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Full Length Research Paper

Cloning of *nis* gene and Nisin purification from *Lactococcus lactis* subsp. *lactis* Fc2

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The present study was focused on nisin produced by *Lactococcus lactis* subsp. *lactis* (Fc2 isolate). The isolate was identified by 16S rRNA using specific universal primer. The obtained cell-free supernatant inhibits the growth of *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* by using agar diffusion assay. Nisin structural gene was detected by polymerase chain reaction with nisin gene-specific primer followed by cloning in PGEM-T easy vector, transformation in *E. coli* JM109 and direct sequencing of the construct. The Nis gene which encoded nisin Z showed identical sequences to Nis Z in Genbank with accession number AB727286, as indicated by the substitution of asparagine residue instead of histidine at position 27. In this way, it was established that the nis Z gene for nisin Z production is widely distributed. The purified nisin by chloroform extraction was analyzed on 20% SDS-PAGE and gave sharp band at ~ 3.4 kDa. The 3 dimension structure of the purified Nisin was studied by CPHModels as pdb with chimera program.

Key words: *Lactococcus lactis*, *nis* cloning, 16S rRNA, chloroform extraction and SDS-PAGE.

INTRODUCTION

Nisin, antimicrobial peptide (3.4 kDa), is produced by *Lactococcus lactis* ATCC 11454 during its exponential growth phase (Vessoni-Penna and Moraes, 2002). Nisin is a bacteriocin commercially used as natural agent for food biopreservation. It has recently been considered safe by the World Health Organization (WHO) and by the Food and Drug Administration (FDA), with the denomination of generally recognized as safe (GRAS) (de Vuyst and Vandamme, 1994; Arauz et al., 2009). This bacteriocin has large antimicrobial activity spectrum against Gram-positive bacteria and their spores, but shows little or no

activity against Gram negative bacteria, yeasts or moulds. However, Gram negative bacteria can be sensitized to nisin by exposing to chelating agents (EDTA), sublethal heat and freezing (Vessoni Penna et al., 2006). As a result of its antimicrobial properties, nisin has been accepted as a safe and natural preservative in different areas of food industry and it has also been used as treatment for some health conditions such as stomach and colon ulcers, cosmetic and veterinary products (Delves-Broughton et al., 1996; Liu et al., 2004; Von Satazewski and Jagus, 2008). The bacteriocin nisin,

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which is produced by *L. lactis* subsp. *lactis*, has been extensively studied and used as a food preservative because of its lethal action and wide spectrum of activity (Hurst, 1981; Delves-Broughton et al., 1996). Two nisin variants, which differ only by one amino acid substitution at position 27, have been studied extensively. Nisin A contains histidine and nisin Z asparagine (Mulders et al., 1991). Usually, nisin producer strains are isolated from dairy products (Rauch et al., 1994).

Brotz and Sahl (2000) showed that nisin is the most popular of the lantibiotics. It was first discovered in the early 20th century and has been used for several decades as a natural, safe food preservative, especially in the dairy industry. Nisin's use as a food preservative has been well-studied and well-documented. It is sometimes used as a comparison when studying the effects of other natural antibacterials, such as certain essential oils. Though it is considered a type A lantibiotic, nisin's antibiotic action appears to occur through two separate mechanisms. Study aim to obtain variant strain to enhanced antimicrobial activity of nisin depending on the target microorganism. This enhancement is apparent against Gram positive and Gram negative targets is particularly novel. Further efforts will focus on determining the mechanistic basis for these enhancements.

MATERIALS AND METHODS

Growth media and organism preparation

The isolate *L. lactis* ssp. *lactis* Fc2, was identified by chemical method previously mentioned. MRS had optimum conditions: pH (7), temperature (37°C), Agitation (120 rpm), yeast extract (0.2 g, %), Tween 80 (0.04%) as described previous (Abdel Kareem et al., 2005).

Cultivation of pathogenic strains

Listeria monocytogenes, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* were used as indicator strains. They were cultured on plates containing Luria-Bertani (L.B) agar media. The plates were incubated at 37°C for 24 h.

Detection of nisin

LB broth culture of *L. lactis* ssp. *lactis* Fc2 strain was prepared and pH adjusted to approximately 2 to 3 with HCl. This acidified culture was placed into boiling water bath for 5 min to remove cell bound nisin by hot acid extraction. Broth media were centrifuged at 12,000 rpm for 12 min at 4°C. To exclude the inhibiting activity due to acids, the pH of the cell free supernatant (CFS) was adjusted to 6.5 with 1 M NaOH and filter sterilized through (0.45 µm) cellulose membrane.

