-	None
	F	0/5	-	None
2.0	M	0/5	2-48	Grouping, writhing, tachycardia, convulsions
	F	1/5	2-48	Grouping, writhing, tachycardia, convulsions
5.0	M	3/5	2-48	Grouping, writhing, tachycardia, convulsions
	F	4/5	2-48	Grouping, writhing, tachycardia, convulsions
LD ₅₀ = -				

All treated mice (n=10 in each group; 5 males and 5 females) were carefully examined for up to 14 days after the treatment for adverse effects including behavioral changes, lethality and latency of death. D/T, number of dead mice / number of treated mice; none, no symptoms observed during the observation period; latency, time to onset of effects after the treatment; M, male; FM, female.

intraperitoneal route with the dose of 2.0 g/kg. No changes in organ weights were observed.

Assessment of sub-chronic toxicity

The animals treated with the HE of *A. carambola* L. for 60 days at the doses of 0.125 and 0.25g/kg did not present any behavioral changes during the period of treatment. In

addition, no deaths were recorded during the treatment. In relation to the body weight of the animals, treatment with the HE of *A. carambola* L. did not cause any change in this parameter (Table 5). The profile of food consumption was also found to be unaltered in the treated animals, when compared to the control group. Treatment with the extract of *A. carambola* L. did not lead to any macroscopic changes in the vital organs (kidneys, lung, heart, liver, spleen and pancreas) or to changes in the

Table 2. Effect on body weight in mice after single oral administration of *Averrhoa carambola* L. extract.

Group	Sex	D ₀ (g)	D ₇ (g)	D ₁₄ (g)
Control	M	26.26± 1.09	28.00± 1.32	27.74± 1.14
	F	19.86± 0.78	19.98± 0.70	20.46± 0.75
<i>A. carambola</i> (0.5 g/kg)	M	24.68± 1.41	26.10± 1.66	26.60± 1.69
	F	21.16± 0.89	21.42± 0.90	22.12± 0.92
<i>A. carambola</i> (1.0 g/kg)	M	26.80± 2.91	27.42± 2.16	27.74± 2.24
	F	22.44± 1.90	22.88± 1.80	23.24± 1.76
<i>A. carambola</i> (2.0 g/kg)	M	27.80± 2.46	28.07± 2.32	28.35± 2.15
	F	20.70± 0.71	21.15± 0.47	21.73± 0.49
<i>A. carambola</i> (5.0 g/kg)	M	26.75± 1.65	28.00± 1.95	27.70± 2.25
	F	21.05± 1.30	21.80± 1.37	22.60± 1.38

All treated mice (n=10 in each group; 5 males and 5 females) were carefully examined for up to 14 days after the treatment. Data are expressed as mean ± S.E.M. No significant difference between control and the extract (P≤0.05).

Table 3. Weight of organs from surviving mice 14 days after administration of a single dose of the extract of *Averrhoa carambola* L.

Group	Kidney (g)	Heart (g)	Liver (g)	Spleen (g)
Control	0.41± 0.02	0.13± 0.004	1.64± 0.09	0.10± 0.006
<i>A. carambola</i> (0.5 g/kg)	0.41± 0.02	0.14± 0.004	1.64± 0.10	0.10± 0.006
<i>A. carambola</i> (1.0 g/kg)	0.41± 0.02	0.13± 0.005	1.68± 0.12	0.10± 0.006
<i>A. carambola</i> (2.0 g/kg)	0.40± 0.09	0.12± 0.005	1.29± 0.10	0.11± 0.01
<i>A. carambola</i> (5.0 g/kg)	0.40± 0.08	0.14± 0.01	1.37± 0.04	0.11± 0.007

The values represent the means of the organ weights (in grams) ± S.E.M. All treated mice (n=3-10 in each group; males and females) were carefully examined for up to 14 days after the treatment. No significant difference was between control and the extract (P≤0.05).

Table 4. Effect of single oral and intraperitoneal dose of *Averrhoa carambola* L. extract in rats.

Route – dose (g/kg)	Sex	D/T	Latency (h)	Symptom
Oral route				
Control	M	0/5	-	None
	FM	0/5	-	None
1.0	M	0/5	-	None
	FM	0/5	-	None
2.0	M	0/5	-	None
	FM	0/5	-	None
5.0	M	0/5	-	None
	FM	0/5	-	None
8.0	M	0/5	-	None
	FM	0/5	-	None
LD ₅₀ = -				
Intraperitoneal route				
Control	M	0/5	-	None
	FM	0/5	-	None
0.5	M	0/5	-	None
	FM	0/5	-	None
1.0	M	0/5	-	None
	FM	0/5	-	None

Table 4. Contd.

2.0	M	4/5	2-4	Piloerection, Sedation, Writhing, Convulsions
	FM	5/5	2-4	Piloerection, Sedation, Writhing, Convulsions
3.0	M	5/5	2-4	Piloerection, Sedation, Writhing, Convulsions
	FM	5/5	2-4	Piloerection, Sedation, Writhing, Convulsions

LD₅₀= 1.49 g/kg

All treated rats (n=10 in each group; 5 males and 5 females) were carefully examined for up to 14 days after the treatment for adverse effects including behavioral changes, lethality and latency of death. D/T, Number of dead rats / number of treated rats; none, no symptoms observed during the observation period; Latency, time to onset of effects after the treatment; M, male; FM, female.

Table 5. Effect on body weight in rats after daily oral administration of *Averrhoa carambola* L. extract.

Group	Sex	D ₀	D ₁₅	D ₃₀	D ₄₅	D ₆₀
Control	M	234.6± 14.44	235.4± 15.34	238.9± 13.77	257.0± 18.96	278.6± 14.26
	FM	212.4± 12.07	213.5± 12.07	217.4± 11.54	241.2± 11.70	257.5± 11.88
<i>Averrhoa carambola</i> (0.125 g/kg)	M	245.5± 4.58	248.6± 5.11	252.1± 0.73	242.3± 6.63	276.8± 9.67
	FM	215.6± 4.62	218.7± 4.92	225.4± 3.94	212.4± 6.63	246.9± 9.65
<i>Averrhoa carambola</i> (0.25 g/kg)	M	229.4±7.93	231.5±8.17	235.1±7.98	262.2±6.69	271.6±8.36
	FM	202.3± 5.24	204.9± 5.62	199.5± 3.59	232.5± 6.81	241.9± 8.43

All treated mice (n=6 in each group; 3 males and 3 females) received the extract orally at daily doses of 0, 0.125 and 0.25 g/kg for up 60 days. Data are expressed as mean ± S.E.M. No significant difference was between control and the extract (P≤0.05).

Table 6. Effects of daily oral administration of *Averrhoa carambola* L. extract for up 60 days on the biochemical parameters of rats.

Parameter	Control	0.125 g/kg	0.25 g/kg
COL – T (mg/dL)	57.4±5.62	57.5±5.50	75.7±7.51
GLI (mg/dL)	152.2±14.43	109.5±11.50	186.3±24.6
ALB (g/dL)	2.89±0.18	2.74±0.46	2.78±0.12
TRI (mg/dL)	73.50±15.61	49.33±21.79	88.00±23.64
AST (U/l)	100.3±6.65	130.5±31.50	107.7±20.10
ALT (U/l)	36.25±4.75	39.00±8.00	22.00±7.00
URE (mg/dL)	878.8±50.01	848.5±8.50	778.7±41.33
CRE (mg/dL)	1.03±0.11	1.25±0.05	1.00±0.057
PT (d/dL)	9.08±1.12	7.75±0.95	7.20±0.40
ALP (U/l)	44.25±10.09	29.00±1.00	48.67±7.42
COL – HDL (mg/dL)	9.75±1.65	4.33±2.60	6.00±2.65

Data are expressed as mean ± S.E.M (n=6). COL-T, Total cholesterol; GLI, glucose; ALB, albumin; TRI, triglyceride; AST, aspartate aminotransferase; ALT, alanine amino transferase; URE, urea; CRE, creatinine; PT, total protein; ALP, alkaline phosphatase; COL- HDL, high density lipoprotein. *p≤0.05 compared with the control group. No significant difference was between the control and the *Averrhoa carambola* L. extract (p≥0.05).

weights of the organs. At the end of the treatment, analysis of the blood of these animals showed no statistically significant changes in the selected biochemical parameters (Table 6).

DISCUSSION

In many low and middle income countries, folklore or herbal medicine often represents the popular therapeutic

system to which people are referred for their primary health care. The use of herbal remedies is further substantiated by its affordability, knowledge of medicinal plants and the belief that they are harmless. The increase in the number of users as opposed to the scarcity of scientific evidence on the safety of medicinal plants has raised concerns regarding toxicity and detrimental effects of these remedies. Medicinal plants commonly contain various bioactive principles which have the potential to cause beneficial and/or harmful effects. To optimize their safe use as plant-based medicines, we should, despite the historical experience of application in humans and animals, evaluate the toxicity of these medicinal herbs (Li et al., 2010). The assessment of the extract of *A. carambola* L. in this study was prompted by the fact that this form of plant medicine closely mimics the traditional dosage form, while it also represents a convenient form that can be standardized in terms of its chemical or physical constituents, and also is easily stored. Thus, the assessment of the safety of this dosage form of *A. carambola* L. is greatly facilitated. The present study was designed to investigate the pre-clinical toxicological effects of the hydroalcoholic extract of the leaves of *A. carambola* L. administered by oral (acute and subchronic effects) and intraperitoneal (acute effect) routes in order to determine the safety of the extract.

The goal of tests of acute toxicity is to define the lethal dose of a drug administered in a single dose or in several doses intercalated over a short period of time, as part of the initial pharmacological triage during which the action of the drugs on important functions is observed (Déciga-Campos et al., 2007). In addition, toxicity assessments are carried out with the objective of determining the potential of new substances and products to cause harm to human health. Tests of acute systemic toxicity are used to classify and appropriately label substances according to their lethal or toxic potential as established by relevant legislation (Valadares, 2006).

Among the animals in the control groups (rats and mice), no significant physiological changes were observed in either of the sexes. The mice that received the HE at lower doses (0.5 or 1.0 g/kg) also did not present significant changes. By contrast, the mice treated with HE at doses of 2.0 or 5.0 g/kg presented signs of toxicity, such as convulsions and tachycardia, in the first four hours after the treatment. From the second day on, the animals appeared sedated. These effects occurred in both sexes; however, the only animals that died were two females (Table 1). In the mice that received the HE via intraperitoneal route, behavioral changes were observed with doses of 2.0 or 5.0 g/kg HE in the first four hours after treatment, including writhing, grouping and convulsions. On the second day after treatment, the animals presented sedation and death proportional to the dose employed (Table 1). No significant change in body weight was observed in these animals (Table 2). Macroscopic examination of the kidneys, heart, liver and spleen of the survi-

ving animals did not reveal any relevant changes; likewise, there was no significant change in the weight of these organs (Table 3). The study by Provasi et al. (2001) did not record any death among mice treated with a 3.5 g/kg dose; however, in that study, the period of monitoring of the animals was limited to four hours. This is important since although toxic effects can appear in multiple systems and organs, it is the liver that is most heavily affected both because of its anatomical position and because its cells concentrate the compounds to be metabolized, together with the resulting metabolites and the enzymes responsible for the metabolic process (Mendes, 1988).

In the rats treated orally, there were no behavioral changes nor deaths with the doses employed (Table 4), nor were there macroscopic or weight changes in the organs analyzed after 14 days of observation. The rats that received HE via the intraperitoneal route at doses of 2.0 and 3.0 g/kg presented an index of mortality proportional to the dose used (Table 4), and the mean lethal dose (LD₅₀) of HE for the rats treated via intraperitoneal route was 1.49 g/kg. The signs of toxicity presented by these animals were piloerection, writhing, sedation and depression. Moreover, the animals died in the first two hours after receiving the extract. We know that the value of the mean lethal dose as a fundamental parameter for the definition of chemical toxicity is limited, since this test measures only the acute toxicity produced by a single dose, and not long-term toxicity, and also does not measure individual reactions (Rang et al., 2003). Besides this, doses in excess of the maximum dose for the evaluation of this parameter, which is 2 g/kg (Larini, 1997), were used in this work.

Studies of sub-chronic toxicity are also performed in the pre-clinical evaluation of drugs, in an attempt to identify any damage that the use of the drug under consideration could cause to biological systems in the medium- and long-term (Sorrentino et al., 2006). The animals treated with the HE of *A. carambola* L. leaves for 60 days at doses of 0.125 and 0.25 g/kg did not present any behavioral alterations or deaths in the treatment period. No significant changes in the weight of the animals were noted (Table 6); likewise, there were no significant changes in food consumption in the groups that received HE compared to the control group. There was no apparent effect of the *A. carambola* L. HE on the selected organs, nor did they show any macroscopic alterations or changes in weight.

In the analysis of blood samples, no statistically significant changes were observed in the biochemical parameters in the animals treated with HE, compared to the control group (Table 6). The assessment of the sub-chronic toxicity of *A. carambola* L. involved examining liver function by assay of ALT, aspartate amino transferase (AST) and alkaline phosphatase (ALP), with no changes being found in relation to the control group at the doses employed. These data are similar to those

reported by Provasi et al. (2001), who did not find significant changes in the levels of ALT and AST in rats. In the small animal clinic, abnormalities are commonly detected in the serum levels of these enzymes, which are considered sensitive indicators of hepatobiliary disturbances (Sharon and Center, 1995).

A rise in serum enzyme activities may result from reversible or irreversible changes in cell permeability, the induction of microsomal enzymes or structural damage associated with necrosis, cholestasis or hepatocellular ischemia. Numerous pathological processes involving the liver can lead to proportionally distinct rises in liver enzymes, due to variation in the distribution of each specific enzyme in the hepatic lobule (Sharon and Center, 1995).

The transaminases are enzymes that catalyze the interconversion of amino acids and alpha-keto acids by transfer of the amino group. They are widely distributed throughout the tissues, with AST predominantly found in the liver, heart, cardiac muscle, striated muscle, kidney and pancreas, while ALT is predominantly seen in the liver, kidney and heart. AST is present in the cytosol as well as in the mitochondria of hepatocytes. The plasma activity of this enzyme is controlled by an enzymatic release mechanism localized to the cell membrane of the hepatocyte. The rate of excretion of the transaminases is variable, with AST being eliminated more quickly than ALT (Miller and Gonçalves, 1999). The increased levels of amino transferases in the serum of animals treated with the extract may have resulted from the release of enzymes from the cells of the organ affected, or from a change in cell permeability (Rebecca et al., 2002). With regard to *A. carambola* L., to our knowledge, only one study in the literature has investigated the effect of acute toxicity of the hydroalcoholic extract of the leaves in rats and mice, with the authors showing that the hepatic enzymes (ALT) and AST were unchanged in relation to the control group (Provasi et al., 2001).

ALP is a phosphohydrolase enzyme found in various tissues, with higher concentrations in the liver, the epithelium of the biliary tract and in the bones. In the liver, ALP is secreted by the hepatocytes and by the cells of the mucosa of the biliary tract. In general, any active hepatopathy can increase the values of ALP, but the greatest increases in the levels of the enzyme occur in cases of obstruction of the biliary tract. In the case of hepatotoxic drugs, there is a smaller rise in enzyme levels (Miller and Gonçalves, 1999).

Urea is produced in the liver and excreted by the kidneys. When kidney filtration is insufficient, urea accumulates in the blood. Some drugs are capable of enzyme induction, and can consequently increase toxicity. According to literature, biochemical assays that show increased urea and creatinine in animals subjected to treatment with plant-based drugs may indicate that the effect of the drug interfere with the metabolism and function of the kidney (Baliga et al., 2004). Raised levels of total cholesterol and triglycerides or low levels of HDL are related to

the risk of cardiovascular disease. Subchronic treatment with the HE of *A. carambola* L. did not alter these parameters. This result may be explained by the fact that rats are resistant to the development of hypercholesterolemia and atherosclerosis, possibly as a result of the increased conversion of cholesterol to biliary acids in the liver (Soares et al., 2005).

It should be emphasized that the interpretation of laboratory exams is much more complex than a simple comparison with reference values, classifying the test results as normal or abnormal according to the limits for the reference, and then comparing the results with standards that indicate the presence of certain diseases (Ravel, 1997). With regard to reference data in laboratory animals, the analysis and interpretation of the results are even more difficult, since the scientific literature lacks reference values for laboratory analyses in the animal species used in the present work. Consequently, the results obtained following the treatment of animals with the HE of *A. carambola* L. were compared by statistical methods with the data obtained in the control groups, according to methodology employed by Daher et al. (2006) and Thanabhorn et al. (2006), who assessed the toxicity of plants in animals and used their own control groups as references of normality in the analysis of their results.

Conclusion

The present acute and sub-chronic pre-clinical toxicological study in Wistar rats and Swiss mice using the hydroalcoholic extract of *A. carambola* L. leaves demonstrated that the plant species investigated has relatively low sub-chronic and acute toxicity in these animals.

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Full Length Research Paper

Recovery of water from cacti for use in small farming communities

Corneels Schabort*, Aryna Otto, Morné Bothma, Percy van der Gryp and Sanette Marx

Energy Systems, School of Chemical and Mineral Engineering, North-West University, Hoffman Street, Potchefstroom, South Africa.

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In this study, an extensive investigation was conducted to determine if declared weeds could be used as a source of water for agricultural practices in dry areas. The objective of this study was to determine if declared weeds could successfully be used as a source of water for agricultural practices in dry areas by extracting the water by means of mechanical and chemical methods. The *Cereus jamacura* cactus, also known as Queen of the Night, with a moisture content of 91 wt%, was selected for this study. Both mechanical and chemical extraction methods were used to determine the maximum water yield possible. Juicing, pressing with a hydraulic cold press and pressing with rollers were used as mechanical methods to extract water from the cacti and water yields of 7.0, 4.9 and 2.9 wt% were obtained respectively. The chemical extraction processes entailed the pulping of the cacti and the filtering off of the water. The effect of pectinase, cellulase and a surfactant at a fixed dosage on the amount of water extracted (mass of water per mass of cacti used) was investigated. The quality of the water was also determined. Temperature (30 to 50°C) and pH (2.5 to 6.5) were varied to find the optimum extraction conditions. The highest water yield (55 wt% of total cacti mass) was obtained using pectinase enzymes at a temperature of 40°C and a pH of 3.5 and cellulose enzymes at a temperature of 35°C and a pH of 5.5. This relates to a yield of 550 L of water per ton of cacti, making chemical water extraction a viable option if compared to the pollution created by the annual burning of the cacti. It was concluded from this study that the water that was extracted from the *C. jamacaru* cacti would not be suitable for either domestic or industrial application due to the high levels of potassium (up to 2,650 ppm), phosphates (up to 2,200 ppm), sulphates (up to 3,800 ppm) and nitrates (up to 670 ppm) in the water. The high concentration of phosphates and nitrates, however, makes the extracted water an excellent fertiliser for crops requiring high nitrate and phosphate dosages. Small community farmers could thus benefit by using cacti as a source of water for small scale biofuels production plants while also obtaining an excellent additional fertiliser for crop cultivation.

Key words: *Cereus jamacaru*, water yield, water quality.

INTRODUCTION

The availability of water for domestic, industrial and agricultural application is a growing worldwide concern, with an increasing number of areas being chronically short of water (UNESCO, 2012). Climate change, drought

and desertification have impacted large parts of the Southern African region, especially areas where people are primarily dependant on natural resources (Dougilla et al., 2009). As the 30th driest country in the world, with an

*Corresponding author. E-mail:12380687@nwu.ac.za. Tel:+27 18 299 1760. Fax:+27 18 299 1535.

average rainfall of just 450 mm per annum, South Africa faces the same water scarcity problem as the rest of the world (DEA, 2011). Exploring available water sources in arid areas that are not yet being utilised is thus of utmost importance.

Cacti are succulent plants due to their ability to store water in their tissues. The efficient storage of water in the cacti's stem, body, leaves and roots, as well as the plant's low water consumption, allow cacti to resist extreme desert conditions (Andreote et al., 2013). Certain South African cacti species, like *Cereus jamacura*, *Opuntia ficus-indica*, *Opuntia imbricata* and *Echinopsis spachiana*, are further classified as invasive plants and declared as category 1 weeds according to the South African Conservation of Agricultural Resources Act (43 of 1983). These cacti species are targeted for control due to their serious environmental impact, including higher usage of water, blockage of water passages, erosion, as well as the reduction in the specific environment's biodiversity.

The Thusanang area, situated in South Africa's North-West province, is a very dry region where declared cacti species grow abundantly. Currently both farmers and members of the rural community merely cut down these cacti, dry the leaves and use the dried leaves as fuel for cooking and heating purposes. Not only is the valuable water not recovered from the cacti, but the burning of this plant material increases greenhouse gas emissions.

In previous studies by Costa et al. (2012), Degu et al. (2009) and Gebremariam et al. (2006) cacti were shown to be successfully used as an animal feed supplement. The objective of this study is to determine whether water can be effectively extracted from the cacti using mechanical and chemical extraction processes and whether the extracted water can be utilised for domestic, industrial or agricultural purposes. The focus is placed on *Cereus jamacura*, which is found in the Thusanang area. *Cereus jamacura* is often used as a cattle fence by South African farmers. In studies by Díaz-Medina et al. (2012) and De Albuquerque et al. (2008) the potential of the cactus's medical application was indicated.

Mechanical methods used for recovering juice from fruit include chopping, pressing, diffusion and centrifugal processes. The purpose of these methods is to separate the liquid phase from the solid phase (Hui et al., 2002). It is proposed to use juicing, pressing with a hydraulic cold press and pressing with rollers as mechanical methods to extract water from the cacti. The three processes were selected based on the simplicity, low maintenance and low capital input required.

Juicing using a juicer or an extractor produces a murky liquid product that contains no suspended solids (Barrett et al., 2004). Juicing by rotary methods increases the amount of polysaccharides (including pectin and cellulose) extracted from the pulp to the liquid product (Hui et al., 2002).

The hydraulic cold press is used in small juicing companies and was the only fruit juicing method for many

years (Barrett et al., 2004). Roller presses work by pushing two cylinders or rollers against each other and sending fruit pieces between the two rollers while the rollers are turning (Tzia and Laidakis, 2003). The resulting liquid products of both the hydraulic cold press and the roller press are clearer than the liquid product obtained by rotary methods (Hui et al., 2002).

Chemical extraction techniques, on the other hand, are also well documented in literature and readily available. The combined synergetic use of pectinase and cellulase further increases the extraction yield of the juice (Abbès et al., 2011). In a study by Sreenath et al. (1994) the addition of cellulase and pectinase increased the recovery of juice from pineapples by up to 14%. Demir et al. (2001) did a similar study and found that pectinase increased juice yield from carrots by 17.7%. Furthermore, surfactants also play an important role in breaking down cellulose in plants and fruit. In a study by Yang et al. (2011) it was shown that a surfactant can increase the hydrolysis of cellulose at high rotation speeds.

Based on these successful applications of cellulase, pectinase and surfactants, it is proposed to improve water extraction from the pulped cacti by using these extraction agents. Cellulase and pectinase are both enzymes which are generally used in food processes where juice is extracted. Each enzyme is known for its ability to break down specific components in the structures of fruit and was therefore suitable for the specific study. The surfactant, on the other hand, is a known polysorbate which is used in a variety of industries, including healthcare and detergents.

MATERIALS AND METHODS

C. jamacura cacti were collected from the Thusanang rural community. The cacti pieces used were the leaves chopped off from the full-grown plants. Celluclast 1.5 L (Novozymes) with a cellulose content of 15 wt% and Pectinex Ultra SP-L (Novozymes) with a polygalacturonase content of 5 wt% were used as enzymes. Tween 80 (Merck), with a saponification number of 45 to 55 mg KOH/g and a hydroxyl number of 65 to 80 mg/g, was used as surfactant. NaOH (98%, Labchem) and H₂SO₄ (98%, Labchem) were used to adjust pH. All chemicals were used without further purification.

Moisture content

The moisture content of the cacti was determined by baking the chopped leaves in baking pans in an oven at 105°C for 24 h. The dry mass was subtracted from the wet mass to determine the moisture content.

Mechanical extraction of water

A Russell Hobbs Juice Sensation juicer was used for the juicing of the cacti and the extracted juice's weight recorded. A custom-built hydraulic cold press (Figure 1) was used for pressing the cacti cladodes. The cacti cladodes were cut into chunks to fit into the 8.5 cm diameter cylinder of the cold press. The water was collected in



Figure 1. Custom-built hydraulic cold press.



Figure 2. Custom-built roller press.



Figure 3. Chopped cactus leaves without thorns or bad spots.



Figure 4. Juicing the chopped cactus leaves using a Russell Hobbes juicer.

the collector ring and the weight of the extracted water recorded. A custom-built roller press (Figure 2) was used for roll pressing of the cacti. A glass beaker was placed underneath the rollers to collect the extracted water.

Chemical extraction of water

The experimental procedure consisted of two stages. The first

stage entailed the juicing of the cactus leaves. First of all, the thorns and bad spots were removed from the leaves. The leaves were then chopped into smaller pieces, as shown in Figure 3. For the juicing step, a Russell Hobbes juicer was filled with small cactus pieces (Figure 4) to produce a pulp medium, as shown in Figure 5.

The second stage entailed the extraction of the water from the cactus pulp. The experimental extraction method for all three

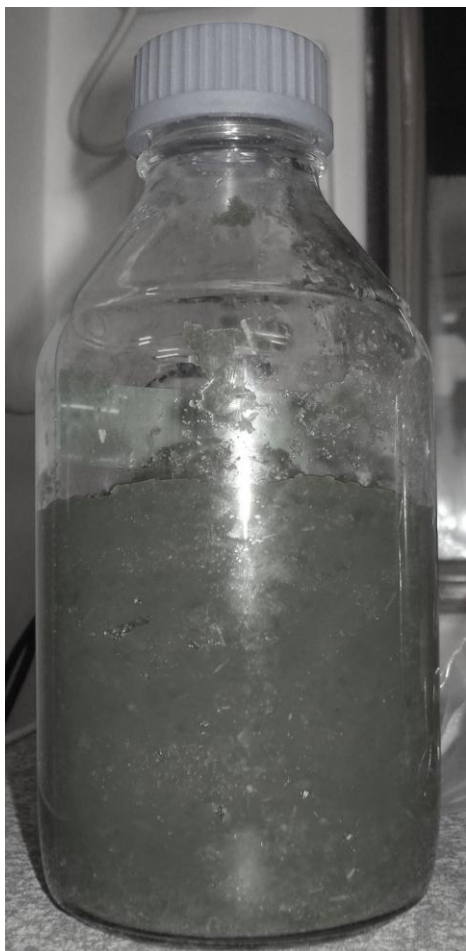


Figure 5. Cactus pulp.

extraction agents (Celluclast 1.5L, Pectinex Ultra SP-L and Tween 80) is identical. Initially, a Duran flask (1000 ml) was filled with 200 g of cacti pulp. A Scientific Series 2000 oven was used to accurately maintain the predetermined temperature during the experiment. The pH was adjusted throughout using NaOH and H₂SO₄.

The optimum extraction conditions were determined by investigating the influence of temperature and pH on the extraction of water from the cacti pulp. All experiments were executed at a constant extraction agent dosage of 14 g for the enzymatic experiments (0.07 wt/wt% for both the Celluclast 1.5L and Pectinex Ultra SP-L) and 0.25 g for the surfactant experiments (1.25 g/kg for the Tween 80).

The pH dependant experiments were executed at a constant temperature of 40°C. The pH was controlled at five specific values, ranging from 2.5 to 6.5. All of the temperature dependant experiments were carried out at a constant pH of 4.5. The temperature was controlled at five specific values, ranging from 30 to 50°C.

The duration of the experiments included an oven time of an hour at the specific temperature and pH. Afterwards the oven temperature was increased to 99°C for 20 min to ensure that the enzymes undergo a process of denaturation. After a cooling period, the water and solid pieces were separated by means of a Büchner filter. A compositional analysis was done on a sample of the filtered water. For the determination of the total dissolved solids (TDS), micro-filtration was performed using 0.22 µm filters.

RESULTS AND DISCUSSION

Moisture content

Most cacti consist of about 90 wt% water (Stuart, 2009). The moisture content of *C. jamaicura* was found to be 90.9 wt% (± 1.28 wt%). The high moisture content is comparable with studies on other cacti species. Gebremariam et al. (2006) found *Opuntia ficus-indica* to have a moisture content of 88.0 wt%.

Mechanical extraction

Poor water yields were obtained by means of mechanical extraction. Juicing yielded 7.0 wt% water, while 4.9 and 2.9 wt% were obtained with the hydraulic cold press and the rollers respectively. The juicer has the highest water yield of the three mechanical extraction methods, even though the liquid product is very thick and slimy. The water extracted with the cold press was a clear liquid, due to the lower cellulose and mucilage polysaccharides (Hui et al., 2002). The very low yield obtained by means of the rollers can be explained by insufficient pressure exerted on the cactus cladodes.

Chemical extraction

The effect of pH

The pH affects the water yield for both the enzymes and the surfactant. In Table 1 it can be seen that in the case of Celluclast 1.5 L, the water yield increased as the pH increased and decreased again beyond the optimum pH of 5.5. The maximum water yield of 55 (± 13.3 wt%) was obtained at a pH of 5.5. The water yield remained constant over the pH range in the case of Pectinex Ultra SP-L, with a maximum yield of 55 (± 22.6 wt%) at a pH of 3.5. Tween 80 shows an opposite trend if compared to that of Celluclast 1.5 L. With an increase in pH, the water yield decreased. The maximum water yield with Tween 80 of 50 (± 9.3 wt%) is obtained at a pH of 3.5.

The temperature also affects the water yield for both the enzymes and the surfactant. In Table 2, it can be seen that the maximum water yield of 55 (± 33 wt%) and 50 (± 24 wt%) was obtained for Celluclast 1.5 L and Tween 80, respectively at a temperature of 35°C. In the case of Pectinex Ultra SP-L, a maximum yield of 51 (± 31.1 wt%) was obtained at 40°C.

Water quality

The quality of the extracted water was analysed to determine whether the water would be suitable for domestic, industrial or agricultural use. The concentration of specific constituents, the total dissolved solids (TDS),

Table 1. Effect of pH on the water extraction yield.

pH	Celluclast 1.5 L	Pectinex Ultra SP-L	Tween 80
2.5	39	50	48
3.5	50	55	50
4.5	51	49	41
5.5	55	53	45
6.5	51	54	44

Table 2. Effect of temperature on the water extraction yield.

Temperature (°C)	Celluclast 1.5 L	Pectinex Ultra SP-L	Tween 80
30	36	35	45
35	55	49	50
40	46	51	46
45	40	43	42
50	40	50	39

Table 3. Chemical analyses of extracted water.

Parameter	Celluclast 1.5 L	Pectinex ultra SP-L	Tween 80	Domestic standard	Industrial standard	Agricultural standard
TDS (mg/L)	12,239	11,039	11,639	< 450	< 1600	<10,000
Total hardness (mg/L)	2,073	2,278	2,184	< 100	< 1000	Not available
Ca (mg/L)	1,407	1,572	1,559	< 32	Not given	Not given
Mg (mg/L)	666	706	625	< 30	Not given	Not given
Sodium adsorption Ratio	0.04	0.02	0.02	Not given	Not given	< 2.00
Na (mg/L)	34	14	12	< 100	Not given	Not given
K (mg/L)	2,643	2,221	2,483	< 50	Not given	Not given
PO ₄ (mg/L)	2,128	1,067	689	Not given	Not given	Not given
SO ₄ (mg/L)	3,827	3,709	1,896	< 200	< 500	Not given
NO ₃ (mg/L)	402	667	14	< 6	Not given	< 10
NO ₂ (mg/L)	0	0	0	< 6	Not given	Not given

the total hardness of the water, as well as the sodium adsorption ratio, are given in Table 3. The water quality corresponds to the water extracted at the optimum pH and temperature for each one of the extraction agents.

The total dissolved solids (TDS) refer to the amount of inorganic salts dissolved in the water. The TDS levels for all the samples are well above the target ranges, making it unsuitable for domestic, industrial and agricultural uses (DWAf, 1996). However, there are commercial crops that reveal higher salt tolerance. These include amongst others asparagus, barley, cotton, rye, sugar beet and wheat (Maas and Kotze, 1990).

The total hardness of water can be defined as the sum of the magnesium and calcium concentrations present in the water. The total hardness levels of the extracted

water of all the samples were well above the assigned target ranges for both domestic and industrial use (DWAf, 1996). As no guidelines are provided for agricultural application, it may prove to be beneficial when used for specialised irrigation applications for crops with a deficiency in calcium and magnesium.

The sodium adsorption rate (SAR) serves as an indication of the potential of water to induce sodic soil conditions. It also illustrates the level at which the soil's exchangeable sodium percentage (ESP) will stabilize after a long period of irrigation. This is only considered for the agricultural application of the water for irrigation purposes. The SAR is calculated using the measured concentrations of sodium, calcium and magnesium. The SAR remains well below 2 for all the samples. The extrac-

ted water can therefore be used for irrigation.

There is a further guideline with regards to the sodium level in the water. In all three samples the sodium level meets the requirement for domestic use. Potassium plays a prominent role in plant growth. Potassium nitrate and various other potassium compounds are quite often used as fertilisers. Guidelines for potassium are only provided for domestic use. The concentration of potassium is well above the desired level, making the water unsuitable for domestic use. However, potassium-rich water will be suitable for agricultural irrigation purposes.

No guidelines are provided by the Department of Water Affairs and Forestry for the concentration of phosphates in water (DWAF, 1996). Plants do have a phosphorous need which has to be satisfied and this result in the use of fertilizers containing phosphorous. The possibility of using the water for agricultural irrigation purposes is therefore attractive.

The concentrations of sulphates present in the water are well above the given ranges for both household and industrial uses (DWAF, 1996). However, sulphates are a good source of sulphur, which is required by plants for growth. The use of fertilizers containing sulphur is used to increase the yields of crops and to reduce the necessity of sulphur leaching (Scherer, 2001).

The concentration of nitrates and nitrites is limited in water for domestic use due to the impact it may have on human health (DWAF, 1996). Target ranges for the application of agricultural irrigation exist and the presence has an effect on the quality and yields of crops (DWAF, 1996). None of the extraction agents was able to successfully reduce the nitrates level below 10 mg/L, but Tween 80 managed to reduce the nitrates level to 14 mg/L, which is much lower than the 402 mg/L and 667 mg/L of the Celluclast 1.5 and Pectinex Ultra SP-L.

Conclusions

In the study, mechanical and chemical extraction methods were used to determine the highest water yield possible from cacti. Mechanical methods proved to be unsuccessful and a maximum yield of 7 wt% was obtained by using a juicer.

Chemical extraction methods proved to be more efficient. Celluclast, Pectinex Ultra SP-L and Tween 80 were added to cacti pulp at different process conditions. The optimum process conditions for the highest water yield were obtained as follows:

- i. Celluclast 1.5L:55 wt% (T = 40°C and pH = 5.5)
- ii. Pectinex Ultra SP-L:55 wt% (T = 40°C and pH = 3.5)
- iii. Tween 80:50 wt% (T = 40°C and pH = 3.5)

The quality of the water was evaluated with regards to

the concentration of the major constituents. It can be concluded that the extracted water is not suitable for domestic or industrial application. The water is, however, suitable for agricultural use without further treatment with regards to the total hardness, the sodium adsorption ratio, potassium, phosphates and sulphates.

The extracted water has a total dissolved solids content higher than specified for agricultural application. The extracted water will either need to be filtered additionally or only used for higher salt tolerant crops. Nitrates are the only other concern with regards to agricultural application and will require additional treatment to counter a possible decrease in the quality and yield of crops, especially in the water extracted using Celluclast 1.5L and Pectinex Ultra SP-L.

It can thus be concluded that the extracted water is suitable for specialised agricultural irrigation purposes.

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Full Length Research Paper

Comparative antioxidant and hypoglycaemic effects of aqueous, ethanol and n-hexane extracts of leaf of *Vitex doniana* on streptozotocin-induced diabetes in albino rats

Yakubu, O. E.^{1,2*}, Ojogbane, E.³, Nwodo, O. F. C.², Nwaneri-Chidozie, V. O.⁴ and Dasofunjo, K.¹

¹Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

²Department of Medical Biochemistry, Cross River State University of Technology, Calabar, Cross River State, Nigeria.

³Department of Biochemistry, Kogi State University, Anyigba, Kogi State, Nigeria.

⁴Department of Biochemistry, Salem University, Lokoja, Kogi State, Nigeria.

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Several herbal preparations are used to treat diabetes, but their reported hypoglycemic effects are complex. This study therefore was designed to evaluate the effect of aqueous extract of *Vitex doniana* leaves on oxidative stress and lipid peroxidation in streptozotocin-induced diabetic and non-diabetic rats. Diabetes was induced intraperitoneally using 50 mg/kg streptozotocin, while diabetic rats were treated in 12 h cycles for four weeks with 100 mg/kg of the extract and glibenclamide (2.5 mg/kg). Nondiabetic control rats received distilled water. The levels of fasting blood sugar (FBS), thiobarbituric acid reactive substance (TBARS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), catalase (CAT) and superoxide dismutase (SOD) activities total, conjugated and unconjugated bilirubin concentration were assayed. The results indicate that the concentrations of TBARS, ALT, AST, ALP and bilirubin were significantly increased while the activities of SOD and CAT were reduced in the diabetic animals ($p < 0.05$). The extract significantly increased CAT and SOD activity and reduced FBS, TBARS, ALT, AST, ALP and bilirubin concentrations significantly ($p < 0.05$) compared to normal. However, glibenclamide treatment showed slight modification in the changes observed compared to the extract. The study concluded that the extract reversed diabetes and diabetes-induced oxidative changes in the hepatocytes, thus suggesting its use for the management of diabetic complications.

Key words: *Vitex doniana*, lipid peroxidation, streptozotocin-induced diabetic.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by degeneration of carbohydrates, protein and fat metabolism (O'Brien and Granner, 1996). Such alterations result in increased blood glucose, which causes long-term complications in many organs.

Oxidative stress in cells and tissues results from the increased generation of reactive oxygen species and/or from diseases in antioxidant defense potential (Gumieniczek et al., 2002). Lipid peroxidation of cellular structures, a consequence of free radical activity in turn

seemed to play an important role in aging and late complications of diabetes (Ugochukwu and Cobourne, 2003; Hunkar et al., 2002) disrupting natural antioxidant defence systems and altering antioxidant enzyme activities in various tissues like the liver (Rauscher et al., 2000; Rauscher et al., 2001). On the other hand, an increase in circulating lipids may be a reason for increased lipid peroxidation in diabetes. Currently, there is a renewed and growing interest in the use of plant-based products as drugs or as 'leads' in the manufacture of more potent drugs (Ogbonnia et al., 2008). Several secondary plant metabolites have been shown to modify biological processes, which may reduce the risk of chronic diseases in humans (Ugochukwu et al., 2003). Globally, the prevalence of diabetes mellitus is increasing. The increase in prevalence has accelerated due to the aging population structure in the developed countries and due to the globally increasing obesity, as well as stressing life style.

Diabetes mellitus is the sixth leading cause of death globally (Nash et al., 2001). *Vitex doniana* sweet, (family *Verbanaceae*) is a perennial shrub widely distributed in tropical West Africa, and some East African countries including Uganda, Kenya and Tanzania, and high rainfall areas. It is found in the middle belt of Nigeria particularly Kogi, Benue, and parts of the savannah regions of Kaduna, Sokoto and Kano states (Etta, 1984). It is variously called *vitex* (English), *dinya* (Hausa), *dinchi* (Gbagyi), *uchakoro* (Igbo), *oriri* (Yoruba) *ejiji* (Igala) and *olih* (Etsako) (Burkill, 2000). *V. doniana* is employed in the treatment of a variety of diseases. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea dysentery and diabetes (Irvine, 1961; Etta, 1984) indicating that the plant's leaves may possess antidiabetic properties among others. The roots and leaves are used for nausea, colic and epilepsy (Bouquet et al., 1971; Iwu, 1993). In North-Central and eastern parts of Nigeria, the young leaves are used as vegetables or sauces and porridge for meals, especially for diabetic patients.

MATERIALS AND METHODS

Collection and preparation of plant materials

Fresh leaves of *V. doniana* were collected from its natural habitat in Ankpa, Kogi State, and it was identified and authenticated by the ethnobotanist in the Department of Medicinal Plant Research and Traditional Medicine of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. A voucher specimen number NIPRD/H/6415 was deposited at the herbarium of the department. The plant material was dried in the laboratory at room temperature and pulverized using laboratory mortar and pestle.

Aqueous extraction

About 400 g of the pulverized sample was soaked in 2 L of distilled

water (1:5 W/V) and was allowed to stand for 24 h at room temperature. The extract was filtered and the filtrate was concentrated using rotary evaporator under reduced pressure. It was allowed to dry at room temperature and stored in refrigerator prior to usage.

Ethanol / n-hexane extraction

About 400 g of the pulverized sample was soaked in 2 L (1:5 w/v) of ethanol/n-hexane (2:1 v/v) for 24 h. The extract was filtered under reduced pressure using filter paper, membrane filter and vacuum pump. Ethanol extract was separated from the n-hexane extract using separatory funnel and the filtrates were concentrated using rotary evaporator under reduced pressure respectively. The extracts were reconstituted freshly in distilled water at appropriate concentrations for the various experimental doses using the equation of Tedong et al. (2007):

$$V(\text{ml}) = (D \times P)/C$$

Where D = dose used (g/kg body weight); P = body weight (g); C = concentration (g/ml) and V = volume.

Animal management

Male albino rats (7 to 8 weeks old) were purchased from the animal house of the Department of Biosciences, Salem University, Lokoja, Nigeria. They were acclimatized for two weeks prior to commencement of experiment. They were kept at room temperature and maintained *ad libitum* on growers mash (feed) and weighed prior to experiment.

Induction of diabetes

Rats were fasted overnight and experimental diabetes was induced by intraperitoneal injection of streptozotocin (STZ) with a single dose of 50 mg/kg body weight. STZ was dissolved in a freshly prepared 0.1 M cold citrate buffer of pH 4.5 (Rakieten et al., 1963). Control rats were similarly injected with citrate buffer. Because STZ is capable of inducing fatal hypoglycemia as a result of massive pancreatic insulin release, STZ treated rats were provided with 10% glucose solution after 6 h for the next 24 h to prevent severe hypoglycemia. After 3 days for development and aggravation of diabetes, rats with moderate diabetes (that is, blood glucose concentration 250 mg/dl) that exhibited hyperglycemia were selected for experiment (Canepa et al., 1990).

Experimental design

In the experiment, the rats were divided into nine groups of five rats each and treatment was carried out orally. Group 1: (N. control) normal rats (non-diabetic, no treatment); Group 2: (D. control) diabetic rats, no treatment; Group 3: (D. STD) diabetic rats treated with 2.5 mg/kg glibenclamide; Group 4: (D. aqueous) diabetic rats treated with 100 mg/kg aqueous extract; Group 5: (D. ethanol) diabetic rats treated with 100 mg/kg of the ethanol extract; Group 6: (D. hexane) diabetic rats treated with 100 mg/kg of the n-hexane extract; Group 7: (N. aqueous) non-diabetic rats treated with 100 mg/kg aqueous extract; Group 8: (N. ethanol) non-diabetic rats treated with 100 mg/kg of ethanol extract; Group 9: (N. hexane) non-diabetic rats treated with 100 mg/kg of n-hexane extract.

On the 28th day of post-treatment, the animals were fasted overnight, anesthetized with chloroform and sacrificed by humane

decapitation. Blood was collected in centrifuge tubes, and serum collected after centrifugation at 2,000rpm for 10 min and stored in deep-freezer prior to analysis. Fasting blood glucose and packed cell volume was monitored weekly. Liver and kidneys were surgically removed, immediately washed with ice-cold normal saline and used for the assay of TBARS and antioxidant enzymes.

Tissue preparation

Weighed liver and kidney samples were homogenised separately in 10 parts (w/v) of ice-cold 50 mM Tris-HCl, (pH 7.4) using a homogeniser (Janke and Kunkel, Germany). The homogenates were centrifuged at 3,000 rpm for 15 min and the supernatants were collected. The supernatants were used for measurement of scavenging enzyme activities and lipid peroxides (TBARS).

Determination of fasting blood sugar

Fasting blood sugar (FBS) was determined using Accu-Check Advantage glucometer.

Determination of biochemical parameters

Thiobarbituric acid reactive substances (TBARS)

Hepatic lipid peroxidation was determined as thiobarbituric acid reactive substances as described by Torres et al. (2004). Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde, a product which react with thiobarbituric acid. The product of the reaction is a coloured complex which absorbs light at 535 nm. The extinction coefficient, $1.56 \times 10^5 \text{ M}^{-1} \text{ Cm}^{-1}$ was used in the calculation of TBARS and values were expressed as nmol/mg protein.

Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) catalyzes the transamination of aspartate to alpha-ketoglutarate to form glutamate and oxaloacetate, which then reacts with 2,4-dinitro-phenylhydrazine to form hydrazone derivative of oxaloacetate, a coloured complex which can be measured at 546 nm. Aspartate aminotransferase was determined as described by Reitman and Frankel (1957) using assay kits (Agape Laboratories Ltd, UK).

Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) catalyzes the transamination of alanine to alpha-ketoglutarate to form glutamate and pyruvic acid, which then reacts with 2,4-dinitro-phenylhydrazine to form hydrazone derivative of pyruvate, a coloured complex which can be measured at 546 nm. Alanine aminotransferase was determined as described by Reitman and Frankel (1957) using assay kits (Agape Laboratories Ltd, UK).

Alkaline phosphatase (ALP)

Serum alkaline phosphatase was determined as described by Klein et al. (1960). Serum alkaline phosphatase catalyses the hydrolysis of a colourless substrate of phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which, at alkaline pH values turns into a pink colour that can be determined photo-metrically at 550 nm.

Serum bilirubin

This was determined colorimetrically according to the method described by Jendrassic and Grof (1938) using assay kits (Agape Laboratories Ltd, UK). Conjugated bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Assay of enzymatic antioxidant

Superoxide dismutase (SOD)

The activity of superoxide dismutase was measured at 560 nm according to the method described by Martin et al. (1987). Briefly, auto-oxidation of hematoxylin is inhibited by SOD at assay pH, the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range, and was expressed as unit/mg protein.

Catalase (CAT)

Catalase activity was measured using the method of Abei (1974). The decomposition rate of H_2O_2 was measured at 240 nm for 5 min using a spectrophotometer. A molar extinction coefficient of $0.041 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the catalase activity and was expressed in unit/mg protein.

Statistical Analysis

All the values estimations were expressed as mean \pm standard deviation and analyzed for ANOVA and post hoc Duncan's -test using SPSS. Differences between groups were considered significant at $P < 0.05$ levels.

RESULTS AND DISCUSSION

Thiobarbituric acid reactive substances (TBARS) levels

TBARS level was significantly ($p < 0.05$) elevated in the liver of diabetic control rats, when compared with the normal control rats. This increase was reduced significantly ($p < 0.05$) in the extract treated rats as well as in the rats treated with glibenclamide (Table 1).

Effects of the extract on hepatic enzymes

Hepatic enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are shown in Table 2. Glibenclamide caused significant elevation ($P < 0.05$) in the activities of these enzymes in the serum. Treatment with *V. doniana* aqueous extract at the dose of 100 mg/kg significantly reduced the activity of the enzymes compared to the control. Similarly, treatment with glibenclamide was able to reduce ALP activity significantly but non-significant in

Table 1. TBARS levels in normal and diabetic rats treated with *V. doniana* aqueous, ethanol and n-hexane extract and glibenclamide.