Quantitative estimation of nisin prepared from the CFS

This was done after cooling the assay medium to about 45°C (Wolf and Gibbons, 1995). Nisin activity was carried out by critical dilution

assay according to Pucci et al. (1988). Serial two fold dilutions were made from CFS of the *L. lactis* ssp. *lactis* Fc2 culture. Forty microliter (40 µl) was taken from each dilution and carried out on the surface of filter paper disks on LB agar plates; also, the LB was seeded with 1% (v/v) suspension of log phase cells of every indicator strains culture.

Antibacterial assay comparison of test organisms with nisin

The *Lactococcus* plates were incubated in aerobic condition for 24 h for each of the 6 organisms (*L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. cereus* and *E. coli*). A set of standard bio-assay plates (1.5% agar, 1% Tween 20, 7 mm diameter filter paper disks) Lauryl Tryptose Broth (LTB) as assay medium was prepared (stock solution of nisin standard and purified 30 µl was inoculated on sterilized filter paper discs compared with different concentrations of antibiotics discs replicated three times on each set of *Lactococcus* were streaked in the centre of the plates). Plates were incubated for 24 to 48 h at 37°C under anaerobic aerobic conditions, respectively. The zones of inhibition were measured.

Identification of *lactococcus* by 16S rRNA

Full length 16S rRNA (1500 bp) were amplified from isolates (Fc2) by PCR using (universal forward primer P1 and universal reverse primer): Forward: (5'-AGAGTTTGATCCTGGCTCAG-3'), Reverse: (5'-CGGTTACCTTGTTACGACTT-3') under the optimum conditions (denaturation 94°C for 1 min, annealing at 45°C for 30 s and extension at 72°C for 2 min, 35 cycles). Amplified 16S rRNA was purified from 0.8% melting point agarose gel. Bands obtained from polymerase chain reaction (PCR) product were eluted and purified by Qiagen elution kit. PCR instructions, and DNA band desired was excised from ethidium bromide stained agarose gel with a razor blade and transferred to Ependorf tube. DNA was sequenced directly using specific primer with concentration of 20 pmol in Promega company lab. The sequence alignment was prepared with DNA STAR software program. Nucleotides sequences of the products were edited using Bioedit version 5.0.6 (Hall, 1999) and phylogenetic tree by mega 4 program.

Gene isolation

Genomic DNA was extracted by a modification of the method of Engelke et al. (1992). The polymerase chain reaction analysis followed procedure described by Horn et al. (1991) with some modifications. The PCR amplification was carried out in a 50 µl mixture in a DNA thermo cycler. The conditions consisted of 35 cycles of 94°C for 1 min, 55°C for 1 min and 72 h for 2 min. The primers were designed from nis A structural gene, which were complementary to regions 17 bp upstream and 2 bp downstream of the coding region (Dodd et al., 1990; Mulders et al., 1991). The restriction sites EcoRI and KpnI were added at 5 end, respectively, for cloning purpose in other studies elsewhere. Forward and reverse primers were as follows: primer F(5'-CCG GAATTC ATA AGG AGG CAC TCA AAA TG-3') primer R(5'-CGG GGT ACC TAC TAT CCT TTG ATT TGG TT-3'). The amplified PCR products were purified from low melting point agarose and the nucleotide sequences in promega lab, Biotechnology Company. *nisZ* expressed nisin Z (PCR product) was purified and ligated to PGEM®-T easy vector; ligated plasmids were transformed into *E. coli* JM109 and transformants were selected using the blue/white screening procedure. After growing on IPTG/X gal agar plates supplemented with 100 µg/ml ampicillin, screening of recombinants was performed according to Sambrook et al. (1989). Purified plasmids were sequenced using a sequencer in promega lab. The sequence

Table 1. Inhibition zones and MICs (Minimum inhibitor concentrations) obtained for purified and Stander nisin Z, using different nisin-sensitive indicator strains.

Indicator strains	Radius of inhibition zone using purified nisin (mm)	MIC Concentration (ug/ml)	Radius of inhibition zone using stander nisin (mm)	MIC concentration (ug/ml)
<i>Listeria monocytogenes</i>	0.9	0.019	1.4	0.017
<i>Pseudomonas aeruginosa</i>	2.1	0.2	2.7	0.018
<i>Staphylococcus aureus</i>	0.5	0.24	0.7	0.2
<i>Bacillus subtilis</i>	2.9	0.014	3.2	0.012
<i>Bacillus cereus</i>	3.4	0.016	3.7	0.015
<i>Escherichia Coli</i>	1.3	0.015	1.6	0.01

alignment was prepared with DNA STAR software program (DNASTAR, INC., Madison, Wis.) and manually edited with GeneDoc (www.NCBI/blast.com), and determined translation encoded regions at www.expasy.org/cgi-bin/dna_aa. Construct contains nisin Z gene fragment diagramed by NTI vector program. Nucleotides sequence of the products was edited using BioEdit version 5.0.6 (Hall, 1999).

Purification of nisin

Chloroform extract (2 L) of a suitable broth medium was inoculated with 1% of an overnight culture of the nisin producing bacterium *Lactococcus* sp. and incubated for approximately 18 h at 35°C. Cells were pelleted at 7100 g for 15 min in a refrigerated (12°C) centrifuge; the nisin containing supernatant fluid was collected and purification of nisin was carried out according to Burianek and Yousef (2000).

Gel electrophoresis and detection of nisin molecular weight

One milliliter from overnight culture incubated in shaking incubator at 37°C and 120 rpm of *L. lactis* ssp. *lactis* Fc2 was collected by centrifugation at 10,000 rpm for 15 min. Bacterial pellets were resuspended well in 60 µl distilled water then 20 µl sample buffer and 10 µl protein tracking dye were added. Polyacrylamide gels were prepared with unheated samples as described before (Mulders et al., 1991). Centrifugation for 15 min at 10,000 rpm was done and placed on ice until loading. The components of the separating gel were placed in 50 ml beaker and mixed gently to avoid air bubbles. The solution was pipetted into the assembled vertical slab gel unit in the casting mode leaving 2 ml from the front. A layer of distilled water was pipetted on the top of solution. The gel was allowed to polymerize at room temperature and the water was poured from the upper surface. The stacking gel solution was prepared and added onto the top of separating gel where the comb was inserted. The gel polymerization was allowed. The assembled vertical slab containing the polymerized gels were put into the electrophoresis chamber filled with the tank buffer. The protein samples were loaded into the wells after removing the comb by using Hamilton syringe in loading; a protein marker was also loaded (200 volt and tank buffer 1X). For electrophoresis, the gel was stained in 50 ml of staining solution for 45 min with shaking at room temperature and then destained in destaining solution and finally, gel image was taken.

RESULTS AND DISCUSSION

The antimicrobial substance produced by *L. lactis* subsp.

lactis WNC 20 strain had similar enzymatic, pH sensitivity and heat insensitivity pattern to nisin. The identical antimicrobial spectrum and larger inhibition zones in agar diffusion assay of bacteriocin obtained from WNC 20 as compared with those from DL 11, a nisin A producing strain, suggested that the bacteriocin produced by WNC 20 was nisin Z. De Vos et al. (1993) mentioned that the undiluted culture supernatant of NIZO 22186 resulted in much larger inhibition zones than that of NIZO R5 in a standard agar diffusion bioassay. Also, Tramer and Fowler (1964) found the same result with *M. flavus* as an indicator microorganism. Therefore, the antimicrobial activities of the purified and standardized nisin variants were compared. To study possible differences in specific antimicrobial activity, the MICs of lantibiotic nisin were determined in liquid cultures of *M. flavus*. The MICs appeared to be identical (0.015 µg/ml). In this study *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. cereus* and *E. coli* were used as indicator microorganisms against nisin activity of *L. lactis* by using standard agar diffusion bioassay. The minimum inhibitor concentration (MIC) for inhibition zone was 50 µg/ml of nisin solution. Data shown in Table 1 indicate that the sizes of the inhibition zones varied considerably between the different indicator bacteria and were inversely related to the MICs. The most sensitive Nisin was *B. cereus* (inhibition zone was 3.4 and 3.7 mm) with purified and standard nisin, respectively.

The resistance microorganism was *S. aureus* (inhibition zone was 0.5 and 0.7 mm with purified and standard nisin, respectively). De Vos et al. (1993) and Dosler and Gerceker (2012) reported that the physicochemical properties of nisin Z are contributing to the formation of the large inhibition zones. On the basis of the nature of the His27Asn substitution in nisin Z, it is expected that nisin Z solubility is higher than nisin A solubility at pH values above 6 (the pK value of His) since the Asn side chain is more polar than the uncharged His side chain.