Treatment	Tbars concentration (nmol/mg protein)		
	Serum TBARS	Liver TBARS	Kidney TBARS
N. control	0.41±0.02 ^c	0.62±0.08 ^b	0.56±0.13 ^b
D. control	0.94±0.26 ^e	1.86±0.23 ^c	1.23±0.11 ^c
D. STD	0.44±0.10 ^c	0.62±0.08 ^b	0.52±0.10 ^b
D. AQ (100 mg)	0.40±0.05 ^b	0.62±0.12 ^b	0.57±0.02 ^b
D. ETH (100 mg)	0.32±0.07 ^a	0.57±0.03 ^{ab}	0.54±0.08 ^b
D. HEX (100 mg)	0.60±0.07 ^d	0.61±0.03 ^b	0.56±0.03 ^b
N. AQ (100 mg)	0.36±0.01 ^{ab}	0.46±0.02 ^a	0.44±0.01 ^a
N. ETH (100 mg)	0.42±0.04 ^c	0.45±0.05 ^a	0.44±0.09 ^a
N. HEX (100 mg)	0.44±0.09 ^c	0.48±0.02 ^a	0.53±0.10 ^b

N. control = Normal control; D. control = diabetic control; D. STD = diabetic standard drug (glibenclamide); D. AQ = diabetic aqueous extract; D. ETH = diabetic ethanol extract; D. HEX = diabetic hexane extract; N. AQ = non diabetic aqueous extract; N. ETH = non diabetic ethanol extract; N. HEX = non diabetic hexane extract.

Table 2. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activity in normal and diabetic rats treated with *V. doniana* aqueous, ethanol and n-hexane extract and glibenclamide.

Treatment	Liver enzymes activity (U/L)		
	ALT	AST	ALP
N. control	35.71±05.63 ^b	34.39±10.58 ^a	32.81±4.00 ^a
D. control	55.10±04.58 ^d	55.10±4.58 ^d	79.20±9.09 ^c
D. STD	42.43±07.70 ^c	44.59±8.74 ^b	39.67±8.53 ^b
D. AQ (100 mg)	36.68±05.84 ^b	34.47±7.46 ^a	35.96±8.80 ^{ab}
D. ETH (100 mg)	35.71±05.78 ^b	38.66±12.16 ^{ab}	30.06±8.85 ^a
D. HEX (100 mg)	36.53±05.36 ^b	48.32±11.64 ^c	36.74±4.73 ^{ab}
N. AQ (100 mg)	31.82±03.77 ^a	37.48±11.18 ^{ab}	26.74±4.73 ^a
N. ETH (100 mg)	33.94±03.77 ^a	47.26±10.56 ^c	29.12±7.79 ^a
N. HEX (100 mg)	34.29±02.66 ^{ab}	42.90±7.60 ^b	28.62±3.01 ^a

N. control = normal control; D. control = diabetic control; D. STD = diabetic standard drug (glibenclamide); D. AQ = diabetic aqueous extract; D. ETH = diabetic ethanol extract; D. HEX = diabetic hexane extract; N. AQ = non diabetic aqueous extract; N. ETH = non diabetic ethanol extract; N. HEX = non diabetic hexane extract.

ALT and AST. However, treatment of non-diabetic rats caused no significant decrease in the activity of the enzymes compared with the normal rats.

Fasting blood sugar (FBS)

Table 3 shows the levels of fasting blood sugar (FBS) in the animals. At day one (before induction), there was no statistical difference in the levels of FBS across the groups. At week two, there was significant ($P<0.05$) elevation of FBS in the diabetic groups compared to the normal groups. Treatment of diabetic animals with the extracts was able to restore FBS to normalcy across the week, with aqueous and ethanol extracts more effective than n-hexane and glibenclamide.

Effects of the extract on enzymatic antioxidants

A significant ($p<0.05$) decrease in catalase (CAT) and superoxide dismutase (SOD) activities were observed in the diabetic untreated rats compared to the normal rats (Tables 4 and 5). Treatment with the extract and glibenclamide showed a significant ($p<0.05$) increase in catalase activity, and glibenclamide treatment also significantly increased SOD activity but was non-significant with extract treatment compared to the normal group. There was no significant increase in the activity of the enzymes in non-diabetic rats treated with the extract compared to the normal.

Effects of the extract on serum bilirubin

Administration of streptozotocin caused significant ($P<0.05$)

Table 3. Fasting blood sugar (FBS) in normal and diabetic rats treated with *V. doniana* aqueous, ethanol and n-hexane extract and glibenclamide.

Treatment	Fasting blood sugar (FBS) (mg/dl)				
	Day 0	Week 1	Week 2	Week 3	Week 4
N. control	104.2±8.6 ^a	108.8±8.6 ^a	110.5±11.5 ^a	104.8±6.7 ^a	108.2±7.3 ^a
D. control	97.4±5.2 ^a	318.7±25.0 ^b	236.5±35.4 ^c	236.3±26.8 ^{cd}	248.5±20.9 ^b
D. STD	96.2±5.2 ^a	365.2±23.7 ^c	268.4±29.1 ^c	170.7±25.3 ^b	153.5±28.0 ^c
D. AQ (100 mg)	96.6±7.0 ^a	381.4±29.8 ^c	255.6±27.8 ^d	220.8±8.8 ^c	104.8±12.9 ^a
D. ETH (100 mg)	101.6±9.6 ^a	399.6±47.0 ^{cd}	330.6±37.3 ^{de}	250.6±27.1 ^d	103.4±12.1 ^a
D. HEX (100 mg)	100.8±8.3 ^a	414.8±76.3 ^d	392.8±41.0 ^e	225.2±8.0 ^c	128.6±36.7 ^d
N. AQ (100 mg)	96.8±8.1 ^a	99.3±10.4 ^a	104.8±4.2 ^a	104.6±16.3 ^a	104.4±12.7 ^a
N. ETH (100 mg)	99.2±6.6 ^a	102.8±8.5 ^a	108.1±6.0 ^{ab}	103.4±13.9 ^a	101.6±12.9 ^a
N. HEX (100 mg)	98.4±2.7 ^a	101.8±5.8 ^a	97.8±4.2 ^b	98.5±7.3 ^a	97.8±5.2 ^a

N. control = Normal control; D. control = diabetic control; D. STD = diabetic standard drug (glibenclamide); D. AQ = diabetic aqueous extract; D. ETH = diabetic ethanol extract; D. HEX = diabetic hexane extract; N. AQ = non diabetic aqueous extract; N. ETH = non diabetic ethanol extract; N. HEX = non diabetic hexane extract. Values are mean ± SD, n = 5. All treated groups are compared with control. Values with different superscript across the group are statistically significant at P<0.05.

Table 4. Catalase (CAT) activity in normal and diabetic rats treated with *V. doniana* aqueous, ethanol and n-hexane extract and glibenclamide.

Treatment	Catalase (CAT) activity (U/mg protein)		
	Serum CAT	Liver CAT	Kidney CAT
N. control	13.66±2.31 ^{ab}	21.29±5.61 ^b	14.26±2.51 ^b
D. control	08.13±1.21 ^a	12.78±0.58 ^a	08.13±1.21 ^a
D. STD	08.73±1.67 ^a	17.68±4.77 ^b	11.08±1.88 ^{ab}
D. AQ (100 mg)	11.50±1.47 ^{ab}	19.24±1.04 ^b	12.26±1.80 ^{ab}
D. ETH (100 mg)	13.50±1.27 ^{ab}	18.09±1.68 ^b	13.87±1.07 ^{ab}
D. HEX (100 mg)	09.41±1.12 ^a	18.31±0.81 ^b	10.96±2.39 ^{ab}
N. AQ (100 mg)	15.33±1.34 ^b	20.43±1.12 ^b	15.12±0.78 ^b
N. ETH (100 mg)	15.48±1.65 ^b	20.54±1.17 ^b	16.04±0.99 ^b
N. HEX (100 mg)	14.75±1.41 ^b	19.83±3.16 ^b	14.56±1.37 ^b

elevation in serum total, direct and indirect bilirubin concentration in the control animals compared to normal (Table 6). Both extract and glibenclamide treatment caused significant reduction in bilirubin concentration in the experimental rats compared to normal. However, administration of extract to normal rat showed no significant increase/decrease in total, direct and indirect bilirubin concentration.

DISCUSSION

Diabetes is currently considered as a vascular disease (Ibrahim and Rizk, 2008). It has also been considered by researchers that hyperglycaemia-induced oxidative stress is a critical pathogenic mechanism that initiates a plethora of cascade metabolic and vascular perturbations (Ibrahim and Rizk, 2008; Housom et al., 2001; Hunt et al., 1988). Studies have revealed the beneficial effects of some secondary plant metabolites that possess antioxi-

dant activity in diabetes management. Lipid peroxidation was investigated in our study by assessing the hepatic levels of TBARS; a significant increase in TBARS levels of diabetic rats was observed when compared to normal control rats. Numerous studies with human and animal models have also shown increased lipid peroxidative status in membranes of different tissues in diabetes (Feillet-Coudray et al., 1999; Kakkar et al., 1998; Aydin et al., 2001; Obresova et al., 2003, Ugochukwu and Courbone, 2003). The extract produced significant decreases in TBARS levels in treated diabetic rats when compared to diabetic control rats. Treatment with glibenclamide also caused a slight decrease in TBARS levels of the treated rats. These reductions could lead to a decrease in oxidative stress and hence a reduction in the rate of progression of diabetic complications in the liver. Table 2 represents the changes in the activities of aspartate transaminase, alanine transaminase and alkaline phosphatase.

In the assessment of liver damage by the determination

Table 5. Superoxide dismutase (SOD) activity in normal and diabetic rats treated with *V. doniana* aqueous, ethanol and n-hexane extract and glibenclamide.

Treatment	Suproxide dismutase (SOD) activity (U/mg protein)		
	Serum SOD	Liver SOD	Kidney SOD
N. control	70.17±9.05 ^d	51.16±6.33 ^c	32.16±9.08 ^b
D. control	40.20±5.16 ^a	25.58±5.16 ^a	25.45±5.12 ^a
D. STD	53.60±9.10 ^c	38.98±5.49 ^b	34.11±7.16 ^b
D. AQ (100 mg)	45.60±8.00 ^b	34.23±3.10 ^b	46.84±3.75 ^{cd}
D. ETH (100 mg)	53.61±4.30 ^c	50.45±6.44 ^c	47.22±5.43 ^d
D. HEX (100 mg)	45.21±1.54 ^b	34.11±8.16 ^b	42.24±1.44 ^c
N. AQ (100 mg)	62.64±4.69 ^d	51.00±5.00 ^c	33.03±3.11 ^b
N. ETH (100 mg)	78.04±11.63 ^d	56.27±2.15 ^{cd}	44.49±4.87 ^c
N. HEX (100 mg)	82.67±5.44 ^d	60.73±5.21 ^d	34.46±3.43 ^b

N. control = Normal control; D. control = diabetic control; D. STD = diabetic standard drug (glibenclamide); D. AQ = diabetic aqueous extract; D. ETH = diabetic ethanol extract; D. HEX = diabetic hexane extract; N. AQ = non diabetic aqueous extract; N. ETH = non diabetic ethanol extract; N. HEX = non diabetic hexane extract. Values are mean ± SD, n = 5. All treated groups are compared with control. Values with different superscript across the group are statistically significant at P<0.05.

Table 6. Serum total bilirubin concentration in normal and diabetic rats treated with *V. doniana* ethanol extract and glibenclamide.

Treatment	Bilirubin (bil) concentration (mg/dl)		
	Total bil	Indirect bil	Direct bil
N. control	0.68±0.14 ^b	0.34±0.07 ^{ab}	0.40±0.07 ^b
D. control	1.66±0.14 ^d	0.89±0.10 ^d	0.77±0.04 ^c
D. STD	0.88±0.24 ^c	0.36±0.03 ^b	0.52±0.04 ^{bc}
D. AQ 100 mg	0.84±0.20 ^c	0.54±0.01 ^c	0.39±0.06 ^{ab}
D.ETH 100 mg	0.67±0.17 ^b	0.37±0.07 ^b	0.39±0.04 ^{ab}
D.HEX 100 mg	0.85±0.13 ^c	0.39±0.09 ^b	0.46±0.04 ^a
N. AQ 100 mg	0.58±0.08 ^a	0.28±0.00 ^a	0.39±0.03 ^{ab}
N. ETH 100 mg	0.51±0.01 ^a	0.27±0.01 ^a	0.34±0.08 ^a
N. HEX 100 mg	0.63±0.18 ^b	0.31±0.04 ^{ab}	0.37±0.08 ^a

N. control = Normal control; D. control = diabetic control; D. STD = diabetic standard drug (glibenclamide); D. AQ = diabetic aqueous extract; D. ETH = diabetic ethanol extract; D. HEX = diabetic hexane extract; N. AQ = non diabetic aqueous extract; N. ETH = non diabetic ethanol extract; N. HEX = non diabetic hexane extract. Values are mean ± SD, n = 5. All treated groups are compared with control. Values with different superscript across the group are statistically significant at P<0.05.

of enzyme, enzyme levels such as aspartate transaminase and alanine transaminase are largely used. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Watkins and Seef, 2006). Hepatocellular necrosis leads to high level of serum markers in the blood, among these, aspartate transaminase, alanine transaminase represents 90% of total enzyme and high level of alanine transaminase in the blood is a better index of liver injury, but the elevated levels of enzymes are decreased to normal levels after treatment with the extract. Alkaline phosphatase concentration is related to the functioning of hepatocytes, high level of alkaline phosphatase in the blood serum is related to the increased synthesis of it by cells lining bile canaliculi

usually in response to cholestasis and increased biliary pressure (Handa and Sharma, 1990). Increased level was obtained owing to streptozotocin administration and it was brought to normal level by the extract treatment. Treatment with *V. doniana* aqueous leaf extract decreased the serum levels of aspartate transaminase, alanine transaminase and alkaline phosphatase towards the respective normal value; that is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by STZ. The aforementioned changes can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchymal cells.

Hyperbilirubinemia was observed due to excessive heme

destruction and blockage of biliary tract. As a result of blockage of the biliary tract, there was mass inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged and dead hepatocytes; this is in line with the report given by Gaw et al. (1999). Administration of extract decreased the level of bilirubin, suggesting that it offered protection. Catalase which has been known to scavenge and detoxify H_2O_2 showed a decreased activity in the diabetic control rats probably due to decreased concentration by H_2O_2 in the system (Esra et al., 2004).

Treatment with the extract significantly ($p > 0.05$) increased the activity in the treated rats indicating a possible attenuation of oxidant stress. SOD activity was observed to decrease in the diabetic rats compared to the normal control rats probably acting in a compensatory mechanism to maintain homeostasis. The increase in SOD activity may also be due to decreased mutation of superoxide anions due to their decreased production at the onset of diabetes. The diabetic rats treated with the extract showed increased SOD activity while treatment with glibenclamide demonstrated less increase in SOD activity. This suggests that the extract may have reduced the production of ROS with a concomitant increase in SOD activity.

Our observations are in well agreement with the reports by several workers that STZ-induced diabetes mellitus and insulin deficiency leads to increased blood glucose (Chaude et al., 2001). It has been reported that STZ at lower doses (50 mg/kg) produce partial destruction of pancreatic β -cells with permanent diabetes condition (Aybar et al., 2002) and there may be more possibility of many surviving β -cells (Cherian et al., 1992). Since a much low dose of STZ was chosen for this study, there may be many surviving β -cells, capable of undergoing regeneration. Prolonged administration of extract may stimulate the β -cells of islets of Langerhans to produce insulin (Cherian et al., 1992).

The antihyperglycemic effect of the extracts was compared with glibenclamide, a standard hypoglycemic drug. Glibenclamide has long been used to treat diabetes, to stimulate insulin secretion from the pancreatic β -cells. From the results, it appears that still insulin producing β -cells are functioning in STZ treated diabetic rats and stimulation of insulin release could be responsible for most of the observed metabolic activities. Further, the observed blood glucose-lowering effect in fasted normal and STZ induced diabetic rats could possibly be due to the increased peripheral glucose utilization. A number of other plants have also been shown to exert hypoglycemic activity through stimulation of insulin release (Pari and Maheswari, 2000; Prince and Menon, 2000).

Our results therefore indicate that the aqueous extract when used for diabetes management may control and or prevent the development of diabetic complications arising from increased oxidative stress and lipid peroxidation.

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Full Length Research Paper

Investigation of some important phytochemical, nutritional properties and toxicological potentials of ethanol extracts of *Newbouldia laevis* leaf and stem

Anaduaka, Emeka G.^{1*}, Ogugua, Victor N.¹, Egba, Simeon I.² and Apeh, Victor O.¹

¹Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.

²Department of Biochemistry, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

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The phytochemicals, nutritional and toxicological potentials of the ethanol extracts of the leaf and stem of *Newbouldia laevis* was investigated in this study. The percentage yields of *N. laevis* ethanol leaf and stem extracts were found to be 7.44 and 3.30% (w/w), respectively. The preliminary phytochemical screening showed that ethanol leaf and stem extracts contains alkaloids, flavonoids and tannins. The quantitative phytochemical analysis showed that the leaf and stem extracts contained respectively: alkaloids (14.74 ± 0.06 and 6.27 ± 0.0 mg/g), flavonoids (15.51 ± 0.04 and 5.18 ± 0.04 mg/g), cardiac glycosides (6.77 ± 0.02 mg/g), tannins (1.74 ± 0.11 mg/g), saponins (4.07 ± 0.06 mg/g), steroids (41.72 ± 0.02 mg/g) and terpenoids (8.67 ± 0.09 mg/g). The following amounts of vitamins and minerals were found in the leaf and stem-bark extracts, respectively; vitamin A (5.19 ± 0.00 and 3.01 ± 0.00 mg/100 g), vitamin C (2.35 ± 0.55 and 1.05 ± 0.08 mg/100 g) and vitamin E (9.33 ± 0.02 and 4.08 ± 0.11 mg/100 g); minerals: Mg (76.12 ± 0.04 and 54.25 ± 0.04 mg/100 g), Fe (16.84 ± 0.06 and 1.19 ± 0.03 mg/100 g) and Se (3.08 ± 0.03 and 0.29 ± 0.07 mg/100 g). The acute toxicity test of the ethanol leaf and stem extracts showed no toxicity up to 5000 mg/kg body weight.

Key words: *Newbouldia laevis*, phytochemical properties, vitamins, minerals, toxicity.

INTRODUCTION

A very large area of Nigeria ecological zones is populated with many plant species which have found their usefulness either directly or indirectly for humans (Oliver-Bever, 1986). The medicinal values of many of these plants cannot be over emphasized in the light of oral traditions and folklores from the distant past that have continued to extol the healing virtues of these plants and their extracts. One of such medicinal plant is *Newbouldia laevis*, whose medicinal values have stood the test of time. Plants contain active components such as anthraquinones, flavonoids, glycosides, saponins, tannins, etc which possess medical properties that are harnessed for the treatment of different diseases (Feher

and Schmidt, 2003). The active ingredients for a vast number of pharmaceutically derived medications contain components originating from phytochemicals in plants. These active substances that contain the healing property are known as the active principles and are found to differ from plant to plant (Galm and Shen, 2007). Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress.

Phytochemicals are naturally occurring and are believed to be effective in combating or preventing disease due to their antioxidant effect (Halliwell and Gutteridge, 1992; Ejele et al., 2012). The medicinal lie in

their component phytochemicals, which produce the definite physiological actions on human body. The most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Iwu, 2000). Some of these naturally occurring phytochemicals are anticarcinogenic and some others possess other beneficial properties, and are referred to as chemopreventers. One of the predominant mechanisms of their protective action is due to their antioxidant activity and the capacity to scavenge free radicals.

N. laevis (*Bignoniaceae*) is commonly known as African Border tree or boundary tree (Gbile and Adesina, 1986). It is called "Aduruku" in Hausa; "Ogirisi" in Igbo; "Ikhimi" in Edo and "Akoko" in Yoruba languages (Ogunlana and Ogunlana, 2008). It grows to a height of about 7.8 (up to 15 m), more usually a shrub of 2 to 3 m, many-stemmed forming clumps of gnarled branches. It is easily recognized by its short branches, coarsely toothed leaflets and purple and white flowers (Iwu, 1983). *N. laevis* is native to tropical Africa and grows from Guinea Savannas to dense forests, or moist and well-drained soils (Burkill, 1984). One remarkable thing about this plant is that it hardly dies hence it is used to indicate boundary marks among the Igbo people of South Eastern Nigeria (Gill, 1992). *N. laevis* have recently attracted research interest because it possesses antioxidant properties against a variety of physiologically relevant free radicals (Ajibolu et al., 2011). Therefore, there is need to continue the investigation of the mechanism by which plants and plant products protect and prevent tissues from damage by chemical compounds that generate reactive oxygen species in the system.

MATERIALS AND METHODS

Collection and identification of plant materials

The leaves and stem of *N. laevis* were used for this study. The leaves and stem of *N. laevis* were collected within University of Nigeria, Nsukka and were identified in the Bioresources Development and Conservation Programme (BDGP) Research Centre, Nsukka, Enugu State. The fresh leaves and stem of *N. laevis* were washed with clean water to remove dirt and sand, drained and chopped. They were dried under shade for several days and then pulverized into fine powder.

Extraction of plant materials

A quantity, 500 g of each of the powdered form of the leaves and stem of *N. laevis* were macerated in 1.5 L of ethanol for 48 h. The solution was filtered with Whatman no. 4 filter paper and the filtrate was concentrated to a semi-solid residue in an oven at 60°C.

Phytochemical screening

The phytochemical analysis of the leaves and stem of *N. laevis* were carried out according to the method of Harborne (1973) and Trease and Evans (2002) to identify its active constituents.

Quantitative phytochemical analysis

Alkaloid determination

The determination of alkaloid was as described by Harborne (1973). A portion (5 g) of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 h. This was filtered and the extract was concentrated on a water bath to ¼ of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract till a precipitate was formed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Flavonoids determination

This was determined according to the method of Harborne (1973). A quantity, 5 g of the sample was boiled in 50 ml of 2M HCl solution for 30 min under reflux. It was allowed to cool and then filtered through whatman No. 1 filter paper. A measured volume of the extract was treated with equal volume of ethyl acetate starting with a drop. The solution was filtered into a weighed crucible. The filtrate was heated to dryness in an oven at 60°C. The dried crucible was weighed again and the difference in the weight gave the quantity of flavonoid present in the sample.

Steroids determination

This was determined by the method described by Edeoga et al. (2005). A known weight of each sample was dispersed in 100 ml freshly distilled water and homogenized in a laboratory blender. The homogenate was filtered and the filtrate was eluted with normal ammonium hydroxide solution (pH 9). The eluate (2 ml) was put in test tube and mixed with 2 ml of chloroform. Ice-cold acetic anhydride (3 ml) was added to the mixture in the flask and 2 drops of conc. H₂SO₄ were cautiously added. Standard sterol solution was prepared and treated as described earlier. The absorbances of standard and prepared sample were measured in a spectrophotometer at 420 nm.

Determination of vitamin contents

The vitamin assay was performed with the method of Pearson (1976).

Vitamin A: A quantity of 1.0 g of ground sample was macerated with 20 ml of petroleum ether. This was decanted into a test tube and then evaporated to dryness. 0.2 ml of chloroform-acetic anhydride (1:1, v/v) was added to the residue. 2 ml of TCA-chloroform in like (1:1 v/v) was added to the resulting solution and absorbance was measured at 620 nm. Vitamin A standard was prepared in like manner and the absorbance taken at 620 nm. The concentration of vitamin A in the sample was extrapolated from the standard curve.

Vitamin E: A quantity of 1 g of the sample was macerated with 20 ml of ethanol and then filtered. 0.2% ferric chloride in ethanol and 1 ml of 0.5% α-α-dipyridine to 1 ml of the filtrate. This was diluted to 5 ml with distilled water. Absorbance was taken at 520 nm. The standard solutions were prepared similarly and the concentration of vitamin E extrapolated from the standard curve.

Vitamin C: A quantity of 1 g of sample was macerated with 20 ml of 0.4% oxalic acid. This was filtered and to 1 ml of the filtrate was added 9 ml of Indolephenol reagent. The standard solution of vita-

vitamin C was prepared similarly and the absorbances of the standard solution and the sample were read at 520 nm. The concentration of vitamin C was extrapolated from the standard curve of vitamin C.