Identification Fc2 by 16S rRNA

Molecular techniques, such as PCR have been used

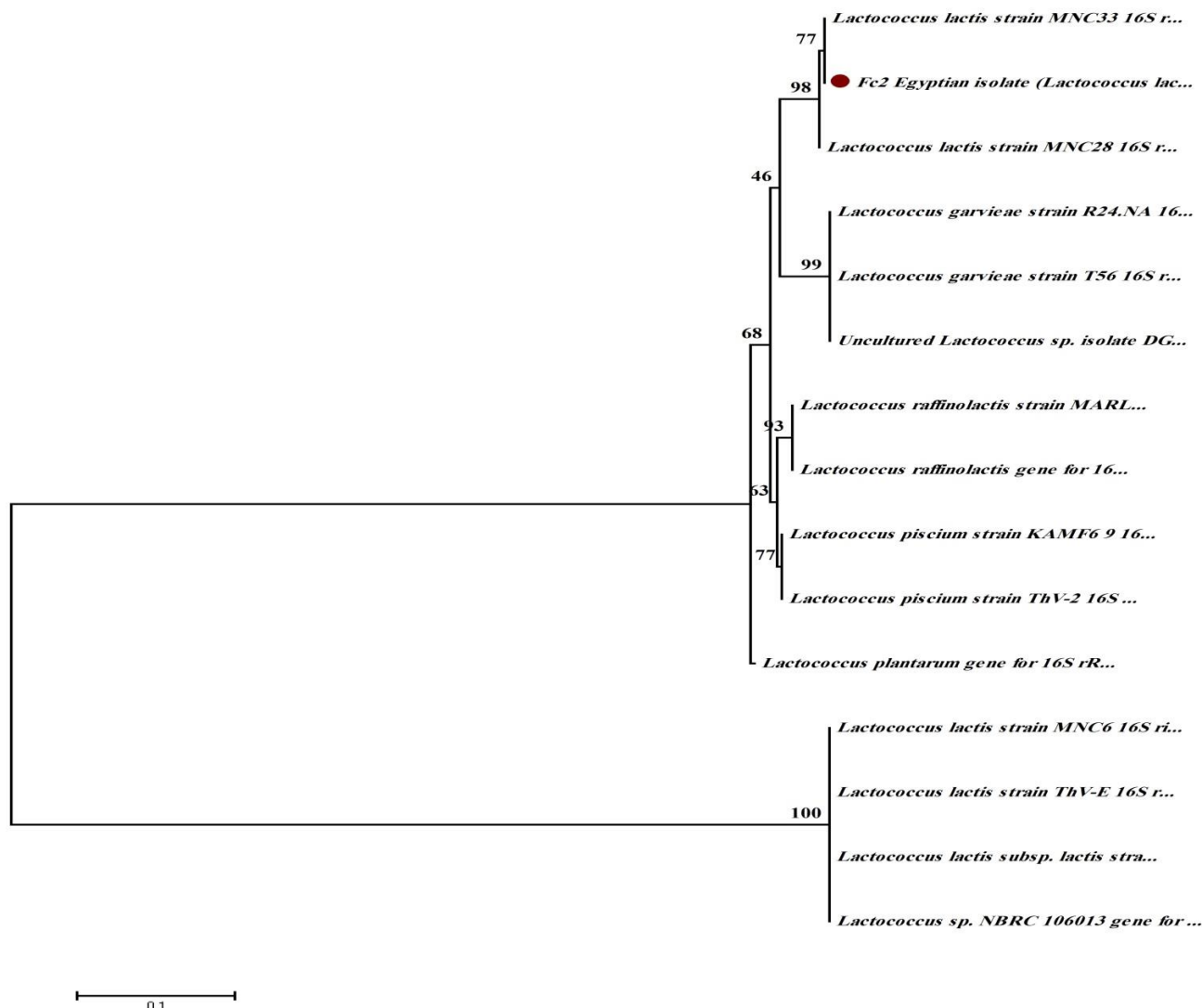


Figure 1. Phylogenetic tree average distance tree using neighbor-joining (NJ) including those of *Lactococcus lactis* spp.

extensively for several years for identification and characterization of food borne pathogens in food samples (Hill, 1996; Wang et al., 1997). Here, we tried to use PCR method based on 16S rRNA gene accurate method for identification and confirmation of Fc2 strain. 16S rRNA gene bands which were detected by specific primer at 1500 bp. The ribosome consists of two unequal subunits, which associate via numerous intersubunit contacts. Medium-resolution structural studies have led to grouping of the intersubunit contacts into 12 directly visualizable intersubunit bridges. Most of the intersubunit interactions involve RNA. The relevant sequences were downloaded and phylogenetic analysis has been carried out as shown in Figure 1. Small rRNA gene sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. First, the construction of a universal phylogenetic tree classifies organisms into

three domains of life: bacteria, Archaea, and Eucarya (Olsen et al., 1992; Olsen and Woese, 1993; Thompson et al., 1994; Arto pulk et al., 2010). Second, it revolutionized the classification of microorganisms, and makes the classification of non-cultivable microorganisms possible (Relman et al., 1990; Relman et al., 1992). Third, it helps to elucidate the relation of unknown bacterial species to known ones.

Gene isolation

Total DNA was isolated from these strains, and the structural nisin gene was amplified by PCR, resulting in a 350 bp DNA fragment. The presence of nis A or nis Z genes was determined by direct sequencing of the PCR product. Sequence ladder was obtained, indicating that a

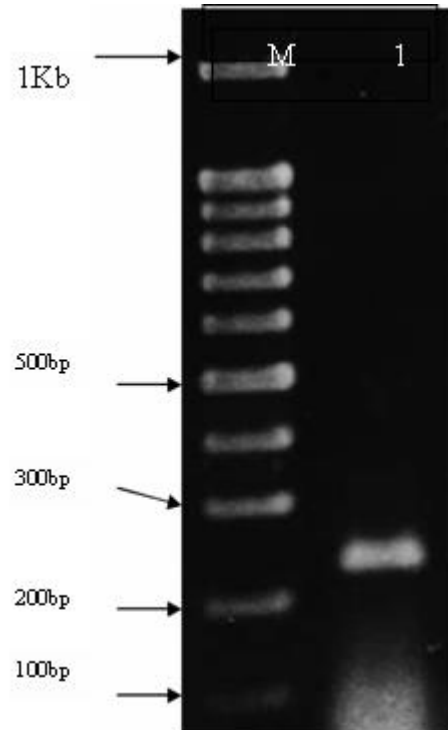


Figure 2. Agarose gel electrophoresis of PCR products with nisin gene-specific primer. Lane 1, shows nisin gene from *Lactobacillus plantarum*; lane M, was loaded with 100 bp ladder DNA markers.

```

atgagtacaaaagat t t t t a a c t t g c a g t t g g t a t c t g t t t c g a a g a a a g a t t c a g g t g c a
M S T K D F N L Q L V S V S K K D S G A
tcaccacccattacaagtatttcgctatgtacaccggttgtaaaacaggagctttgatg
S P P I T S I S L C T P G C K T G A L M
ggagctaacatgaaaacagcaacttgctattgtagatttcacgtaagcaataataagca
G A N M K T A T C H C S I H V S K - - A
    
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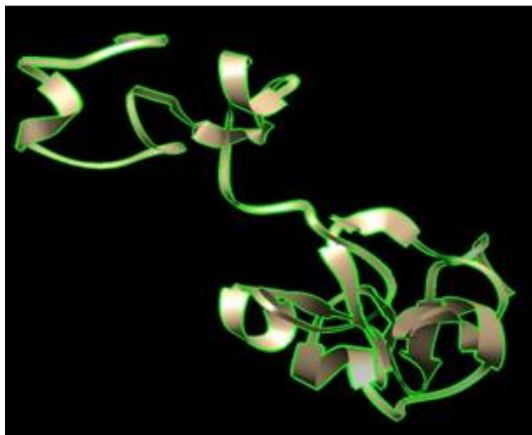
Figure 3. Gene sequence.

single nis gene species was present in the strains. In strain NCDO 2118, a small insertion of 16 bp was detected between positions 180 and 181 (Mulders et al., 1991), which was located just downstream of the nis Z structural gene. This insertion consists of a tandem duplication of the octanucleotide AACCAAAT present at positions 173 to 180. Sequences were as reported for the nis A or nis Z genes, differing only in the nucleotide at position 148 (Buchman et al., 1988 and Mulders et al., 1991). Data appear in Figure 2 illustrated nis gene expressed nisin Z product detect at ~ 227 bp. PCR product was eluted and cloning in PGEM-T easy vector (Figure 5) follow sequenced using alignment via genebank sequence identical nis Z gene expressed nisin Z with similarity 96% with new nucleotide sequence 4%.

Figure 3 showed the gene sequence with 180 bp and 60 amino acids.

Results in this study are in agreement with Noonpakdee et al. (2003) who found that the bacteriocin produced by *L. Lactis* WNC 20 was nisin, PCR analysis using the published sequences of the nisin structural gene (Dodd et al., 1990) was performed. Two primers complementary to sequences occurring proximal to the 3 and 5 ends of the nisin A structural gene were used to amplify nisin gene from the genomic DNA of *L. lactis* DL 11 and *L. lactis* WNC 20. A 227 bp fragment was amplified from the genomic DNA of *L. lactis* WNC 20, which was identical to that amplified from a nisin-producing strain of *L. lactis* DL 11. Results indicated that sequences were 100% identical to that of nisin A except

A



B

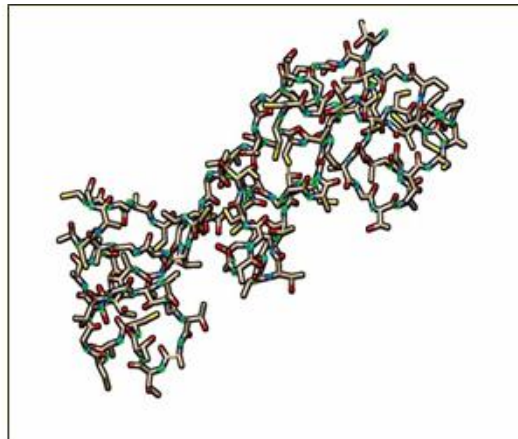