Determination of mineral contents of the leaves and stem of *N. laevis*

The method of AOAC (1970) was used. 2 g of sample was weighed into a crucible and ashed into a furnace at 550°C for 6 h. The ash was cooled, 6N HCl was added and boiled for 10 min, while covering the crucible with a watch glass. After boiling the sample, it was allowed to cool and filtered into 100 ml volumetric flask. The crucible was washed with distilled water and the washings added to the ash filtrate. The ash filtrate was then made up to 100 ml with distilled water. An aliquot of the filtrate was aspirated into the atomic absorption spectrophotometer and the absorbance values corresponding to different minerals recorded. The percentage of the elements in the samples was calculated from the absorbance values of the samples and standard solutions.

Determination of magnesium

A precipitate formed in the previous test was removed by filtration and made strongly alkaline with ammonia. A volume of 1 cm³ of 10% sodium phosphate solution was added. The formation of a crystalline precipitate indicated the presence of magnesium.

Determination of ferric iron

Several cubic centimeters of the solution was acidified with hydrochloric acid, and 1 cm³ of 10% ammonium thiocyanate was added. The formation of a red colour indicated the presence of ferric iron. If negative, take a second portion and a few drops of hydrogen peroxide and warm. This will oxidize any ferrous iron (iron II) to ferric, which can be detected as aforementioned.

Acute toxicity test of the ethanol extracts of the leaves and stem of *Newbouldia laevis*

The method of Lorke (1983) was used for the acute toxicity test of the leaves and stem-bark of *N. laevis*. Thirty six (36) albino mice were utilized in this study. The test involved two stages. In stage one, the animals were grouped into three groups of three rats each and were given 10, 100 and 1000 mg/kg body weight of the extracts respectively and in the second stage, 1600, 2900 and 5000 mg/kg body weight of the extracts were administered to the animals. The administration of the extracts was done orally. The median lethal dose (LD₅₀) was calculated from the second phase.

Statistical analysis

Data were mean of three replicates ± SD. Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 19. One way analysis of variance was adopted for comparison, and the results were subjected to post hoc test using least square deviation (LSD). The data were expressed as mean ± standard deviation. P < 0.05 was considered significant.

RESULTS

Table 1 shows that both leaves and stem ethanol extracts

Table 1. Preliminary phytochemical screening of ethanol extracts of *N. laevis* leaves and stem.

Variable	Leaves	Stem
Alkaloids	++	+
Flavonoids	+++	+
Steroids	+	-
Saponins	+	-
Glycosides	++	-
Terpenoids	+	-
Tannins	+	+

+ slightly present, +++ highly present, ++ moderately present, - absent.

of *N. laevis* contain alkaloids, flavonoids, tannins, steroids, terpenoids, cardiac glycosides and saponins were not detected in the ethanolic stem extract but were present in the leaves extract. There were slight differences in the alkaloids and flavonoids contents in the leaves and stem extracts. Table 2 shows that the leaves extract contains higher amounts of phytochemicals to the stem extract. Table 3 shows that both leaves and stem extracts contain vitamins A, C and E but in different quantities. The mineral content of the leaf and stem were respectively found to be 76.12 ± 0.04 and 54.25 ± 0.04 mg/100 g for magnesium; 16.84 ± 0.06 and 1.19 ± 0.03 mg/100 g for iron; 3.08 ± 0.03 and 0.29 ± 0.07 mg/100 g for selenium (Table 4). The acute toxicity test of ethanol extracts of *N. laevis* leaves and stem showed no death up to 5000 mg/kg body weight (Table 5).

Effects of ethanol extract of *N. laevis* leaves and stem on organ histology

Histopathology explanation

Microscopic examination of the liver and kidney sections of the control rat showed normal morphological structure of the central vein and kupffer cells respectively as shown in Figure 1. On the other hand, microscopic investigation of the liver and kidney section of diabetic untreated rat demonstrated various areas of hepatocyte degeneration of the liver and mild congestion of the glomerulus of the kidney (Figure 2). Investigation of the liver and kidney sections of diabetic treated with glibenclamide (standard drug) revealed normal histological structure of the tissues as shown in Figure 3. Treatment with the leaves extract in the dose of 200 mg/kg body weight showed partial restoration of normal histological structure of the liver and kidney with few disturbances in the liver and kidney cells arrangements (Figure 4). Treatment with the leaves extract in the dose of 400 mg/kg body weight showed complete restoration of normal histological structure of the liver and kidney with no disturbance in the cell arrangements (Figure 5) when compared to group 2

Table 2. Quantitative phytochemical constituents of ethanol extracts of *N. laevis* leaves and stem.

Phytochemical constituent (mg/g)	Leaves (mean±SD)	Stem (mean±SD)
Alkaloids	14.74 ± 0.06	6.27 ± 0.02
Flavonoids	15.51 ± 0.04	5.18 ± 0.04
Steroids	41.72 ± 0.02	-
Saponins	4.07 ± 0.06	-
Tannins	1.74 ± 0.11	0.21±0.01
Cardiac glycosides	6.77 ± 0.02	-
Terpenoids	8.67 ± 0.09	-

Table 3. Vitamin constituents of ethanol extracts of *N. laevis* leaves and stem.

Vitamin constituent (mg/100 g)	Leave (mean±SD)	Stem (mean±SD)
A	5.19 ± 0.00	3.01 ± 0.00
C	2.35 ± 0.55	1.05 ± 0.08
E	9.33 ± 0.02	4.08 ± 0.11

Table 4. Mineral constituents of ethanol extracts of *N. laevis* leaves and stem.

Mineral constituent (mg/100 g)	Leaves (mean±SD)	Stem (mean±SD)
Mg	76.12 ± 0.04	54.25 ± 0.04
Fe	16.84 ± 0.06	1.19 ± 0.03
Se	3.08 ± 0.03	0.29 ± 0.07

Table 5. Phases I and II of the acute toxicity (LD₅₀) test of *N. laevis*.

Parameter	Dosage (mg/kg body weight)	Mortality (leaves stem)
Phase I		
Group 1	10	0/3 0/3
Group 2	100	0/3 0/3
Group 3	1000	0/3 0/3
Phase II		
Group 1	1600	0/3 0/3
Group 2	2900	0/3 0/3
Group 3	5000	0/3 0/3

(diabetic untreated rat).

Moreover, the treatment with the stem extract in doses of 200 and 400 mg/kg body weights showed normal histological structure of the portal areas of the liver and the glomerular tufts of the kidney (Figures 6 and 7).

DISCUSSION

Preliminary and quantitative phytochemical constituents of the leaves and stem were investigated as well as their vitamin and mineral contents and toxicological potentials. The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids and tannins in both the leaf and stem extracts. Steroids, saponins, glycosides and terpenoids were only present in the leaves extract. The presence of tannins, terpenoids, flavonoids, steroids and cardiac glycosides in the leaf extract was in line with the reports of Usman and Osuji (2007) and Azando et al. (2011). Several available literature reports are discordant on the phytochemical composition of the plant. Usman and Osuji (2007) did not detect the presence of alkaloids and saponins in their study while Dandjesso et al. (2012) reported the absence of alkaloids, flavonoids, saponins and steroids on the leaf extract. Ejele et al. (2012) reported in their work the absence of flavonoids and steroids while Akerlele et al. (2011) reported the presence of saponins and steroids in the stem extract but was not detected in this study. Differences in these reports could be due to environmental factors, time of collection and handling. The quantitative phytochemical analysis in this study indicates higher concentrations of phytochemicals on the leaf extract compared to the stem as shown in Tables 1 and 2. Phytochemicals are secondary plants metabolites responsible for many observed bioactivity of plant extracts. They are known to possess antioxidant, anti-inflammatory, antibacterial, immunomodulatory and antisickling activities (Egba et al., 2012). The presence of those metabolites no doubt is indication of the potential medicinal usefulness of the plant extracts.

The result of this work shows that the two extracts possess some biologically active compounds which could serve as potential sources of drugs. Saponin has been shown to have immense significance as antihypercholesterol, hypotensive and cardiac depressant properties (Price et al., 1987). Presence of tannins as shown in the result suggests the ability of this plant to play a major role as antidiarrhoeic and antihaemorrhagic agent (Asquith and Butler, 1986). Flavonoids have been shown to have antibacterial, anti-inflammatory, anti-allergic and antiviral antineoplastic activity (Alan and Miller, 1996). Many of

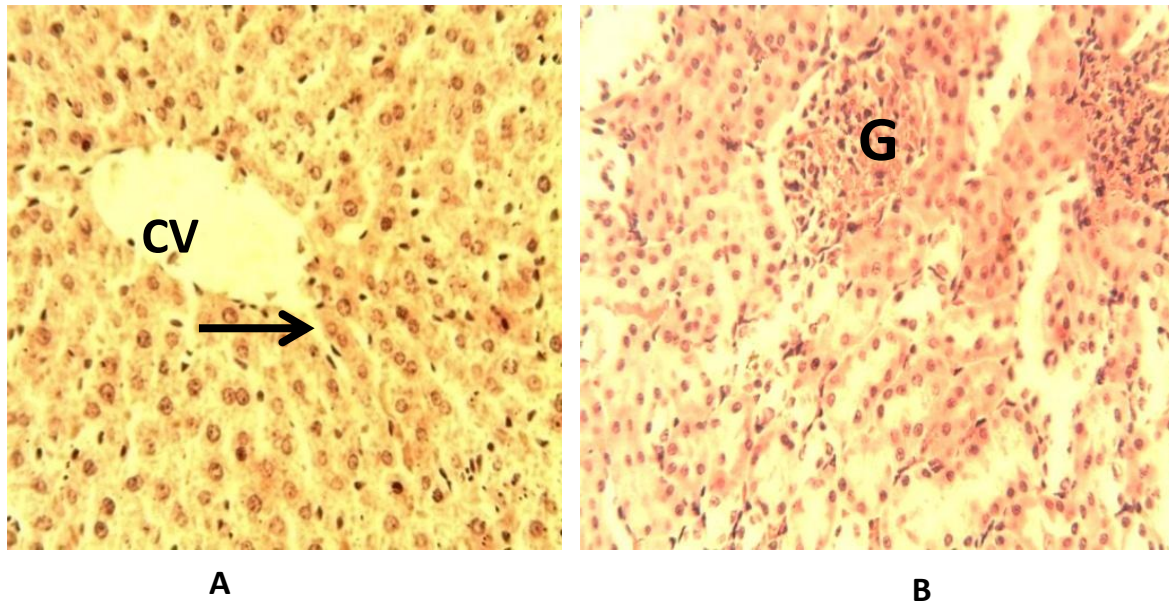


Figure 1. Photomicrograph of sections of organs from control rats showing, A) Normal liver with its central vein (CV) and Kupffer cells along the sinusoids (arrow), B) Kidney showing normal glomerulus (G) and renal tubules H&E $\times 400$.

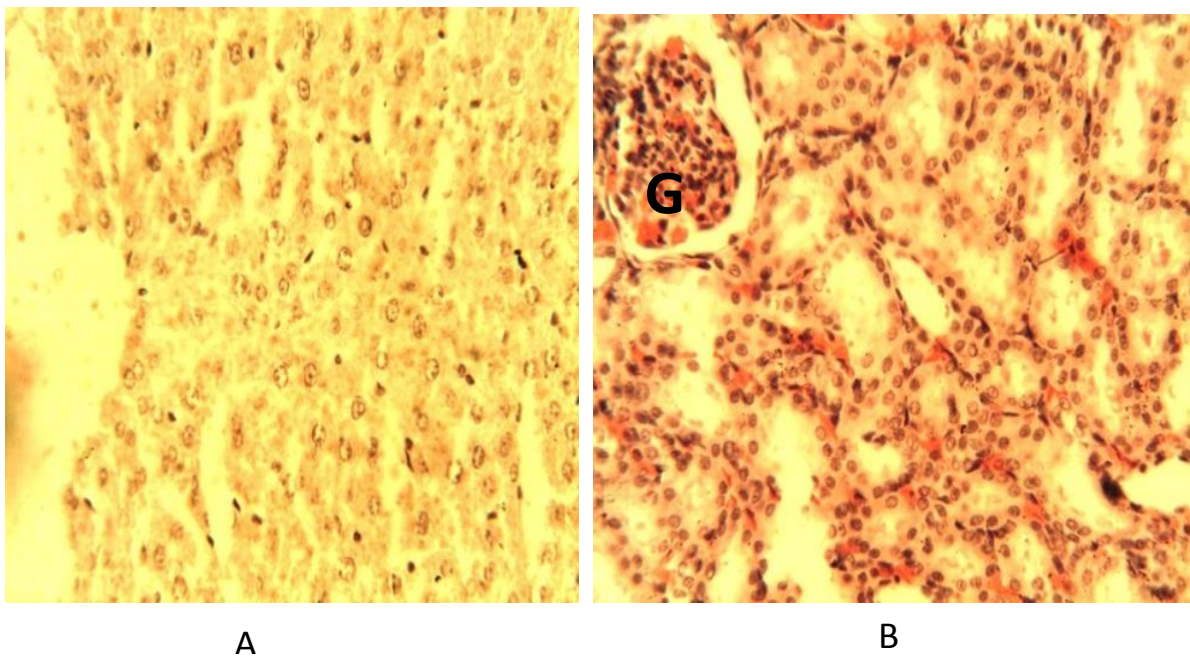


Figure 2. Histologic sections of organs from untreated diabetic rats. A) Liver with focal areas of hepatocyte degeneration, B) kidney having mild congestion of the glomerulus (G) H&E $\times 400$.

these alleged effects have been linked to their known functions as strong antioxidant, free radical scavenger and metal chelators (Nakayama et al., 1993). Steroidal compounds are of importance in pharmaceuticals because of their relationship with compounds used as sex hormones (Okwu, 2001). The terpenoids have also been shown to decrease blood sugar level in animal studies (Kuzuyama

and Seto, 2003). Glycosides may be crucial in the transduction of intracellular signals mediated by neurotransmitters, hormones, and neuromodulators receptors (Neer, 1995), activated by certain biological enzymes through hydrolysis, resulting in the separation of the sugar portion. When activated, these molecules can act on different intracellular targets (glycoside-linked signal trans-

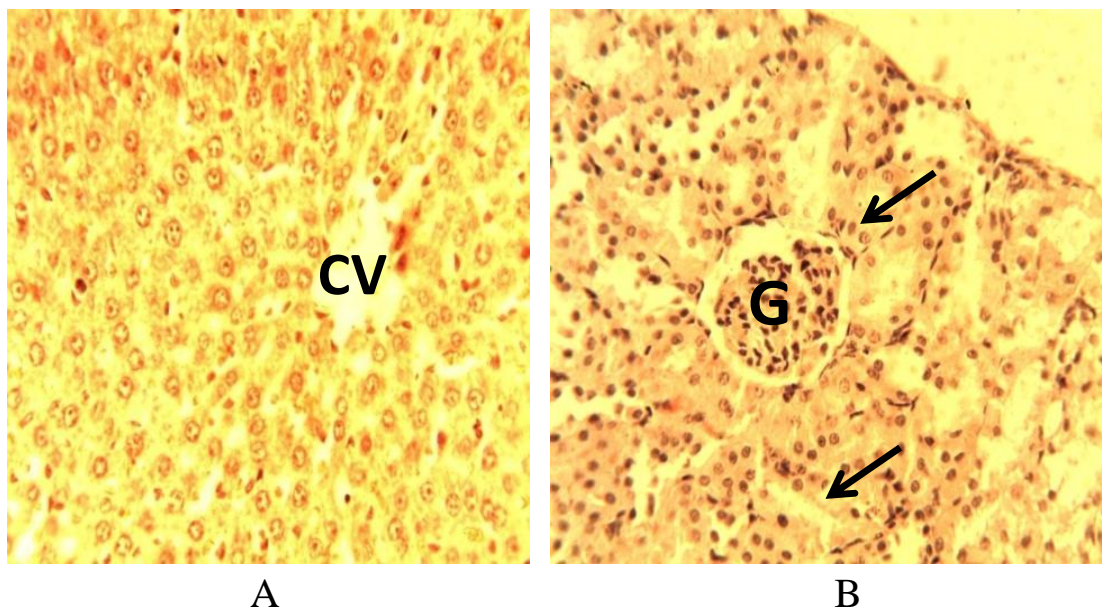


Figure 3. Photomicrograph of sections of organs from rats treated with glibenclamide showing A) Liver with the central vein (CV), and B) The kidney with no observable histologic change [the glomerulus (G) and renal tubules 'arrow']. H&E ×400.

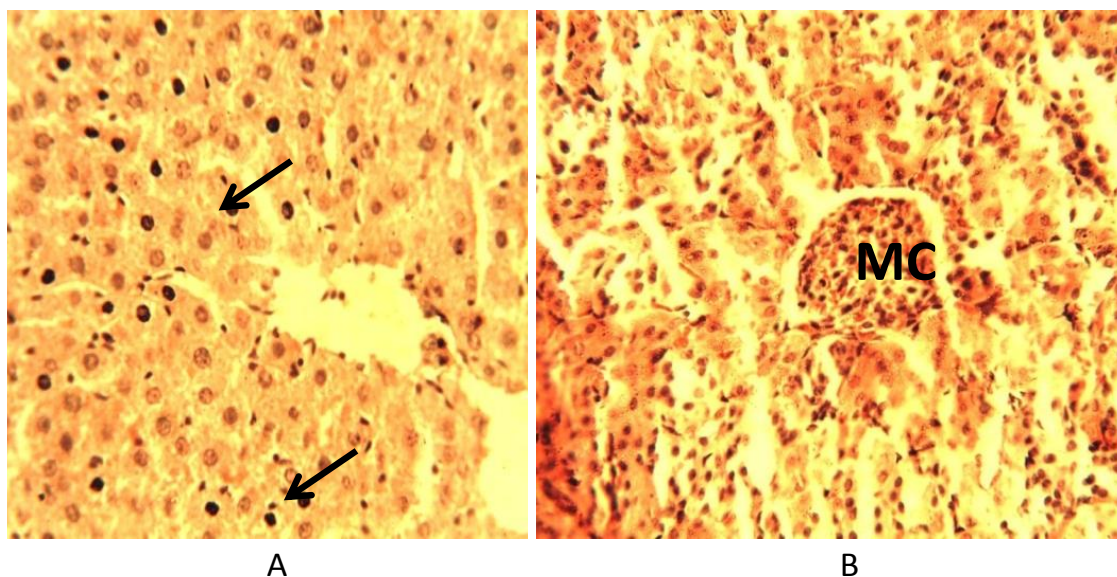


Figure 4. Histologic sections of organs from rats treated with 200 mg/kg of ethanol leaf extract showing A) Liver with apoptotic cells (arrow) and B) kidney with mild congestion (MC) of the glomerulus. H&E ×400.

duction proteins) (Dwivedi, 2006).

The result shows appreciable vitamin A, C and E content in the plant extracts. Vitamin C and E are potent natural antioxidants that scavenge free radicals and ameliorate their deleterious effects. The presence of these vitamins in the extracts suggests their possible role in curbing the incidence of oxidative stress in humans and animals. Calcium is a major factor sustaining strong

bones and plays a vital role in muscle contraction and relaxation, blood clotting, synaptic transmission and absorption of vitamin B₁₂. Potassium and magnesium are known to decrease blood pressure. Potassium plays a role in controlling skeletal muscle contraction and nerve impulse transmission. Patients with soft bone problems are usually placed on high calcium and potassium vegetables meals (Mensah et al., 2008). The acute toxicity (LD₅₀)

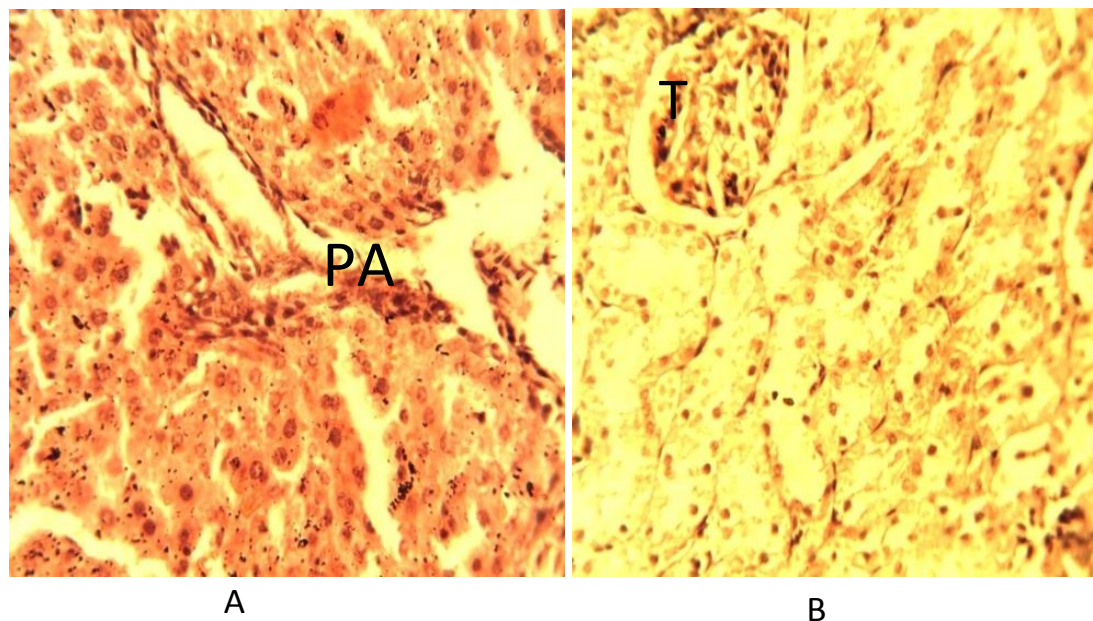


Figure 5. Photomicrograph of sections of organs from rats treated with 400 mg/kg ethanol leaves extract. A) Liver showing the portal area (PA) with no remarkable histologic change and B) Kidney with normal tubules and glomerular tufts (T). H&E $\times 400$.

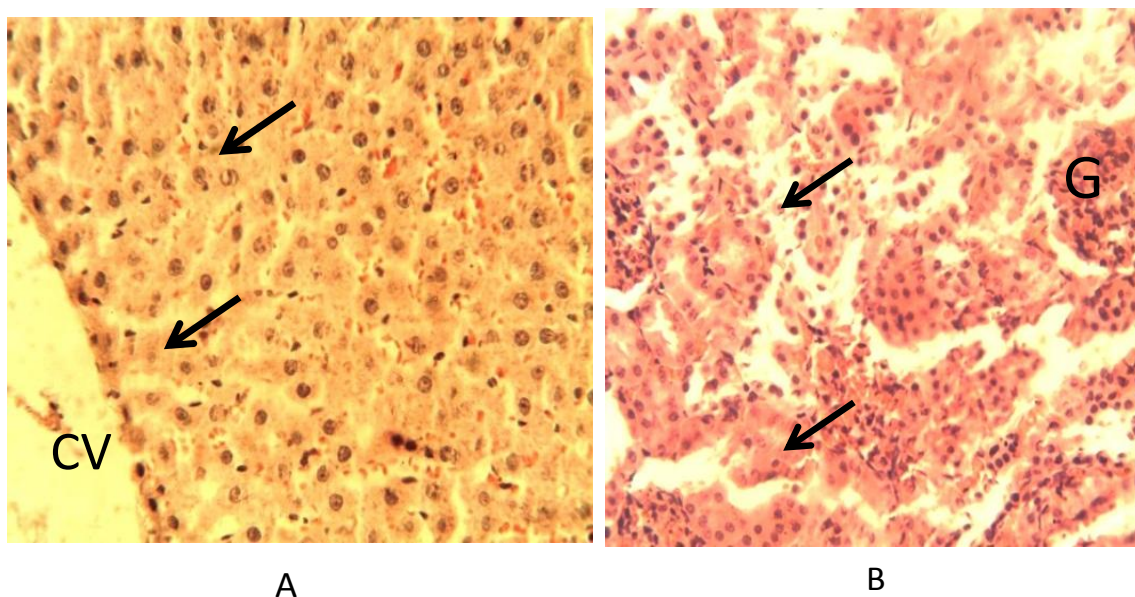


Figure 6. Photomicrograph of sections of organs from rats treated with 200 mg/kg of ethanol stem extract. A) Liver showing the central vein (CV) and normal plates of hepatocytes (arrows), B) kidney showing mild congestion of the glomerulus (G) and interstitium (arrows). H&E $\times 400$.