Figure 4 (a). Nucleotide sequence and deduced amino acid sequence of the *nisZ* gene isolated from *Lactococcus . lactis subsp. lactis* (Fc2). The amino acid sequence is shown below the coding sequence. **(b).** 3 D structure of purified nisin Z from *lactococcus*.

for a C-to-A transversion at position 148. This resulted in an asparagine (AAT) residue at position 27 of the nisin peptide, instead of histidine (CAT). This indicates that the bacteriocin produced by *L. lactis* is nisin Z. The gene sequence (180 bp) had a similarity of 96% with *L. lactis* nisin (*nis Z*) gene. Hence, the purified Nisin from Egyptian strain which was identified by 16S rRNA in this study. The 3 Dimension structure of it obtained by CPHModels as pdb with Chimera program 1.7rc is as indicated in Figure 4b.

Nisin Z purification

Purification of nisin by chloroform was an easy method that gave high yield of nisin. Data shown in Table 2 indicated that nisin activity and specific activity were increased after chloroform extraction. Nisin yield was 0.5 g/l in MRS medium. Purification fold was also higher after using chloroform extraction than the culture supernatant. Solubility of chloroform in water is only 0.815%, therefore, chloroform is a solvent with intermediate polarity and is immiscible with water. These properties make chloroform most suitable for bacteriocin concentration from culture media. Nisin is amphiphilic peptide with high affinity towards lipid membrane (Breukink and Kruijff, 1999; Burianek and Yousef, 2000). Similar results were obtained by (Yang et al., 1992; Meghrouf et al., 1997).

Gel electrophoresis and detection of nisin molecular weight

Nisin had a molecular weight of 3.4 kDa. Kuipers et al. (1992) reported that nisin separation SDS-PAGE use

certain amount of purified nisin and use coomassie blue have molecular weight of 3 kDa. Monomer could arise as reaction products from hydroxyl addition to dehydro groups or from intramolecular additions involving nucleophilic groups within the peptide. Electrophoresis of purified nisin after storage for two weeks gave the same result but nisin appeared in dimmer form means that it has molecular weight of 7 kDa. Nisin on SDS-PAGE with coomassie blue stain give one band, after storage for two weeks or more, produced several bands, some of which were diffused, nisin as monomer very closely to 3.5 or 7 kDa as other references on the other hand Hansen et al. (1990) and Yildirim and Johnson (1997) nisin have 2.5 kDa. Figure 6 showed that the chloroform extraction gave a purified nisin from *L. lactis* (Fc2) had ~ 3.4 kDa.

Conclusion

The purified Nisin by cheap method (chloroform extraction) from Egyptian strain was identified by 16S rRNA (producer nisin) with high activity compared with other data published previously 14000 u/ml, so we can consider this strain as variant to enhanced antimicrobial activity depending on the target microorganism. This enhancement is apparent against Gram positive and Gram negative targets is particularly novel. Further efforts will focus on determining the mechanistic basis for these enhancements. Also, nisin was studied in 3 Dimension structure of it by CPHmodels as PDB with Chimera program 1.7rc, *nis* gene which encoded nisin Z in; Egyptian strain was isolated, sequenced and submitted in GenBank with accession number AB727286, indicated by the substitution of asparagine residue instead of histidine at position 27. In this way, it was established that the *nis Z* gene for nisin Z production is widely distributed.

nisZ gene expressed Nisin Z product

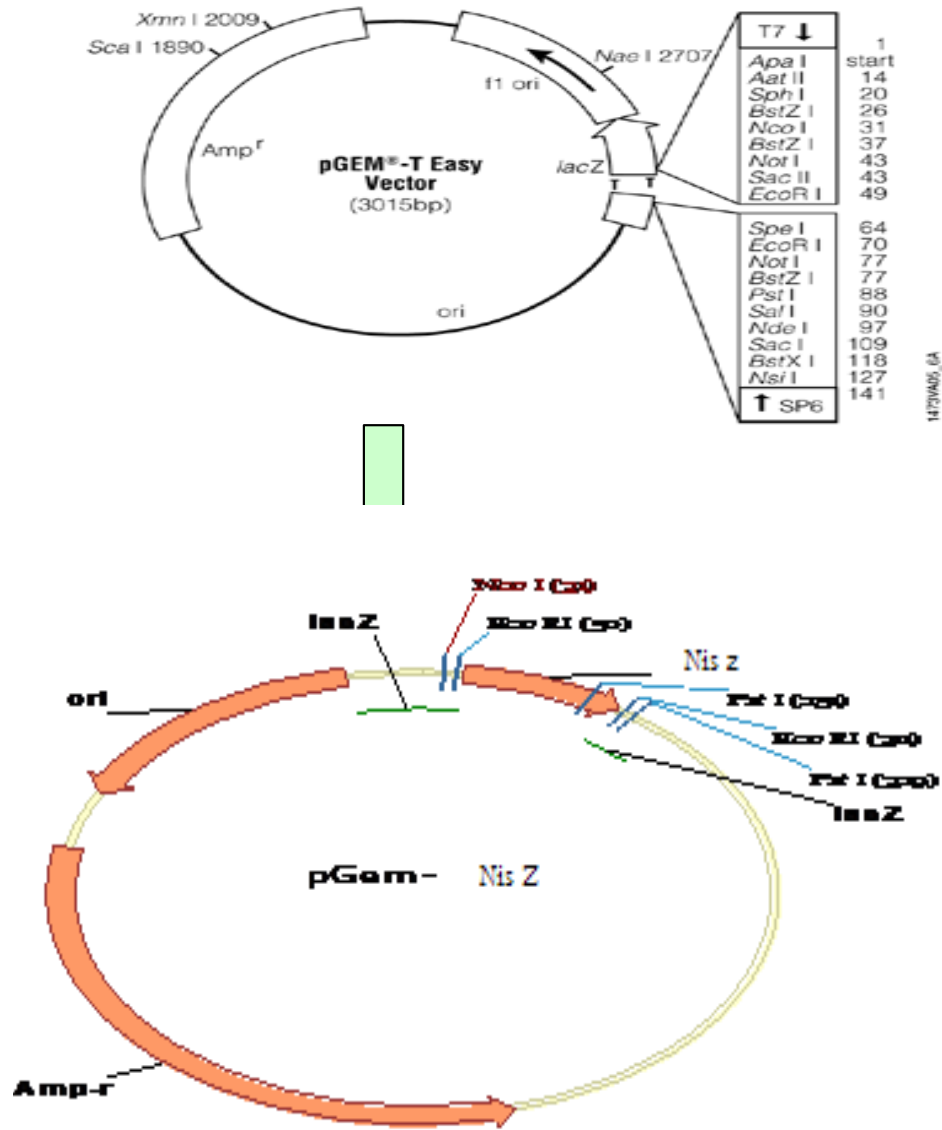


Figure 5. Diagram indicating the ligation process for nisZ gene fragment into PGEM –T-easy vector (construct). Nisin Z purification.

Table 2. Nisin Z purification at different stages of purification.

Purification steps	Nisin activity	Specific activity	Purification	Purified nisin
	(Au/ml)	(Au/mg)	Fold (%)	(g/l)
Culture supernatant	10000	20	1.00	--
Chloroform extraction	14000	28	1.4	0.5

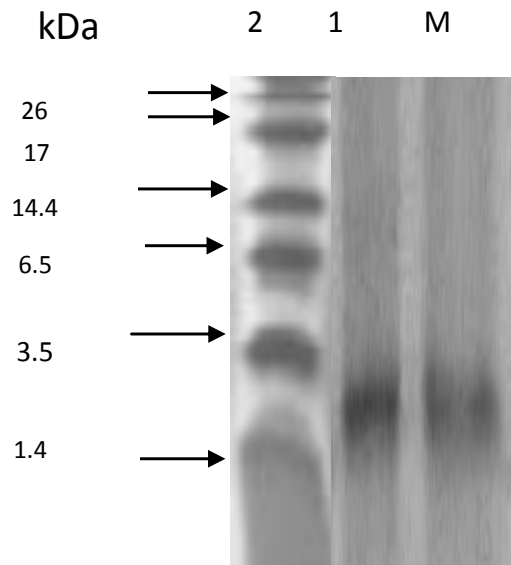


Figure 6. SDSPAGE purified nisin from *L. Lactis* sp lactis. Lane M, Polypeptide SDSPAGE Standards (1.4 to 26.6 kDa); lane 1 Standard nisin; lane 2, purified nisin Z by chloroform extraction.

Conflict of Interest

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

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Full Length Research Paper

Promoting increased *Chlorella sorokiniana* Shih. et Krauss (Chlorophyta) biomass production using *Moringa oleifera* Lam. leaf extracts

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Chlorella sorokiniana Shih. et Krauss, a unicellular green alga was assayed to assess its to promotion potentials response of aqueous and ethanolic leaf extracts of *Moringa oleifera* Lam. *C. sorokiniana* grown in 200 ml aliquots of modified basal medium for two weeks: was treated with the aqueous and ethanolic extracts at 10, 20, 30, 40 and 50% concentrations, respectively. A control was set up without *Moringa* extract and each treatment replicated thrice. The increases in biomass (individuals/mL) were monitored at two - day intervals. Both aqueous and ethanolic leaf extracts had appreciable promoter effects (61.49 and 95.55%, increases respectively) over the control. The ethanolic exhibited more growth activity (21.09%) than the aqueous extract. The promoter effects of both extracts increased proportionally with the increase in levels of concentration with time. There were significant differences in the interactive effects of the extracts, concentration and day (duration) ($p < 0.05$). The findings of this study indicate the potential use of the aqueous and ethanolic leaf extracts of *M. oleifera* as possible enrichments for *C. sorokiniana* growth medium.