(LD₅₀) test of the ethanol extracts of *N. laevis* leaf and stem shows that the plant extracts were not toxic up to 5000 mg/kg body weight. This indicates that the leaves and stem extracts are safe for human and animal consumption and compliments earlier studies (Owolabi et al., 2011). This observation is supported by the histopathological examination which showed clear restoration

of diabetes induced pathological changes in tissue sections. The plants studied here can be seen as a potential source of useful drugs. Further studies are going on in this plant in order to identify, characterize and elucidate the structure of the bioactive compounds. There is equally need to study the toxicological effect of the plant with prolonged usage.

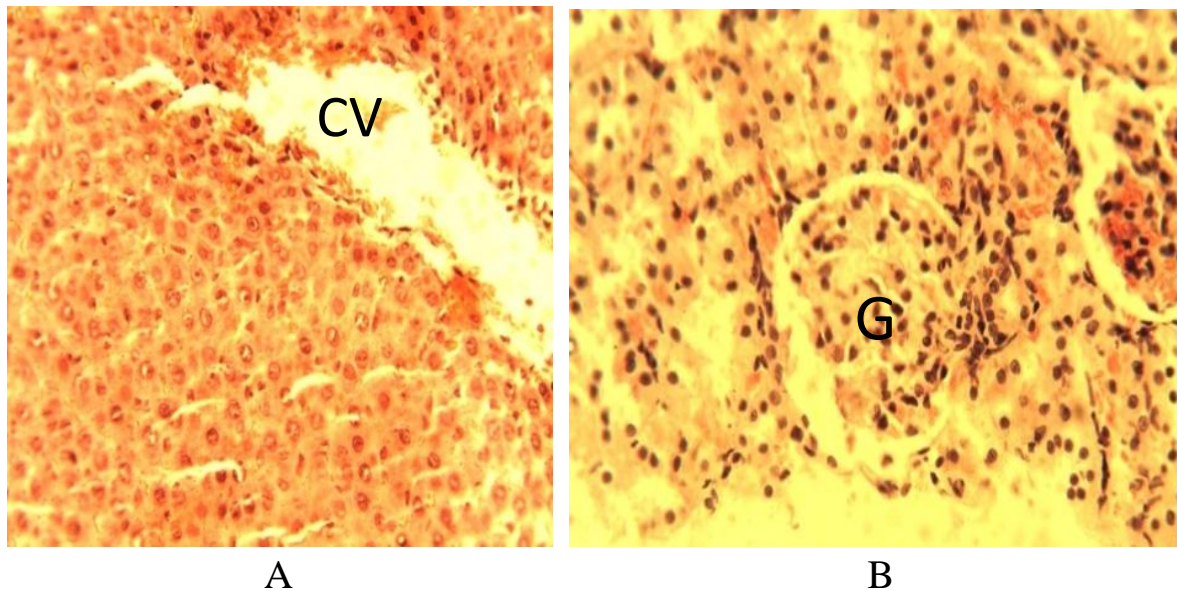


Figure 7. Histologic sections of organs from rats treated with 400 mg/kg ethanol stem extract. A) Liver showing central vein (CV) and normal hepatocytes, B) kidney showing mild hypercellularity of the glomerulus (G). H&E x400.

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Full Length Research Paper

Water and energy saving bioprocess for bioethanol production from corn grain applying stillage liquid part recirculation

Małgorzata Lasik*, Małgorzata Gumienna, Katarzyna Szambelan and Zbigniew Czarnecki

Institute of Food Technology of Plant Origin, Department of Fermentation and Biosynthesis,
Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland.

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The distillery stillage is a major and arduous byproduct generated during ethanol production in distilleries. The liquid part of this stillage was proved that can be recycled in the ethanol production from corn, without disturbing the fermentation process. The corn seeds were fermented employing the conventional non-pressure method for gelatinizing as well as including a novelty: Recirculation system of distillery stillage liquid part instead of process water. The efficiency of fermentation and main chemical parameters of stillage were estimated. The liquid part of stillage was recycled 28 times. At these conditions distillery yeast *Saccharomyces cerevisiae* efficiently produced ethanol yielding 79.80% of the theoretical, keeping the vitality and quantity on the same level. However, recirculation of the liquid part of stillage caused protein and potassium increase in the wet cake what makes this product more attractive for fodder supplementation. It was proven that the addition of stillage liquid fraction to the mashing process instead of process water and 28 recirculation cycles in ethanol production from corn constitutes the way which could significantly reduce the water and energy consumption, what essentially reduce whole general production costs without ethanol efficiency decreasing.

Key words: Corn, stillage liquid part, recirculation, ethanol yield.

INTRODUCTION

Bioethanol is the most promising biofuel and the starting material for various chemicals production. The addition of bioethanol in motor fuel enhances its octane number and decreases the negative ecological effects. Increase in the demand for ethanol as a fuel additive resulted in an increase in the amount of corn used for ethanol production. There are many technologies and materials applied for effective bioethanol production and stillage utilization (Mwithiga, 2013; Anwar et al., 2012; Marx et

al., 2012; Shanavas et al. 2011; Mojovic et al., 2010; Sun et al., 2010; Gibreel et al., 2009; Krzywonos et al., 2009a; Nolicic et al., 2009; Cibis et al., 2006a). Corn is characterized by high crop (8.0 t ha^{-1}) and ethanol yield (417 L t^{-1}) from ha. Corn grain is very important cereal material containing starch. This cereal contains over 60% of starch and is easy to handle as a material for fermentation. Corn can be converted into ethanol by either wet or dry milling method (Belyea et al., 2004;

*Corresponding author: E-mail: lasik@up.poznan.pl; Tel. +48618487288; Fax: +486184387314.

Abbreviations: DDGS, Dry distiller's grains with solubles; CDS, condensed distillers' solubles; WDGS, wet distillers' grains with solubles; DM, dry matter.

Devantier et al., 2005; Kwiatkowski et al., 2006). The hydrolyzing process must be preceded by gelatinization of the starch. Pressure cooking is very effective for further fermentation of starchy materials but production costs are high due to the high energy consumption in the cooking process. The processes to reduce the high production costs are required and non-pressure cooking fermentation system has also been successfully used and reached a fermentation efficiency equal to that of the conventional pressure cooking system (Shigechi et al., 2004; Mojovic et al. 2009; Nicolic et al. 2009; Shunavas et al. 2011).

However, the bioethanol production also generates arduous byproducts. The great influence on distillery industry situation has a raw material cost, expense of energy, water and rational utilization of byproducts. In distillery industry major byproducts are carbon dioxide and stillage. Stillage contains residual oligosaccharides, organic acids and non volatile metabolic byproducts of the fermentation (Cibis et al., 2006b; Kim et al., 2008). Its certain part can be dried together with spent grains to produce dry distiller's grains with solubles (DDGS) and used as ingredient in fodder production. The stillage liquid part can be concentrated to produce syrup called condensed distillers' solubles (CDS) or the wet distillers' grains is combined with the syrup giving wet distillers' grains with solubles (WDGS). Often the wet form of stillage is used as animal feed because of energy-consuming drying which makes the process more costly (Kim et al., 2008; Mojović et al., 2009). Stillage has to be chemically analyzed, among others for mineral substances which can be used as fertilizer components (Cibis et al., 2006b). But high water content in stillage gives many problems like transporting such product to farms or storage (Krzywonos et al., 2009b).

There is high surplus of stillage and that is why a lot of research is conducted aiming at creating technology for utilization or reusing this byproduct (Cibis et al., 2006b; Gibreel et al., 2009; Pejin et al. 2009; Mojovic et al., 2010; Gumienna et al., 2011). To lower the costs of water the distillery applies recirculation of liquid part of stillage. The whole stillage is usually centrifuged to produce a liquid fraction and a solid fraction (wet cake) than the liquid fraction can be recycled instead of process water. Replacing water by the liquid part of stillage makes possibility to save great number of water, energy and decreases sewage quantity (Czupryński, 2004; Pejin et al., 2009; Gumienna et al., 2011). The remaining wet cake, mostly after drying, can be sold as an animal feed.

Composition of stillage and its fractions has been recently of great interest in the area of ethanol production (Mojović et al., 2009). Kim et al. (2008) calculated that corn DDGS and wet distillers (wet cake) grain were rich in glucan, xylan and arabinan, protein, crude fat and crude fibre. The liquid fraction of stillage can not be recycled continuously. During the recirculation the substances are accumulated what causes disturbances in the production

process. In different systems, using various raw materials the ethanol fermentation process with stillage recirculation can give various effects and the limit of recirculation cycles is therefore unknown.

In the research, corn grain was tested as a raw material for ethanol production applying the technique of multiple recirculation of the liquid part of stillage. The aim of this study was to evaluate the effect of stillage liquid fraction recirculation in the mashing process for non-byproducts production and decreasing the amount of used water. The number of recirculation cycles which can be applied without disturbances in the ethanol fermentation process was determined.

MATERIALS AND METHODS

Raw material

Corn grain was obtained from Poznań University of Life Science Experimental Station in Swadzim, harvested in 2010. Raw material was milled before all analysis.

Microorganisms and enzymes

Distillery yeast, *Saccharomyces cerevisiae* ("Ethanol Red", Lesaffre, France) was used for fermentation experiments (0.5 g of the dry yeast per L⁻¹ of mash). Spezyme Ethyl (EC 3.2.1.1), heat stable α -amylase from *Geobacillus stearothermophilus* was used for ground corn liquefaction. Fermentzyme L-400 (EC 3.2.1.3), *Aspergillus niger* glucoamylase was applied for starch saccharification. The enzymes were added in the amounts according to the producer (Genencor International) recommendation.

Fermentation

The ground corn grains were mixed with liquid part of stillage (75%) and water (25%) before hydrolysis process. The ethanol fermentation was performed in 500 ml glass flasks. Non-pressure cooking (100°C, 1 h) was used for gelatinizing the ground corn. Then liquefaction (80°C, 20 min) and saccharification (55-60°C, 100 min) were performed. Fermentation media after hydrolysis were inoculated with *S. cerevisiae* in the form of yeast milk. To assure a sufficient nitrogen and phosphate sources, the fermentation media were enriched with diammonium phosphate, in the amount of 0.4 g l⁻¹. Fermentation was run for 72 h at 30°C batch in stationary culture. After fermentation the distillation process was applied. The remaining stillage was centrifuged (4000 r min⁻¹, 15 min) to obtain the liquid fraction for next fermentation cycle.

Analytical methods

Dry matter was determined directly by drying at 130°C for ground seeds and two-step at 90°C and 110°C for stillage, liquid part of stillage and wet cake, to constant weight. The starch content was estimated with enzymatic method according to Holm et al. (1986). The content of reducing sugars was determined by DNS-method (Miller, 1959). Total protein content was analyzed by Kjeldahl method and potassium content with dr Lange cuvette test. Ash content was determined by combustion of the sample (Krełowska-Kulaś, 1993). The ethanol concentration was assayed, in

Table 1. The efficiency of ethanol fermentation process from corn using stillage liquid part recirculation (75% instead of water).

Number of recirculation cycle	Starch saccharification (%)	pH of the fermented mash	Yeast vitality (%)	Ethanol		
				(L kg ⁻¹ starch) ^a	(% of theoretical yield)	(L 100 kg ⁻¹ corn)
0	90.50	4.69	93.00	60.88	84.64 ^c	38.26
1	96.41	4.28	99.00	54.96	76.44 ^a	33.08
7	96.81	4.35	93.72	58.49	81.35 ^b	35.74
14	80.85	4.35	99.00	60.38	83.98 ^c	37.14
21	82.52	4.63	97.44	60.97	84.80 ^c	38.26
28	77.24	4.59	91.00	58.29	81.07 ^b	36.82

The coefficient of variation was below 5% in all cases. Means within column with different letters are significantly different ($p < 0.05$). ^aThe ethanol yield in L kg⁻¹starch was calculated to total starch.

accordance with polish national standards after distillation using areometric method. The composition and purity of the obtained raw distillates were checked on a Hewlett Packard HP gas chromatograph, using Supelcowax-10 (60 m x 0.53 mm x 1.0 μ m) column and a FID detector.

The organic acids profile of fermented mashes was measured by high performance liquid chromatography (HPLC) method using Waters Alliance, HPX-87H BIO-RAD column with a RI detector, 30°C, flow speed 0.6 cm³ min⁻¹ as previously described (Lasik et al., 2008). This method with the same conditions was also used to confirm the results of ethanol concentration evaluated by traditional areometric method. All experiments were carried out in triplicates. Significances and standard deviation were calculated using the analysis of variance ANOVA, Statistica 6.0 software ($\alpha = 0.05$).

RESULTS AND DISCUSSION

The chemical composition of corn grain was characterized. The protein content in corn was determined at 8.41%. According to the study of Aufhammer et al. (1996), substrates for ethanol production should contain no more than 11% of protein. Starch concentration in the corn grain used in the performed experiment was found at 631 mg g⁻¹, reducing sugars at 7.48 mg g⁻¹ and ash at 1.3%.

In order to define the ethanol production efficiency in fermentation, using 75% stillage liquid part instead of process water for recirculation, there were 28 cycles conducted. Starch saccharification in sweet mashes reached average 84.96% what demonstrated good starch conversion to reducing sugars for further fermentation yield (Table 1). Ethanol yield from corn fermentations during all cycles ranged between 76.44 and 84.80% of theoretical yield (Table 1). Pejín et al. (2009) obtained higher ethanol yield, ranging 96.36-101.16% of theoretical yield, in the ethanol production from corn with liquid fraction of stillage recirculation. However, in their experiments the recirculation was repeated only six times with 10, 20 and 30% of liquid fraction instead of water.

In the present study, *S. cerevisiae* fermented corn mashes yielding average 82.05% of theoretical yield (differences statistically not significant at $p > 0.05$, besides

first recycling). It was observed that liquid part of stillage recirculated 28 times in 75% instead of process water did not cause ethanol fermentation yield decrease (Table 1). Increasing the number of recirculation cycles up to 28 times did not decrease ethanol production efficiency what could be explained by the fact that the liquid fraction enriched the medium with amino acids, vitamins and the products of yeast cells degradation. Gumienna et al. (2011) researched possibility of liquid part of stillage recirculation in bioethanol production from triticale. They also found high ethanol yield (mean 86.68% of theoretical yield) even the number of recirculation cycles was increased up to 40 times. The application of stillage liquid fraction recirculation (up to 28 times) did not statistically significantly ($p > 0.05$) inhibit the yeast growth and activity. In the present experiments, yeast vitality always exceeded 91% (Table 1).

The analyses of volatile compounds in raw distillates were also performed. The percentage of ethanol in the distillates was always higher than 99% of all volatile compounds detected. The most common byproducts found were higher alcohols (3.011-5.383 g L⁻¹ 100% spirit), esters (0.042-0.341 g L⁻¹ 100% spirit), aldehydes (0.033-0.323 g L⁻¹ 100% spirit) and methanol (0.031-0.066 g L⁻¹ 100% spirit) (Table 2). Till the 28th recirculation cycle the quantity of higher alcohols and methanol decreased whereas the concentration of aldehydes and esters increased (differences statistically significant at $p < 0.05$). The distillates obtained after fermentation with liquid part of stillage recirculation were characterized with volatile compounds. It was found that besides methanol, exceeded values acceptable for raw spirits in polish distillery industry were present. The requirements for raw spirits are very important when the spirit is intended for consumption. Taking into account application of the spirit for other needs, for example, bioethanol, higher content of volatile byproducts is not of great importance.

HPLC analysis was conducted for determination of organic acid profile in fermented mashes. The analysis showed the increase of acids quantity along with the

Table 2. Ethanol and byproducts content of corn raw distillates from fermentations with stillage liquid part recirculation (75% instead of water).

Recirculation cycle number		0	1	7	14	21	28
Aldehydes	(g L ⁻¹ 100% spirit)	0.160 ^c	0.033 ^a	0.097 ^b	0.323 ^e	0.201 ^d	0.321 ^e
	(% Total compounds)	0.02	0.01	0.01	0.04	0.03	0.04
Esters	(g L ⁻¹ 100% spirit)	0.052 ^d	0.095 ^b	0.273 ^e	0.137 ^c	0.042 ^a	0.341 ^f
	(% Total compounds)	0.01	0.01	0.04	0.02	0.01	0.04
Higher alcohols	(g L ⁻¹ 100% spirit)	5.383 ^f	3.255 ^b	3.329 ^c	3.011 ^a	3.897 ^d	3.948 ^e
	(% Total compounds)	0.68	0.41	0.42	0.38	0.49	0.50
Methanol	(g L ⁻¹ 100% spirit)	0.066 ^d	0.031 ^a	0.048 ^c	0.042 ^{bc}	0.044 ^c	0.035 ^{ab}
	(% Total compounds)	0.01	0.01	0.01	0.01	0.01	0.01
Ethanol	(% Total compounds)	99.28 ^a	99.56 ^c	99.52 ^c	99.55 ^c	99.46 ^{bc}	99.38 ^{ab}

The coefficient of variation was below 10% in all cases. Means within rows with different letters are significantly different ($p < 0.05$).

Table 3. Composition (mg mL⁻¹) of fermented corn mashes from fermentations with stillage liquid part recirculation (75% instead of water).

Recirculation cycle number	0	1	7	14	21	28
Lactic acid	0.087 ^a	0.064 ^a	0.138 ^b	0.292 ^d	0.293 ^d	0.217 ^c
Acetic acid	0.044 ^b	0.011 ^a	0.010 ^a	0.387 ^e	0.138 ^c	0.199 ^d
Propionic acid	0.406 ^a	0.477 ^{ab}	0.528 ^b	0.457 ^b	0.450 ^{ab}	0.445 ^{ab}

The coefficient of variation was below 10% in all cases. Means within rows with different letters are significantly different ($p < 0.05$).

increased number of stillage liquid part recirculation cycles (Table 3). Lactic acid content ranged from 0.064 to 0.293 mg mL⁻¹, acetic acid from 0.010 to 0.387 mg mL⁻¹ and propionic acid from 0.406 to 0.528 mg mL⁻¹ (differences statistically significant at $p < 0.05$). However, acid content detected in fermented mashes was not significantly important for further fermentations yield. Graves et al. (2006) reported that corn mashes contain at least 40 g L⁻¹ of lactic acid caused final ethanol yield reduction.

It was observed that dry matter content increased significantly ($p < 0.05$) with successive recirculation cycles both for stillage and its liquid part or wet cake (Table 4). Dry matter in stillage increased from 3.50% for the sample with no recirculation to 8.22% for 28th cycle; wet cake was characterized with an increase of dry matter from 14.54 to 21.69%, respectively. The present results showed that the recirculation of liquid fraction caused the protein content increase (differences statistically significant at $p < 0.05$) in the stillage (from 14.00 to 45.74% dry matter (DM)) and wet cake (7.81 to 32.21% DM) (Table 4). Such protein condensation makes the wet cake a good product for fodder production. The liquid fraction of stillage was also characterized with higher quantity of protein after 28th recirculation cycle which increased from 8.99% DM to 38.73% DM (Table 4). Potassium is one of

the most important macroelements occurred in stillage and its fractions. It was observed an increase in potassium content along with increasing amount of recirculation cycles. The liquid fraction of stillage from non recycled sample was characterized with 1.27% DM potassium and after 28th cycle 2.32% DM (differences statistically significant at $p < 0.05$) (Table 4).

Conclusion

The liquid part of stillage was proved that can be recycled in the process of bioethanol production from corn with no significant influence on ethanol production yield. A mean yield during all 28 tested recirculation cycles reached the level of 81.83% of the theoretical yield. Investigated solution of distillery byproducts utilization (liquid part of stillage) constitutes the way which could significantly reduce the bioethanol production costs due to both process water consumption and wastewater production.

ACKNOWLEDGEMENTS

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Table 4. Total protein and potassium content in corn stillage after fermentation (30°C, 72 h) with liquid fraction recirculation (75% instead of water).

Recirculation cycle number		Dry matter		Total protein		Potassium	
		(%)	(%)	(% DM)	(%)	(% DM)	
0	Stillage	3.50 ^a	0.49	14.00 ^a	0.03	0.75 ^a	
	Liquid part	1.50 ^a	0.14	8.99 ^{ab}	0.02	1.27 ^a	
	Wet cake	14.54 ^a	1.14	7.81 ^b	0.05	0.33 ^a	
1	Stillage	5.41 ^{bc}	0.79	14.60 ^a	0.07	1.33 ^c	
	Liquid part	2.72 ^b	0.21	7.72 ^a	0.07	2.65 ^e	
	Wet cake	20.13 ^c	1.13	5.61 ^a	0.07	0.35 ^a	
7	Stillage	6.84 ^d	0.91	16.52 ^{ab}	0.09	1.30 ^c	
	Liquid part	3.74 ^{cd}	0.41	9.86 ^{ab}	0.09	2.33 ^d	
	Wet cake	19.18 ^b	1.33	5.70 ^a	0.09	0.47 ^b	
14	Stillage	5.60 ^{bc}	1.07	19.04 ^b	0.09	1.61 ^d	
	Liquid part	4.16 ^d	0.41	14.47 ^b	0.08	1.92 ^b	
	Wet cake	23.27 ^e	1.86	7.97 ^b	0.11	0.47 ^b	
21	Stillage	7.76 ^e	3.69	47.49 ^c	0.09	1.22 ^{bc}	
	Liquid part	3.60 ^{cd}	1.51	41.95 ^d	0.08	2.21 ^c	
	Wet cake	19.92 ^c	5.86	29.39 ^c	0.10	0.50 ^b	
28	Stillage	8.22 ^e	3.76	45.74 ^c	0.09	1.09 ^b	
	Liquid part	3.46 ^c	1.34	38.73 ^c	0.08	2.32 ^d	
	Wet cake	21.69 ^d	6.99	32.21 ^d	0.10	0.46 ^b	

The coefficient of variation was below 10% in all cases. Means within columns (among the same samples) with different letters are significantly different ($p < 0.05$).

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Full Length Research Paper

Biodigestion of cassava peels blended with pig dung for methane generation

Oparaku, N. F., Ofomatah, A. C* and Okoroigwe, E. C.

National Center for Energy Research and Development, University of Nigeria, Nsukka, Enugu State, Nigeria.

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Biogas production from cassava (*Manihot esculentus*) peels and pig dung under a mesophilic temperature condition was investigated. Three blends of the wastes and a control labeled as B1, B2, B3 and C representing blend 1 (50:50 peel/dung), blend 2 (30:70 peel/dung), blend 3 (10:90 peel/dung) and control (pig dung alone) were used, respectively. Biodigestion of the wastes blends and control was carried out simultaneously under the same environmental and operational conditions of 30 days retention period using four metallic biodigesters of 32 L capacity each. The biogas yield result shows that blend 2 yielded the highest cumulative biogas of 78.5 L, while the least yield of 61.7 L was obtained by blend 3. When compared with the control set up and biodigestion of cassava waste alone from literature, there was blending effect resulting in increase in yield of biogas over the sole digestion of cassava peel or pig dung. Methane production leading to the combustibility of the biogas started at 6th, 5th, 5th and 4th days for B1, B2, B3 and C, respectively. This, in agreement with earlier studies show that better handling of cassava peels for energy production would be achieved by blending it with animal wastes in the right proportion.

Key words: Cassava peel, biogas, co-digestion, anaerobic digestion, wastes blends, lag days.

INTRODUCTION

One of the main environmental problems of today's society is the continuous increase in production of organic wastes which are harmful to human existence. In many developing countries, sustainable waste management and reduction have become major issue due to lack of adequate technology and methodology to handle wastes generated from daily living activities. Some of the waste types which are posing serious environmental threats to human and animal existence in these nations come from agriculture due to their degradable nature and lack of profitable technique to convert these 'wastes' to better manure quality or other useful means such as energy. With increase in farm (agricultural) operations, greater waste production is proposed.

Cassava is one of the major root crops produced in sub-Saharan Africa. World cassava production in 2002 was

estimated at 184 million tonnes (Odoemenem and Otanwa, 2011). As at 2002, Africa exported only one ton of cassava annually (FAO, 2001) but by 2007, out of "more than 228 million tonnes of cassava produced worldwide, Africa accounted for 52% and Nigeria produced 46 million tons making it the world's largest producer of cassava" (IITA -1). It has been projected that total world cassava utilization would hit 275 million tons by 2020 (IFPRI, 2008; Arowolo and Adaja, 2012) while some researchers estimate this number closer to 291 million tons.

Currently, there is increase in campaign for enlarging the cassava production scale in Nigeria. The implication of this is increased waste production from cassava processing. According to FAO (2001), about 250 to 300 kg of cassava peels is produced per tonne of fresh cassava

*Corresponding author. E-mail: ofomatony@yahoo.co.uk. Tel: +2348034010807.

root processed. This suggests huge sum of waste production in form of peels from cassava production and processing. Hence, there is need to design and adopt a system capable of handling the huge waste accruing from this development and anticipated problems such as unpleasant odour production.

One of the ways by which cassava peels can be managed in addition to using it as animal diet (Okeudo and Adegbola, 1993) is by anaerobic digestion for methane and bio-fertilizer production. From the literature, it is obvious that attempts have been made to convert cassava peels to energy. Adeyanju (2008) demonstrated the effect of adding wood ash to the biodegradation of mixture of piggery wastes and cassava peels in a laboratory scale. It was found that the wood ash addition increased the biogas production of either the biodegradation of piggery wastes and cassava peels only or in combination of both wastes in different proportions. The period it takes to produce methane was not stated probably because it was only a laboratory set up. Ofoefule and Uzodimma (2009) compared the biogas production potential of cassava peels alone to the blends of the waste with animal wastes. It was found that cassava peels alone produced a total of 68.70 L of biogas which could burn after 58 days of digestion. When blended with cow dung, poultry droppings and pig dung, the volume of biogas produced increased to 146.5, 166.50, and 169.60 L, respectively, while the flammable gas was produced after 9 days in the first 2 and 11 days in the later. These imply that anaerobic digestion of cassava peels alone is by no means economical. However, in this work, the blending with animal waste was on equal ratio. The effect of different mix ratios in biogas production potential and flammability of the biogas is a gap to be filled up by further investigation. Ezekoye and Ezekoye (2009) combined cassava peels with rice husk in the ratio of 1:5 for biodegradation of the wastes. It is also gathered that this set up was inoculated with cow dung mixed with water. From the result, flammable biogas was produced after 30 days with a total of volume of 3.450 m³. It is not certain which of the substrates is responsible for the long retention period of 70 days and large volume of biogas recorded. However, it is obvious that combination of two plant biomass is not a favourable combination in anaerobic digestion since animal protein is important for microbial activity of the methanogens.

Adelekan and Bamgboye (2009) have done a more detailed investigation from the existing works. The biogas productivity of cassava peels mixed in different ratios with fixed amount of animal wastes was investigated. In this work, 1, 2, 3 and 4 parts of cassava peels were mixed with one part each of poultry, piggery and cattle wastes. It was found that there was statistically significant effect of the mix ratios over biogas production. In this work also, it was found that the equal mass combination in all the animal waste types produced the highest biogas by volume when compared with other ratios. The effect of

mix ratios on how long it takes to produce flammable biogas was not covered.

Combination of cassava waste with other biomass species of agricultural origin has continued to attract the interest of scholars. In addition to the above, llaboya et al. (2010) blended cassava peels with pineapple and plantain peels in a laboratory set up. One of the aims of their work was to monitor the effect of alkaline addition to the substrate in biogas generation potential of the mixture. It was observed that addition of NaOH (alkaline) solution resulted in increase in biogas production over no alkaline addition. Also, there was positive effect in increase of biogas generation by different ratios of alkaline mixture. However, this work did not also address the effect of mixing the cassava peels in different ratios of other wastes.

In order to fill some gaps existing in previous co-digestion of cassava peel with other waste types, there is need to study the effect of varying the mix ratio of cassava peels vis-a-vis mix ratio of other wastes. In this work, quantity of cassava peel is varied with varying quantity of piggery dung in a batch anaerobic digestion. It is aimed at determining the mix combination that will yield the largest volume of biogas and the number of days it would take to produce flammable biogas while working with a particular waste. However, varying cassava peels in blends of multiple animal waste types can be investigated in another work.

Biodegradation of agricultural wastes is important as a way of maximizing the output of agricultural practice of developing nations. Multiple products (benefits) can be derived. This includes energy and biofertilizer production. It has been found that biodegradation of agricultural wastes produces better manure than using the wastes as raw manures in farm practice (Okoroigwe, 2007; Okoroigwe et al., 2008).

MATERIALS AND METHODS

The raw materials (cassava peels and the piggery waste) were obtained within University of Nigeria, Nsukka in Enugu State of Nigeria. Prior to the biodegradation of the wastes, the cassava peels were partially fermented by soaking in water for 7 days in order to reduce the acid content of cassava peels which could be detrimental to the microbes. Cyanide is an inhibitor to microbial activities (Cuzin and Labat, 1992; Cuzin et al., 1992). Standard methods were used to determine the proximate (moisture, ash) and chemical analyses of the samples prior to digestion. The cassava peels and the piggery dung were mixed in the ratio of 50% by weight of cassava peels to 50% by weight of piggery dung. This serves as blend 1 (B1). The second and third blends were achieved by 20% reduction and 20% increase in weight of cassava peel and piggery dung, respectively, in preceding blend. Hence, B2 and B3 were 30:70 and 10:90 (cassava peels: piggery dung), respectively. A control set up contained only piggery dung mixed with water at equal weight. All the samples were added with equal weight of water to the combined weights of the waste types. This is required to achieve 5 to 10% total solids (TS) concentration. The set up was monitored for daily gas production by measuring the volume of gas using downward displacement method. In order to achieve

Table 1. Proximate composition and physicochemical properties of the various wastes.

Parameter	Composition (%)			
	B1	B2	B3	C
Crude protein	2.89	3.23	3.57	3.74
Crude fat	1.17	0.87	0.57	0.42
Crude fibre	1.17	0.87	15.05	0.42
Ash	18.45	18.79	19.13	19.30
Moisture	26.65	21.79	16.93	14.50
Carbohydrate	37.69	41.22	44.75	46.52
Total solids	63.43	67.86	72.29	74.50
Volatile solids	35.00	35.07	35.15	35.19
Carbon	22.72	21.22	19.71	18.96
Nitrogen	0.46	0.52	0.87	0.60
C:N	49	41	23	32

homogeneity of the slurry and discourage scum formation in the system, daily stirring was carried out using the inbuilt stirring mechanism in the digesters.

Both slurry and ambient temperatures were measured at morning and afternoon hours. The average of both readings for any day becomes the reported slurry or ambient.

RESULTS AND DISCUSSION

The physico-chemical parameters of the waste streams are presented in Table 1. It shows that these wastes are good bioresources for methane generation. These values are close to values obtained by other researchers. The values however differ slightly because different waste combinations will give different composition of nutrients. It has been shown that biogas yield from AD of wastes depends on a number of factors such as pH, HRT and CN ratio (Yadika et al., 2004). CN ratio is an important indicator for controlling biological treatment systems (Wang et al., 2012). It has been pointed out that high C/N ratio indicates rapid nitrogen consumption by methanogens and leads to lower gas production while low C/N ratio results in ammonia accumulation and an increase in pH values, which is toxic to methanogenic bacteria (Zhang et al., 2013). This is partly reflected in the biogas yield of the blends as B3 with the lowest C/N ratio yield the least gas among the blends and the B1 with largest C/N ratio yields moderate biogas as compared to B2 according to the explanation above but Yadika et al. (2004), pointed out that during anaerobic digestion, microorganisms utilize carbon 25 to 30 times faster than nitrogen. Thus, to meet this requirement, microbes need a 20 to 30:1 ratio of C to N. This may justify the values presented in Table 1 as good for biogas generation.

The pH of the blend substrates and the control are presented in Figure 1. The result shows that the range of the pH values was 5 to 7.3 from the 3rd day to the 30th

day. It shows that the B1 and C had initial acidic condition. This agrees with Zhang et al. (2013) explanation on relationship between high C/N ratio and toxicity of the reacting medium. Similarly, the pH values of other blends were responsible for the high biogas yield experienced by B2 and B3. Figure 1 also shows that the reactions in all the blends and control have initial acid condition which neutralized as the reactions progressed to the 30th day.

Table 2 presents the cumulative biogas yield of the different blends and the control, while Figure 1 shows daily biogas production pattern of the waste blends and the control. From the result, blend 2 (B2) yielded the largest quantity of biogas (78.5L) followed by B1 (73.5L) while the least yield of 61.7L was obtained from the 3rd blend (B3). When compared with the control set up of biodigestion of piggery dung alone, there was blending effect leading to the increase in biogas yield. This shows that anaerobic digestion of piggery waste can be enhanced by combination with other wastes such as plant biomass. The result is different if combustibility of the generated gas is of interest. Biogas is not useful if it cannot be burnt in a combustor for energy and power production. Even though, the lag day (period to produce flammable gas) is shortest in the control experiment, it is necessary to blend it with other wastes as there is not much difference in the lag day of B2 which produced the largest volume of biogas. The low volume of biogas in biodigestion of pig dung alone is due to high concentrations of NH₄-N which inhibits the process of degradation of organic matter, causing a decrease in volume of biogas produced.

When compared with literature value of anaerobic digestion (AD) of cassava peels only, the least blending yield of 61.7 L (B3) obtained in this work was close to 68.70 L obtained by Ofoefule and Uzodimma (2009). The higher yield however, could be because of larger volume of biodigester and pretreatment used by them. But it has been pointed out that cassava peels alone is a poor feedstock for AD due to its high acid content (Cuzin and Labat, 1992; Cuzin et al., 1992). The results obtained here conform with other reports from other scholars (Adeyanju, 2008; Adelekan and Bamgboye, 2009) showing blending effect. This result has shown that blending the cassava peel with animal wastes should be better done at co-variation of both waste types to obtain an optimum mix ratio that will yield larger volume of gas.

The effect of microbial load (Table 3) can be observed in the pH stabilization (Figure 2) as the reactions progressed from 6th to 30th day. The multiplication of microbes in the system from 6th to 21st day is responsible for acid neutralization as there was progressive consumption of nutrients in the substrates. This was also responsible for the increase in the biogas yield in all the blends as microbial count increased. The peak production of biogas (Figure 1) is observed around the 21st day in blend 2 due to peak microbial count about that

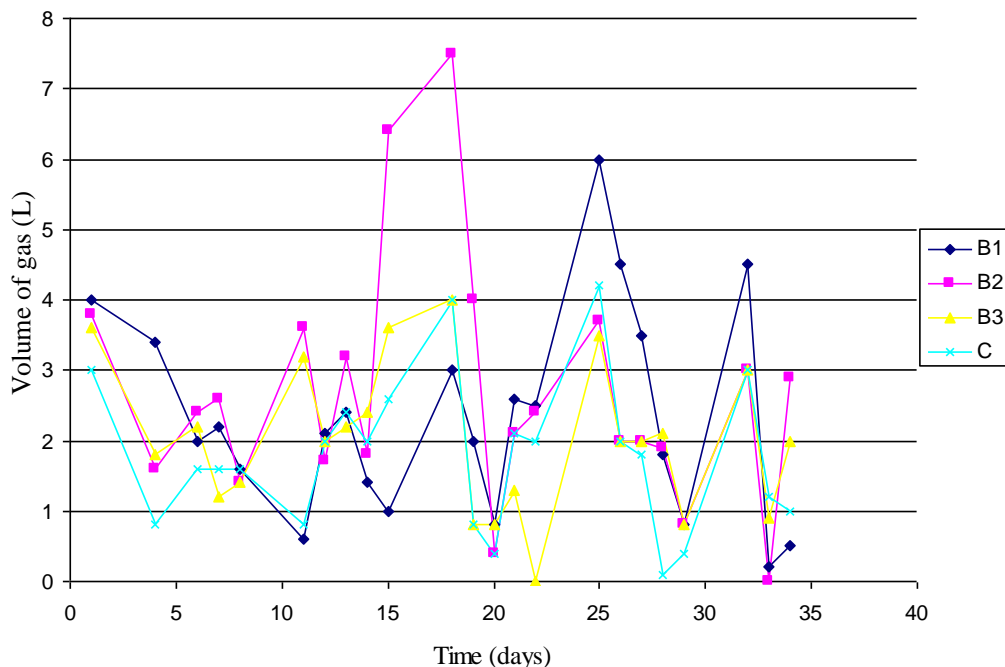


Figure 1. Daily biogas production pattern in the wastes.

Table 2. Cumulative biogas yield pattern of the wastes.

Parameter	Value			
	B1	B2	B3	C
Lag days	6	5	5	4
Cum biogas yield (L/gS)	73.50	78.5	61.7	55.1
Mean biogas yield	2.45	2.62	2.05	1.83

Table 3. Microbial load of the different waste blends.

Day	Microbial count ($\times 10^4$)			
	B1	B2	B3	C
1	2.0	2.2	1.9	1.9
6	2.2	2.3	2.1	2.0
11	2.3	2.5	2.3	2.2
16	3.8	3.9	3.2	3.0
21	2.0	22.0	1.3	12.0
26	3.2	3.3	2.2	2.0
30	2.5	1.7	1.9	1.9

time (Table 3). The microbial load began to reduce from the 21st day until the 30th day due to extensive consumption of nutrients in all the blends as well as in the control.

Figure 3 shows the temperature distribution pattern in both waste slurries and the ambient. The temperature

values were with 25 to 35°C in all cases within the 30 day test period. This is a mesophilic temperature range which enabled the micro-organisms to thrive favourably in the system for maximum performance. It therefore means that the volumetric result of biogas production is at its utmost yield condition.

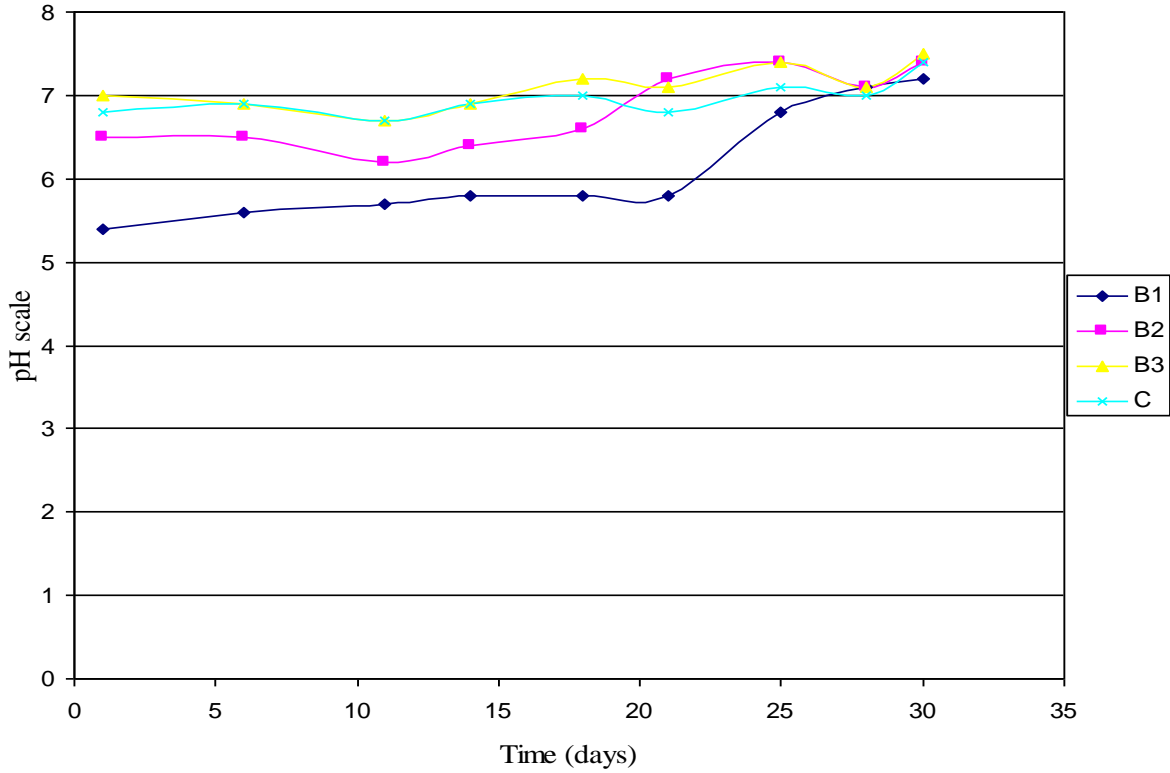


Figure 2. pH variation in the wastes.

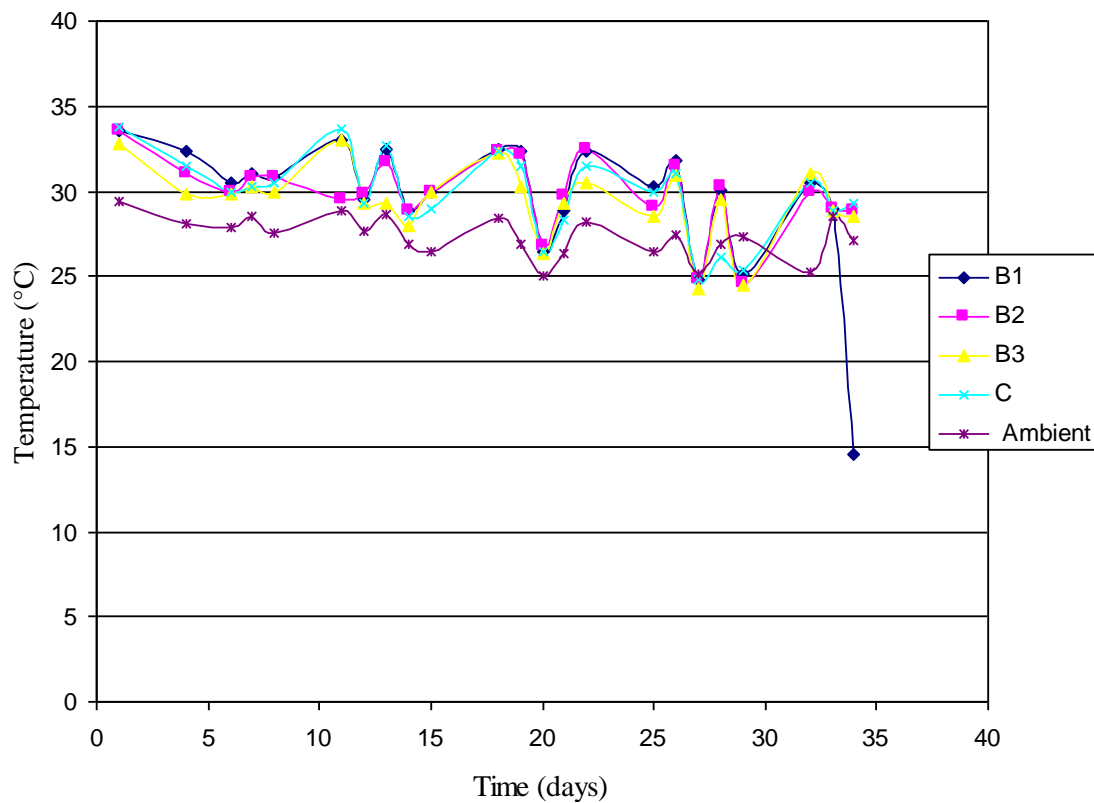


Figure 3. Average slurry and ambient temperature variation.

Conclusion

In this work, co-biodigestion of cassava peels with piggery waste was carried out in varying quantities of both wastes; there was blending effect over single biodigestion either of the plant and animal wastes. The B2 blend showing the combination of 30% by weight of plant waste (cassava peels) with 70% by weight of animal waste (piggery dung), yielded the largest volume of biogas on cumulative basis. Flammable biogas (methane rich biogas) was produced on the 5th day even though biodigestion of piggery waste alone produced flammable biogas on the 4th day. The blending improved the methane production of cassava peels alone from about 59 days (literature value) to five days in combination with piggery dung.

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Full Length Research Paper

The use of earthworm flour for lactic acid biomass production

Liliana Serna Cock^{1,2*}, Carlos Andrés Rengifo Guerrero² and Miguel Angel Rojas Restrepo²

¹Carrera 32 Via Candelaria, Palmira, Valle del Cauca, Colombia.

²School of Engineering and Management, National University of Colombia, Palmira Campus, Colombia.

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The potential use of Californian red earthworm flour as a nitrogen source for the production of lactic biomass was assessed. Three fermentation substrates, earthworm flour (EF), earthworm flour + yeast extract (EF + YE) and a commercial substrate MRS (control) were used. The substrates were formulated using 60 g L⁻¹ of glucose as the carbon source and 34 g L⁻¹ of earthworm flour as the nitrogen source. *Weissella confusa* was used as lactic acid bacterium. Nine batch fermentations were performed at 32°C and 100 rpm for 4 h, and the kinetics of the biomass concentration, lactic acid concentration and substrate consumption were compared. No differences were observed in the biomass concentration of the EF and EF + YE substrates, and final concentrations of 1.36 and 1.47 g L⁻¹ were obtained, respectively. The lactic acid concentration did not differ significantly between EF + YE and the commercial substrate, and values of 4.79 and 4.33 g L⁻¹ were obtained, respectively. These results suggest that earthworm flour can be a low-cost alternative for lactic acid biomass production.

Key words: Earthworm flour, *Weissella confusa*, lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB) are a very important group of microorganisms that have been used for centuries in the production of fermented food; these bacteria have been used in preservation processes, as they can prevent the growth of pathogenic microorganisms (Divya et al., 2002; Gillor et al., 2008). In fermented food products, LAB are responsible for acidification due to the production of acids such as lactic and acetic acids, and the production of bioprotector compounds such as bacteriocins and hydrogen peroxide (Gulahmadov et al., 2006). Studies have highlighted the benefits of using LAB in probiotic products, for example, LAB produce bacteriocins that prevent the growth of the microorganisms responsible for disease and food decay (Serna et al., 2010; Zhu et al., 2009; Divya et al., 2012). LAB are isolated from sources rich in

nutrients such as plants, animals, fermented foods, human and animal gastrointestinal tracts and the female genital tract (Zhu et al., 2009). These microorganisms are considered to be nutritionally demanding and require complex substrates for their growth (Lee et al., 2011; Savijoki et al., 2006). In the fermentation industry, the substrates for growth usually constitute the majority of the costs of microbial biomass production and its bioproducts (Kurbanoglu and Algur, 2002). In the biotechnological production of LAB, different substrates have been used such as glucose, lactose and starch (Sheng and Xia, 2006). However, these substrates are not economically viable, not only because the pure substrates are expensive and require the addition of complex nitrogen sources but also because natural polysaccharides require physi-

*Corresponding author. E-mail: lserna@unal.edu.co.

Abbreviations: EF, Earthworm flour; EF + YF, earthworm flour + yeast extract.

cochemical or enzymatic pre-treatment before they can be fermented (Young- Jung et al., 2004).

In the LAB and lactic acid production process, 68% of total production costs are associated with the cost of the raw material, and 38% of those costs correspond to yeast extract (Djukic - Vukovic et al., 2012). Therefore, nitrogen sources tend to be the most expensive constituent of the culture media.

Vasquez et al. (2004) indicate that the problem associated with lactic acid bacteria and their metabolites production on industrial scale, is due to the high demand for nutritional sources such as commercial culture medium (MRS, TGE, APT). The culture medium containing peptones, bactopectonas and meat extract are expensive. The search for alternative substrates that would allow for the optimum LAB growth while maintaining favourable production costs has been the subject of study of several authors (Qi and Yao, 2007; Serna et al., 2007; Guerra et al., 2001). Guerra et al. (2001) evaluated the ability of *Lactococcus lactis* and *Pediococcus acidilactici* strains to produce bacteriocins in diluted and concentrated serum by evaluating the effect of the total content of sugars, nitrogen and phosphorous. Anthony et al. (2009) evaluated the influence of the substrate composition and the culture conditions on the production of LAB and its bacteriocins using a *Bacillus licheniformis* AnBa9 strain. For producing bacteria, Taskin and Kurbanoglu (2011) evaluated chicken feathers hydrolysates as nitrogen source of low cost, and Kurbanoglu and Canli (2011) assessed as nitrogen source, sheep horns hydrolysates for economic production of glucose oxidase.

The Californian red earthworm (*Eisenia foetida*) flour could become an economically feasible alternative nitrogen source in fermentative processes such as lactic acid biomass production because it has high contents of nitrogen and proteins and a high reproduction rate (Vielma et al., 2007). Vermiculture is an activity that uses the Californian red earthworm as an alternative for recycling organic waste from different sources and as a nonconventional source of proteins and other nutrients at low cost (Vielma et al., 2003). The practice of vermiculture has a century old, and it has been used with environmental objectives such as waste management, soil detoxification and regeneration, and sustainable agriculture (Sinha et al., 2002).

Accordingly, the objective of this work was to evaluate the potential use of Californian red earthworm flour as a nitrogen source for the low-cost production of lactic acid biomass. Its potential use was measured in terms of its nutritional composition and kinetic parameters such as the specific growth rate (μ), biomass concentration, biomass yield ($Y_{x/s}$) and substrate consumption. In addition, concentration and lactic acid yield ($Y_{p/s}$) was measured.

The values of the mentioned variables were compared with the kinetic parameters obtained from a commercial substrate.

METHODOLOGY

Earthworm flour extraction

The Californian red earthworm (*E. foetida*) was obtained from Rancho J, a commercial vermiculture facility located in Buga, Cauca Valley, Colombia. 2 kg of adult worms was obtained from a composting pile composed of cattle and pig excreta. The earthworms were treated and sacrificed according to the methodology proposed by García et al. (2009) and Sogbesan and Ugwumba (2008). The earthworms were washed twice with tap water and left for 24 h in a container with distilled water and constant oxygen bubbling to remove the waste in their digestive tracts. They were sacrificed through a thermal treatment at -20°C (Electrocool LG, Mexico - Ciudad de Mexico) for 18 h. Afterwards, they were dried in a convection oven (Binder ED115, Germany) at 60°C for 24 h. The sample was then removed and milled (Fritsch Germany, particle size of 1 mm, 8000 rpm) to obtain the earthworm flour (EF).

Determination of the chemical composition

The earthworm flour contained dry matter, ash (AOAC, 1990, 942.05), minerals, organic carbon (Walkley and Black method, 1934), phosphorous and boron (Dawson, 1986), (UV-Vis spectroscopy), potassium, calcium, magnesium, sodium, other minor elements such as copper, iron, zinc and magnesium (Varga and Kolodziej, 1974), (atomic absorption spectroscopy), and crude protein (Kjeldahl, 1883) and ether extract (Soxhlet, AOAC, 1990, 920.39).

Lactic acid bacterium

A cryopreserved strain of *Weissella confusa* obtained in studies by Serna et al. (2010) was used. The strain was attached to each substrate after three successive generations. 24 h cultures, incubation temperature of $32 \pm 0.5^{\circ}\text{C}$ and 10% of inoculum, with respect to working volume were used.

Fermentation substrates

Three fermentation substrates were formulated: a control substrate (the MRS commercial substrate, Merck, Germany), the EF substrate (34 g L^{-1}) and EF + YE substrate [34 g L^{-1} of earthworm flour + 4 g L^{-1} of yeast extract (Oxoid, UK)]. All of the substrates were supplemented with 60 g L^{-1} of glucose as a carbon source. Nine batch fermentations were performed (three for each fermentation substrate). Once the substrates were prepared, they were placed in 1000-ml Erlenmeyer flasks with a working volume of 200 ml and were sterilised for 15 min at 121°C (Essem, 250A). The Erlenmeyer flasks remained under elliptical agitation at 100 rpm for 4 h at $32 \pm 0.5^{\circ}\text{C}$ (VWR Incubating Orbital Shaker VWR model 5000I, USA). During the course of the fermentations, the pH was adjusted to pH 6.0 using 1 M NaOH, according to the methodology described by Serna et al. (2010).

Kinetics of the substrate consumption, biomass formation and lactic acid concentration

For each substrate, 10-ml samples were aseptically taken at 0, 1, 2, 3 and 4 h of fermentation (time 0 corresponding to the time the inoculum was added). The samples were centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge - 5804R, Germany). The supernatant was used to measure the lactic acid concentration and

Table 1. Chemical and mineral composition of the earthworm flour (*Eisenia foetida*).

Parameter	Value
Dry matter (%)	93.7
Protein (%)	55.6
Nitrogen (%)	8.91
Ether extract (%)	8.09
Ashes (%)	8.69
P (%)	2.40
K (%)	1.58
Ca (%)	1.05
Mg (%)	0.81
Na (%)	0.73
Cu (ppm)	0.26
Fe (ppm)	5.07
Zn (ppm)	1.38
Mn (ppm)	0.22
B (ppm)	13.9

substrate consumption kinetics. The lactic acid was measured by reflectometry (Reflectoquant RQFlex plus 10 Merck, Germany) using reaction test strips. The substrate consumption was determined by spectrophotometric measurements (Genesys 10UV, EEUU) of the reducing sugars using the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). The precipitate was washed two times with a 0.9% NaCl solution and used to determine the biomass by dry weight (AOAC, 1990, 923.03). With the data obtained for the biomass concentration, lactic acid concentration and reducing sugars, the following kinetic parameters were calculated: the specific growth rate (μ) which is calculated from the biomass formation kinetic curve, and the biomass yield ($Y_{x/s}$) and the product yield ($Y_{p/s}$), which are calculated using Equations 1 and 2, respectively. The percentage of reducing sugars consumed (RSC) was calculated using Equation 3:

$$Y_{x/s} = \frac{X - X_0}{S_0 - S} \text{gg}^{-1} \quad (1)$$

$$Y_{p/s} = \frac{P}{S_0 - S} \text{gg}^{-1} \quad (2)$$

$$RSC = \frac{(S_0 - S) * 100}{S_0} \% \quad (3)$$

Where X_0 , is the initial biomass concentration (g L^{-1}); X , is the final biomass concentration (g L^{-1}); S_0 , is the initial reducing sugars concentration (g L^{-1}); S , is the final reducing sugars concentration (g L^{-1}); and P , is the maximum concentration of lactic acid (g L^{-1}). The final concentrations were determined when P was maximal.

Statistical analysis

A unifactorial model with three replicates was used, where the type

of substrate was a factor with three levels (control, EF and EF + YE). The response variables were the biomass concentration, the lactic acid concentration and the substrate consumption, which were measured at 0, 1, 2, 3 and 4 h of fermentation. In addition, the kinetic parameters specific growth rate (μ), product yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$) were calculated. The results were analysed with the statistical package SAS 9.2 for Windows (SAS Institute Inc.(1993), Cary, NC, USA). The average values were compared with the Tukey test, with a probability of $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of earthworm flour

The results for the percentages of dry matter, nitrogen, protein, ashes, ether extract and earthworm flour minerals are shown in Table 1. The earthworm flour had high protein content (55.6% dry matter). Similar results were reported in previous studies, where the importance of the nitrogen and protein content of earthworm flour was highlighted (Vielma et al., 2007; Sogbesan and Wgwumba, 2008; Garcia et al., 2009). Horn et al. (2007) indicated that the nitrogen content is the most important parameter in the culture media. Likewise, Haq et al. (2008) mentioned that nitrogen is an important quantitatively bioelement, which is required by many microorganisms during fermentation for protein synthesis. Cellular growth is directly influenced by the composition of the fermentation substrate.

According to Vielma et al. (2007), earthworm flour has a good content of essential amino acids, vitamins and minerals that are necessary for optimal cellular growth of lactic acid bacteria. The ash content in earthworm flour was high, and similar to ash content reported by Vielma et al. (2007). Authors such as Horn et al. (2007) and Djukic - Vukovic et al. (2012) used cod viscera and liquid vinasse in lactic acid fermentation, and they found that the ash content was 10.1 and 14.4%, respectively. According to Reddy et al. (2011), high ash contents in the sample could be an advantage because it provides mineral elements that are necessary for the growth of microorganisms. The mineral composition of earthworm flour showed contents of phosphorous, potassium, calcium, zinc and boron. It is significant that the earthworm flour provides elements such as phosphorous because it favors the synthesis of phospholipids, nucleic acids and proteins (Taskin et al., 2011).

In addition, calcium and magnesium can have a protective effect on the cell, favoring its viability (Xue et al., 2008); however, authors such as Chotineeranat et al. (2010) claim that the presence of high concentrations of calcium in the fermentation substrate has an adverse effect on the cell, lowering its growth and biomass yield rate. Furthermore, elements such as Mg, Ca, Mn, Fe and Zn, stimulate the lactic acid production and the growth of lactic acid bacteria (Djukic - Vucokic et al., 2012). Kurbanoglu et al. (2001) report that element such as iron has a toxic effect on microorganisms when used in high

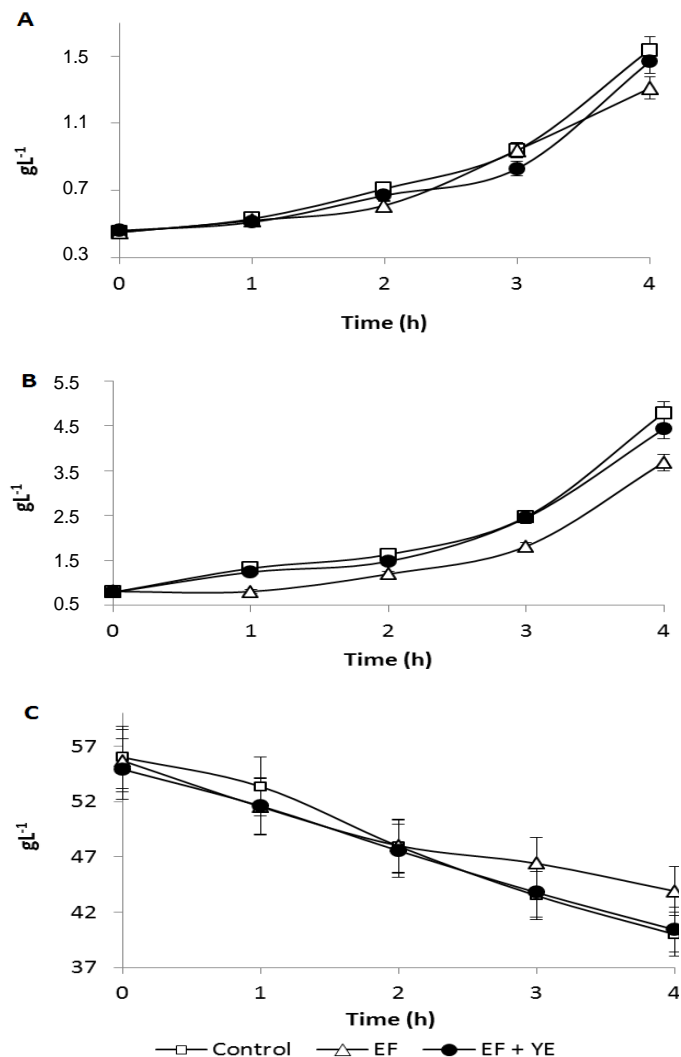


Figure 1. a) Kinetics of the biomass concentration; b) kinetics of the lactic acid concentration; c) kinetics of the *Weissella confusa* substrate consumption for the three evaluated substrates.

concentrations, inhibiting the cellular growth. According to Djukic - Vukovic et al. (2012), the optimal concentration of Fe and Ca for growth of LAB is ≤ 4 and ≤ 8000 mg L⁻¹, respectively. When using 34 g L⁻¹ of earthworm flour, the contribution of Fe and Ca is 0.17 and 357 mg L⁻¹, respectively; this is an optimal range for growth of LAB. Van Niel and Hahn - Hagenrdal (1999) showed in a strain of *L. lactis*, that when the culture medium is supplemented with 2 to 4 ppm of Fe, biomass and product yields are improved.

Formulation of substrate and kinetic parameters

The earthworm flour did not show good solubility during the preparation of the fermentation substrate. When preparing the fermentation broth with earthworm flour as

the nitrogen source, the formation of precipitates occurred after sterilization. Vielma and Medina (2006) claim that the earthworm flour contains proteins with different percentage (%) of solubility that depend on the pH of the medium. Garcia et al. (2010) suggest that when the peptides that constitute the culture medium are smaller, the precipitation and turbidity effects caused by the sterilization will be lower. These authors also claim that the precipitation effect could be caused by the combination of calcium and phosphate ions present in the peptones. Biomass values were corrected by subtracting the fraction of earthworm flour precipitated during centrifugation.

The fraction of earthworm flour was calculated determining its weight in trials where no inoculum was used. Figure 1 shows the kinetics of the biomass concentration, lactic acid concentration and substrate consumption. Table 2 shows the specific growth rate (μ), biomass yield ($Y_{x/s}$), product yield ($Y_{p/s}$) and the percentage of reducing sugars consumed (RSC). Table 3 shows the analysis of the averages obtained by the Tukey test. The substrate consumption did not exhibit significant differences between the three treatments ($p > 0.05$) at any of the fermentation times. The highest consumption value corresponded to the control substrate, followed by the EF + YE substrate (Table 3). The highest biomass concentration (Figure 1a) was obtained at the fourth hour of fermentation using the control substrate (1.54 g L⁻¹), followed by EF + YE (1.47 g L⁻¹) and EF (1.31 g L⁻¹). The statistical analysis did not show statistically significant differences between the three types of substrates ($p > 0.05$). The lactic acid concentration (Figure 1b) did not show significant differences between the control substrate and the EF + YE substrate. The highest lactic acid concentration was obtained with the control substrate (4.79 g L⁻¹), followed by EF + YE (4.33 g L⁻¹) and EF (3.68 g L⁻¹), at the fourth hour of fermentation.

As shown in the Figure 1a and Table 2, the biomass concentration, biomass yield ($Y_{x/s}$) and the product yield ($Y_{p/s}$) were slightly higher with the commercial substrate ($p > 0.05$), followed by EF + YE and EF (Table 2). This growth assumes that when using earthworm flour as the fermentation substrate, the required nutrients are provided, such as nitrogen, vitamins and some amino acids that are necessary for bacterial growth (Altaf et al., 2005). In general, all of these elements are supplied as complex constituents such as the yeast extract. However, the results show that using earthworm flour as the source of nitrogen and minerals, high biomass yield and biomass concentration are obtained, even without the use of yeast extract. Serna et al. (2010) found similar biomass and product yields using a *W. confusa* strain (0.062 and 0.46 g g⁻¹, respectively) and a commercial substrate. However, the same authors found higher yields when using milk supplemented with yeast extract, with yields of 5.98 g g⁻¹ for the biomass and 0.73 g g⁻¹ for the product. Mondragon-Parada et al. (2006) found similar yields

Table 2. Specific growth rate (μ), biomass yield ($Y_{x/s}$) lactic acid yield ($Y_{p/s}$) and percentage of reducing sugars (RSC) of *Weissella confusa* in control, EF + YE and EF substrates.

Substrate	μ (h^{-1})	$Y_{x/s}$ (gg^{-1})	$Y_{p/s}$ (gg^{-1})	% RSC
Control	0.3034	0.069 \pm 0.011	0.301 \pm 0.055	28.59
EF + YE	0.2811	0.068 \pm 0.007	0.298 \pm 0.030	26.41
EF	0.2729	0.065 \pm 0.001	0.253 \pm 0.006	21.17

Table 3. Tukey test* (average comparison).

Substrate	Substrate consumption	Lactic acid	Biomass
Control	48.360 \pm 6.6560 ^a	2.279 \pm 1.5332 ^a	0.830 \pm 0.4372 ^a
EF + YE	47.022 \pm 5.8291 ^a	2.065 \pm 1.4077 ^a	0.793 \pm 0.4079 ^a
EF	49.241 \pm 6.6560 ^a	1.656 \pm 1.2037 ^b	0.756 \pm 0.3573 ^a

*Averages with the same letter are not significantly different ($p > 0.05$).

biomass using *Lactobacillus casei*, and milk serum as fermentation substrate (0.063 gg^{-1}). Vasquez et al. (2004) reported yields of biomass 0.057 and 0.089 gg^{-1} using hydrolyzed squid viscera for growth of strains of *L. lactis* and *P. acidilacti*, respectively. Djukic - Vukovic et al. (2012) using liquid vinasse reported higher yields of lactic acid (0.90 g g^{-1}).

Authors such as Nancib et al. (2001), Altaf et al. (2005) and Gao et al. (2006) have emphasised the importance of vitamins as a microbial growth factor. Authors such as Berry et al. (1999) evaluated the growth of *Lactobacillus rhamnosus* in a similar medium as the MRS commercial substrate. They highlighted the importance of the presence of amino acids such as cysteine, asparagine and glutamine in the fermentation substrate, because these compounds provide elements such as sulphur and nitrogen, which are necessary in the reproduction stage. Vielma et al. (2007) found in their study that earthworm flour contains vitamins such as biotin and riboflavin; amino acids such as leucine, glutamic acid and aspartic acid; and elements such as magnesium, sodium and potassium, that favor cellular growth and metabolism. Recent studies have focused on the search for agro-industrial wastes and non-conventional raw materials with biotechnological potential for fermentation processes to lower the cost of the raw materials. Due to the nutritional value of earthworm flour, this raw material can be used as a nitrogen source for low-cost lactic biomass production. Horn et al. (2007) reported that a culture medium capable of stimulating the growth of lactic acid bacteria is probably, a good substrate for the growth of other microorganisms, which have similar nutritional requirements or lower compared with requirements of lactic acid bacteria.

In this study, earthworms were obtained from vermiculture activity; a process in which the worms were fed with agricultural wastes, therefore, production costs of the worm was not significant. In energetic terms, the use

of these raw materials requires an adaptation of the previous process. For example, to prepare 1 kg of earthworm flour under laboratory conditions, the total required energetic demand was 41.42 kW h^{-1} and was distributed for the following processes: freezing (2.52 kW h^{-1}), drying (38.4 kW h^{-1}) and grinding (0.5 kWh^{-1}). From the aforementioned, the approximate cost of earthworm flour production is 8.96 USD/kg. Therefore, when 34 gL^{-1} of earthworm flour as fermentation substrate is used, the cost of raw materials for the production of lactic biomass is reduced by 60%, compared to the cost of using commercial substrate. However, the cost estimation was based on operations conducted at a laboratory scale; thus, subsequent studies should be aimed at scaling up this process to the industrial level and determining the economic viability of such a process.

Conclusions

The use of earthworm flour as a fermentation substrate considerably reduces production costs and produces acceptable biomass levels for use in the generation of probiotic products. In addition, the biomass can be used in the food industry for the production of bacteriocins that prevent the growth of pathogens and microorganisms that cause food decay. Thus, Californian red earthworm flour has important biotechnological and economic potential in lactic acid biomass production.

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Full Length Research Paper

Isolation and characterization of anticoagulant compound from marine mollusc *Donax faba* (Gmelin, 1791) from Thazhanguda, Southeast Coast of India

N. Periyasamy*, S. Murugan and P. Bharadhirajan

CAS in Marine Biology, Faculty of Marine Science, Annamalai University, Parangipettai – 608 502
Tamil Nadu, India.

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Glycosaminoglycans (GAGs) are linear polysaccharides found in the extracellular matrix and biological fluids of animals where they interact with hundreds of proteins and perform a variety of critical roles. There are five classes of animal GAGs: heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA). Many biological functions can be monitored directly by their impact on GAG quantity. In the present study, glycosaminoglycans were isolated from marine bivalve *Donax faba*. The amount of crude GAG was estimated as 12 gm/kg and of tissue in *D. faba*. After purification using gel chromatography, the yield was found to be 0.83 mg/kg. The bivalve showed the anticoagulant activity of the crude and purified samples 58 and 114 USP units/mg correspondingly in *D. faba*. The structural characterization of anticoagulant GAG was analyzed by Fourier transform infrared spectroscopy. Among the marine bivalve, *D. faba* purified showed more anticoagulant activity than that of crude sample. The results of this study suggest that the GAG from *D. faba* could be an alternative source of heparin.

Key words: *Donax faba*, GAGs crude and purified, anticoagulant activity, Fourier transform- infra red (FTIR).

INTRODUCTION

Glycosaminoglycans (GAGs) are long, linear, disaccharide repeats of hexosamine and highly sulphated galactose or hexuronic acids, and are usually found covalently attached to a protein 'core' to form proteoglycans (Kjellen and Lindahl, 1991; Silbert et al., 1997). GAGs are an extremely heterogeneous group of molecules that can be divided into several different general classes, such as haparan sulphate (HS) (and its model analogue, heparin), dermatan sulphate (DS) and chondroitin sulphate (CS), depending on the composition of the sugar backbone and the degree of sugar modification. Proteoglycans/GAGs are expressed by all nucleated cells and several bacterial pathogens such as *Bordetella pertussis* (Menozzi et al., 1994), *Mycobacterium* sp (Menozzi et al., 1996), and

Listeria monocytogenes (Alvarez-Dominguez et al., 1997), encode surface proteins that recognize GAG, that is, GAG- binding adhesions (Rostand, 1997). The GAGs, HS and heparin have been implicated as participants in a variety of physiological processes including cell-cell recognition, blood coagulation, infection, and cell growth and differentiation. Both are polysaccharides that consist of repeating disaccharide units composed of uronic acid and an amino sugar. HS, which is sulfated lesser than heparin, is nearly ubiquitous in the animal kingdom and is often a cell surface marker. On the other hand, heparin is found only in Chordata, Mollusca and Arthropoda (Nader et al., 1999) and over 80% of its glucosamine residues are N-sulfated (Gallagher and Walker, 1985). Heparin

*Corresponding author. E-mail: vntamil@gmail.com. Tel: 91-9790145943.

has been used for anticoagulant therapy for many years. Its anticoagulant effect appears to be mediated mainly through antithrombin III (AT III), which is a plasma protein and the main coagulation inhibitor in the blood.

Antithrombin III or heparin cofactor, as it is also called, inhibits thrombin and the activated forms of the coagulation factors IX, X, XI and XII. In the absence of heparin, the inhibition reactions are slow, but the addition of heparin strongly accelerates them. Several models have been proposed to explain the effect of heparin on the inhibition of thrombin by ATIII. Heparin is known to bind the ATIII; one widely accepted model assumes that heparin forms a complex with AT III and transforms it into a more rapidly acting inhibitor (Holmer et al., 1979). Currently, commercial heparin preparations are obtained from mammalian sources, either from porcine, bovine intestine or bovine lung. Non-animal sources of heparin for pharmaceutical use are currently not available. However, the occurrence of the heparin is not restricted to mammals. Several heparin and heparin like polymers have been described in invertebrate animals such as crustaceans (Hoving and Linker, 1982), mollusks (Cavalcante et al., 1985) and ascidians (Cavalcante et al., 2000) also. It is real fact that the importance of marine organisms as a source of new substance is growing with marine species comprising approximately a half of the total global biodiversity, the sea offers an enormous resources for novel compounds, and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity. A very different kind of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive and aggressive surrounding very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules (Aneiros and Garateix, 2004).

Molluscs contribute significantly to the total marine fish catch of the world. Marine bivalves are abundant in coastal and estuarine waters of India. The bivalve's fishery is constituted mainly by clams, mussels and oysters; molluscan fishery is not well-organized along the Indian coast. Molluscs are exploited in large quantities by traditional methods and sold live in the market for human consumption. The economically important species of marine bivalves are green mussel (*Perna viridis*), estuarine oyster (*Crassostera madarasensis*) and clam (*Meretrix casta*, *M. meretrix*, *Donax* sp. *Phapia malabarica*, *Villorito cyprinoids*) (Chattergi et al., 2002). Among the mollusks, some have pronounced pharmaceutical activities or other properties useful in the biomedical area. It is surprising that some of these pharmacological activities are attributed to the presence of polysaccharides, particularly those that are sulfated (Arumugam and Shanmugam, 2004). Hence, the attempt has been made to isolate, characterize and anticoagulant activity of the GAG from the marine bivalve *Donax faba* using chromatography. The GAGs

were purified by gel chromatography using sephadox G-100 column. The structure analysis of crude and purified GAGs was determined through Fourier Transform- Infra Red spectrum.

MATERIALS AND METHODS

Isolation of glycosaminoglycans

The molluscs *D. faba* (Class-bivalvia; Family- Donacidae) was collected from the Thazhanguda coastal waters (Latitude, 11° 45' 0N; Longitude, 79° 45' 0E) Cuddalore, Southeast coast of India. Shells were opened and whole tissues were taken. They were blended in 0.4 M sodium sulfate solution (Na₂SO₄; 3.5 1/kg of the tissue) and kept at 55°C for 1 h 30 min. The pH was adjusted to 11.5 by adding 10% sodium hydroxide (NaOH) solution. Aluminium sulfate (Al₂(SO₄)₂) crystals (80 mg/kg tissues) were added to this solution, and the suspension was heated to 95°C for 1 h. Cetyl pyridinium chloride (CPC) solution (3 g/100 ml of 0.8 M NaCl) was used to precipitate the crude white heparin complex. The precipitate was redissolved in 150 ml of sodium chloride solution (2.0 M) and was incubated at 30°C for 30 min. The precipitate was washed with ethanol and methanol through centrifugation and vacuum dried.

Purification of GAGs - Gel chromatography

GAGs were purified on a 5 × 90-cm column of sephadex G-100 (Sigma). The elution rate was approximately 60 ml/h and 15-ml fractions were collected. The active fractions were pooled and extensively dialyzed against distilled water and freeze dried (Laurent, 1978).

Anticoagulant activity

The anticoagulant activities of crude and purified GAGs samples were determined by comparing with the concentration necessary to prevent the clotting of sheep plasma using USP (United State Pharmacopoeia) method.

Fourier transform- infra red spectrum analysis (FTIR)

FT-IR spectroscopy of crude and purified GAG sample of *D. faba* relied on a Bio-Rad FT-IR- 40 model, USA. The sample (10 mg) was mixed with 100 mg of dried KBr and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum further.

RESULTS

Estimation of GAGs

The amount of crude GAGs was estimated as 12 g/kg of tissue in *D. faba*. After purification using gel chromatography, the GAGs yield was found to be 0.83 mg/kg (Table 1).

Anticoagulant activity

By United States pharmacopoeia method, the anticoagulant activity of the *D. faba* crude and purified sample was reported to be 58 and 114 USP units/mg (Table 1 and

Table 1. The yield of glycosaminoglycans and their anticoagulant activity from *D. faba*.

Source	Net yield [GAGs (gm/kg)]		Anticoagulant activity [USP (units/mg)]	
	Crude	Purified	Crude	Purified
<i>D. faba</i>	12	0.83	58	114

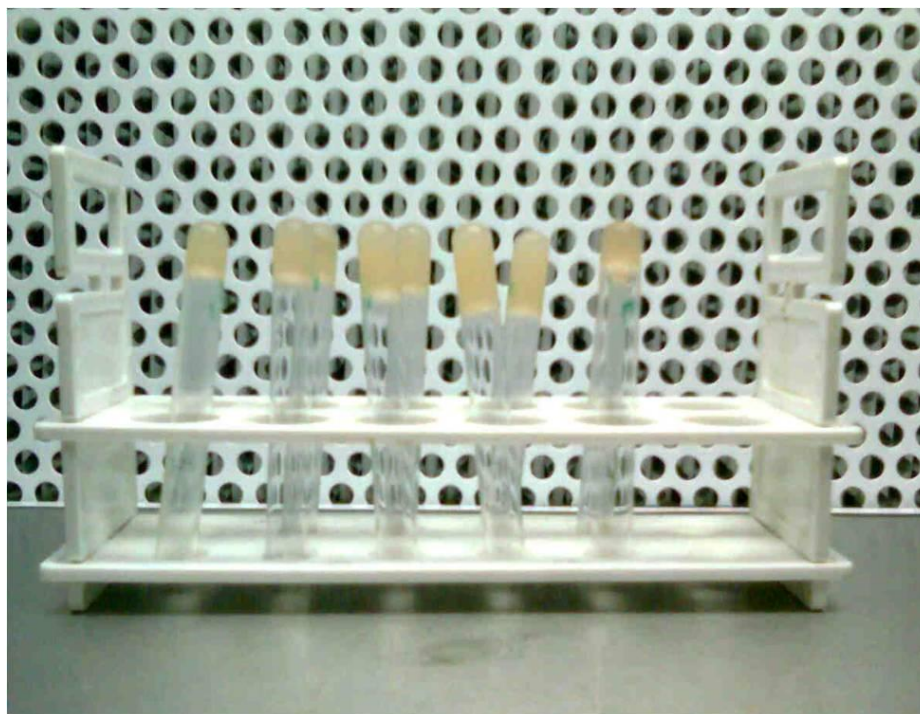
**Figure 1.** Anticoagulant photo.

Figure 1).

Fourier transform – infra red (FTIR) spectral analysis

FT-IR spectrum of GAGs of *D. faba* was compared with standard heparin sulphate (Figures 2, 3 and 4). The IR spectrum of standard heparin sulphate contained 17 peaks at 3973.36 to 545.85 cm^{-1} ; among them 4 major peaks were 3433.29, 1639.49, 1406.11 and 1041.56 cm^{-1} (Figure 2); whereas, the IR spectrum of crude GAG from *D. faba* presents 23 peaks, among them six are major peaks 3437.15, 1639.49, 1251.8, 1197.79, 1128.36 and 1078.21 cm^{-1} (Figure 3). The IR spectrum of purified GAG from *D. faba* presents 17 peaks, and the four major peaks were 3423.65, 1635.64, 1604.77 and 1404.18 cm^{-1} (Figure 4).

DISCUSSION

Heparin and heparin like compounds, which are present in some invertebrate molluscs, showed high anticoagu-

lant activity and share most of the structural properties with mammalian heparins. Similarly, heparin has been prepared from a number of different species including humans (Linhardt et al., 1992), clams (Shuhei et al., 2011), shrimp (Muzaffer Demir et al., 2001) and seaweeds (Mahanama De Zoysa et al., 2008). Heparin and heparin like compounds, which are present in some invertebrate molluscs, showed high anticoagulant activity and share most of the structural properties with mammalian heparins. Homogenization of a 1 kg portion of whole *D. faba* in acetone and subsequent extraction in acetone /petroleum ether resulted in 250 g of defatted tissue. The heparin obtained from defatted tissue described in the present study has all of the features as that of heparin. Similarly, heparin has been prepared from a number of different species including (Linhardt et al., 1992), clams (Pejler et al., 1987; Dietrich et al., 1989) and seaweeds (Wladimir et al., 2000). Extraction of the defatted soft body tissue of the giant African snail and subsequent purification of its GAGs showed that this tissue contained a large amount of GAG and free from impurities (Kim et al., 1996).

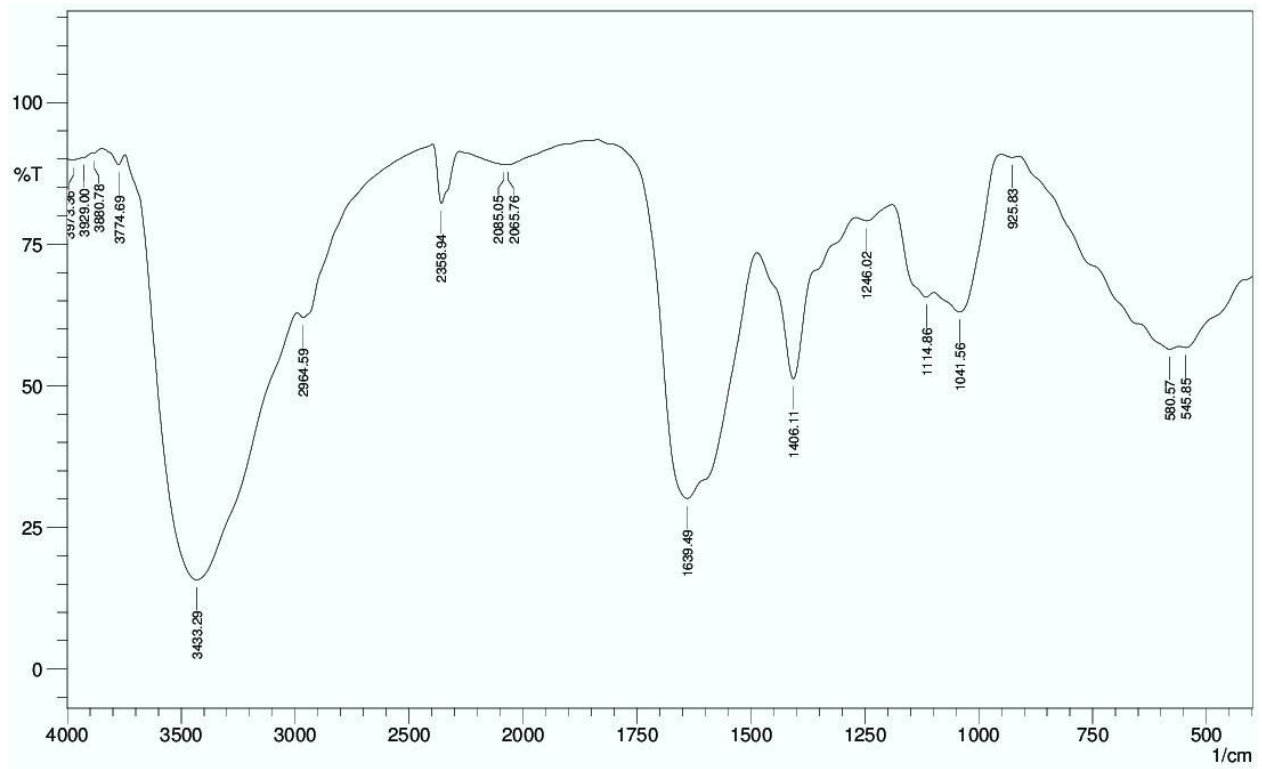


Figure 2. FTIR spectrum of standard.

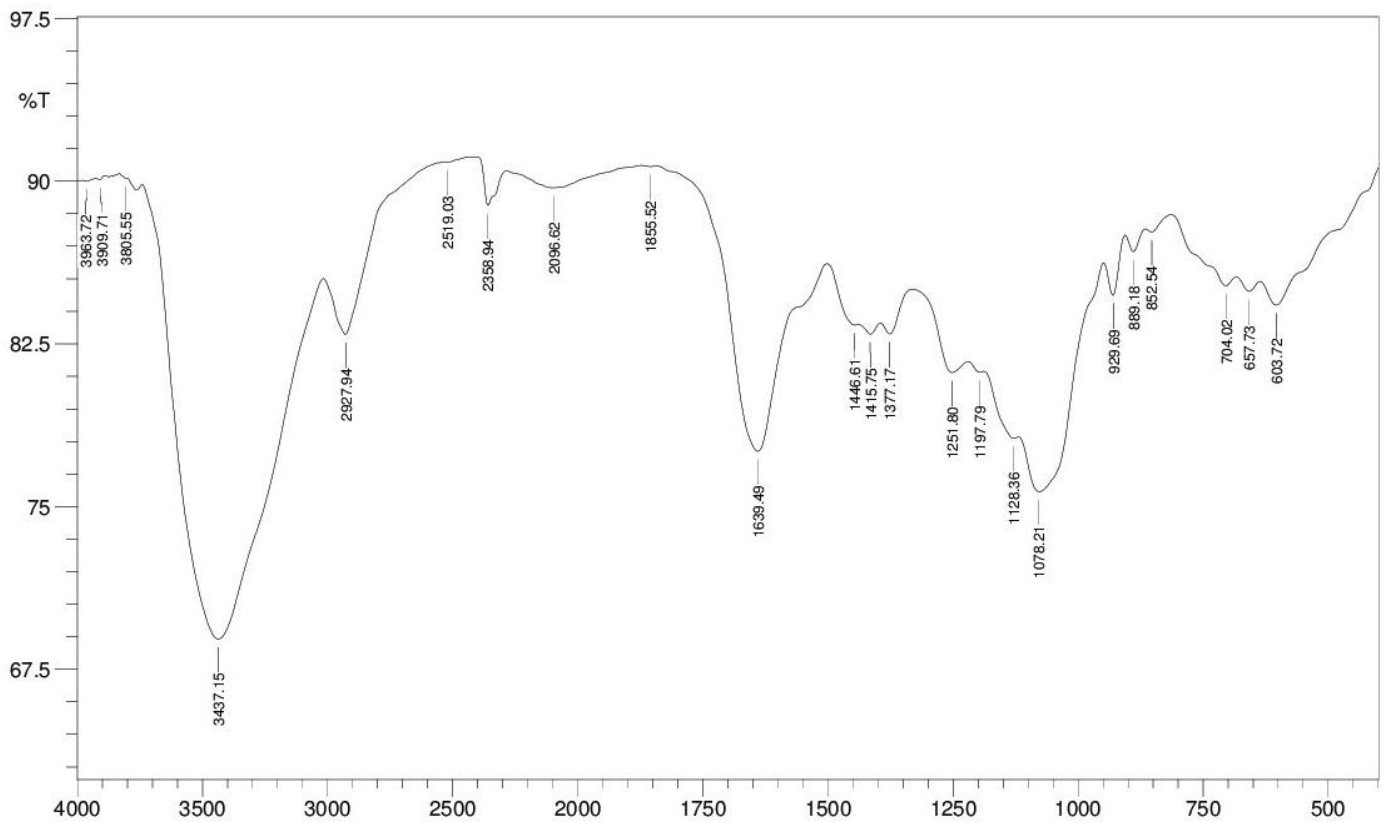


Figure 3. FTIR spectrum of crude GAGs.

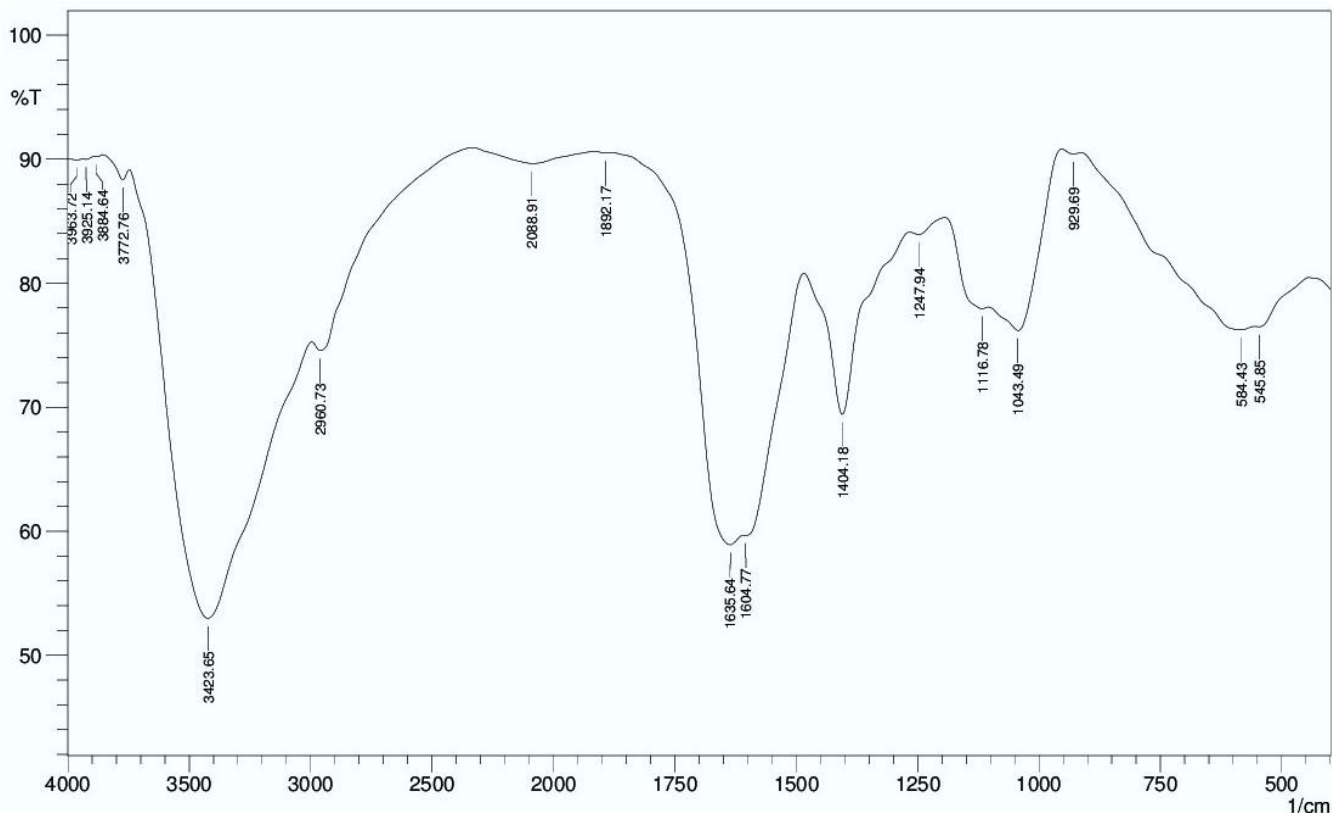


Figure 4. FTIR spectrum of purified GAGs.

The use of cetylpyridinium chloride (CPC) for quantitative separation of sulphated polysaccharides in tissue extracts is preferred. In the present investigation, the yield of crude and purified GAGs was found as 12 and 0.83 gm/kg in *D. faba*. Previously, Dietrich et al. (1989) isolated heparin with a yield of 2.8 and 3.8 g/kg from *Anomalocardia brasiliensis* and *Tivela mactroides*, respectively. Somasundaram et al. (1989) obtained 7.02 gm/kg of heparin like substances in marine molluscs *Katelysia opima*. The purification of heparin-dermatan sulfate and chondroitin sulphate from mixtures by sequential precipitation with various organic solvents was reported by Volpi (1996). However, the cephalopods such as *Sepia aculeate* and *S. brevimana*, and *Loligo duvauceli* and *Doryteuthis sibogae* showed higher net yield of the heparin like sulfated polysaccharides 21.7, 24.0, 16.5 and 8.4 gm/kg, respectively (Mahalakshmi, 2003; Barwin vino, 2003). Arumugam et al. (2008) had quantified the heparin yield as 2.27 and 2.2 g/kg from *Tridacna maxima* and *Perna viridis*, respectively. Vijayabaskar et al. (2008) reported that the isolated glycosaminoglycans (GAG) and purified from both bivalves were estimated as 5.4, 4.1 and 1.4, 1.1 gm/kg wet tissue in *K. opima* and *D. cuneatus*. Vidhyanandhini (2010) reported that the amount of crude GAGs was estimated as 9.85 gm/kg and after purification by using amberlite and barium acetate, the yield was found to be 33 and 148 mg/kg of *K. opima*. Saravanan

and Shanmugam (2010) reported the amount of crude and purified GAG was estimated as 17.2 g/kg and 48 mg/kg of tissue in *A. pleuronectus*. The result of the present study clearly shows that the yield of heparin and heparin-like glycosaminoglycans is higher and lower than those of previously reports. Hence, it could be concluded that the molluscs might be used as potent sources for the extraction of heparin and heparin-like glycosaminoglycans.

The heparin isolated from marine clams and mussels has identical structural features and anticoagulant activity of mammalian polysaccharide (Pejler et al., 1987). Heparin with high anticoagulant activity was isolated from the marine molluscs, *Anomalocardia brasiliensis*, *Donax striatus* and *Tivela mactroides* (Dietrich et al., 1985), which showed similar activity like mammalian heparin but differ in molecular weight; the molluscan heparin have a higher molecular weight and high anticoagulant activity (Dietrich et al., 1989). The biological activity of heparins in invertebrates remains enigmatic. The classes of Crustacea and Mollusca do not possess any blood coagulation system similar to that of mammals and other vertebrates and thus the presence of compounds that all specifically upon the proteins of the blood coagulation system is indeed remarkable (Dietrich et al., 1999). The anticoagulant activity of heparin differs from species to species due to their interaction with enzymes and inhibition of the coagulation

system (Mulloy et al., 2000). The anticoagulant activity of the crude and purified sample of GAG from the whole body tissue of *D. faba* was reported as 58 and 114 USP units/mg. This variation might be due to the presence of non-anticoagulant substance in the samples since the activity of heparin depends upon the amount of impurity carried over in the isolated products. Burson et al. (1956) showed the activity ranging from 130 to 150 USP units/mg for extracted products of *Spisula solidissima* and *Cyprina islandica*. Arumugam and Shanmugam (2004) reported the anticoagulant activity crude and fractionated sample of GAG from *T. attenuate* - 37 and 78 USP units/mg in *T. attenuate*. The anticoagulant activity of crude and purified sample of GAG from *A. pleuronectes* were 15.38 and 83.99 USP units/mg (Suganthi, 2007).

The crude and purified sample of *E. berryi* were 415 and 483.1 USP units/mg (Shanmugam et al., 2008). Vijayabaskar et al. (2008) reported that the extraction of GAGs from *K. opima* and *D. cuneatus* showed anticoagulant activity of 160 and 154 USP units/mg, and after the partial purification through DEAE cellulose column chromatography, the yields of anticoagulant activity were found to be 180 and 175 USP units/mg (Vidhyanandhini, 2010). The anticoagulant activity of crude and purified samples were estimated at 22.52, 20.00 and 18.60 USP units/mg for amberlite; 86.32, 83.06, and 92.43 USP units/mg for barium acetate and 80.36, 75.92, 89.68 USP units/mg for *K. opima*. From the aforementioned, it could be understood that whole body tissue of *D. faba* is also comparatively a good potential source of anticoagulant compounds. The molluscan GAGs were found to be structurally similar to the standard heparin as assessed by the FT-IR spectra. In the present study, the anticoagulant GAGs from whole body tissue of *D. faba* crude and purified sample showed major peaks at 3437.15, 1639.49, 1251.8, 1197.79, 1128.36 and 1078.21 cm^{-1} which is said to be for the GAGs groups (Saravanan and Shanmugam, 2010).

For FT-IR spectrum of purified sample, the sulfate band stated from 1139.92 cm^{-1} and extended down to 995.12 cm^{-1} . The acetyl amino group was represented by a band at 1474.78 cm^{-1} and the carboxylic group at 1552.66 cm^{-1} . This was also well supported by the study of Rivera et al. (2002) who also claimed that the characterization of traces of contaminants in crude heparin by conventional physico-chemical techniques such as size-exclusion or ion-exchange chromatography is relatively difficult. The peak pattern between the standard heparin and the purified sample were at 3433.29, 1639.49 cm^{-1} and 3437.15, 1639.49 cm^{-1} as indicating the presence of GAGs group in the samples analyzed. The FT-IR spectral analysis of the anticoagulant GAGs from *D. faba* showed more or less same number of peaks, lying within the same range of values of the commercial heparin used as a standard. The results in this finding show that bivalve, *D. faba* tissue had GAGs with high quantity of anticoagulant compounds. Thus, the result of the present investigation provides information about the isolation, purification and

characterization of the heparin and heparin like glycosaminoglycans (heparin and heparin sulfate) compound from their chemical characteristic features. Further, it will pave the way for future researchers to take up research in this line to characterize fully the heparin and heparin like GAGs of molluscan source using chromatography different fraction, NMR, MS and other advanced techniques.

Besides the aforementioned, it could also have a very good anticoagulant activity as the anticoagulant compound from this species proves the possibility of its utilization as an additional potent source for the extraction of such anticoagulant compound since the anticoagulant activity of the presents isolated heparin and heparin like GAGs from *D. faba* was even more than that of many heparins obtained from commercial sources.

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