Key words: *Moringa*, leaf extracts, *Chlorella*, medium enrichment, food supplement.

INTRODUCTION

In recent decades, the world energy crisis has triggered off the race for development of efficient as well as cheap sources of biofuels. The demand for algae as natural sources of food supplements, biofuels and efficient carbon sequestrates has necessitated the need for their efficient mass production at minimal cost. The concept of algae culture or algae farming has been derived as a result, and many forms of algae fuels such as cooking oil, biodiesel, bioethanol and biogasoline are in the process of development. It has been reported that oil productivity of

Chlorella exceeds the yield of the best oil seed crops and its biodiesel yield is 12 000 L/ha compared with 1190 L/ha for rapeseed (Schenk et al., 2008; Sharif - Hossain et al., 2008).

C. sorokiniana Shih. et Krauss is a unique single-celled fresh water micro-alga with grass-like odor caused by high chlorophyll content. In perfect growing conditions *Chlorella* converts inorganic chemical elements to organic matter through photosynthesis (Vashishta et al., 2000). *Chlorella* is rich in fats (85% unsaturated fats), complex

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carbohydrates, amino acids, vitamins and minerals. Moreover, it contains RNA (up to 10%) and DNA (up to 3%); chlorophyll, and carotenoids; an array of phytonutrients and enzymes (including pepsin for digestion); polysaccharides as well as the unique *Chlorella* Growth Factor (CGF). Also, it has one of the highest amounts of beta-carotene among all green products (Chinnasamy et al., 2009), hence its use as a health food supplement. Furthermore, it helps in aeration of water by removing carbon dioxide and restoring oxygen in the process of photosynthesis. The growth of this alga is, therefore, encouraged in sewage disposal plants where it outgrows and suppresses harmful bacteria by its rapid rate of multiplication (Akpor and Muchie, 2010). Mohan et al. (2009) reported that *Chlorella* is used to keep the air in space vehicles pure, supply food in space stations and prolonged space flight trips. The stale air rich in carbon dioxide can be fed into a floodlit container, containing water, mineral nutrients and *Chlorella*; and the alga restores oxygen by photosynthesis.

Moringa oleifera Lam., "Miracle tree", has recently attracted great attention in every field of scientific research. *M. oleifera* (Moringaceae) is cultivated across the tropics and used for a variety of purposes (Jahn, 1984). The seed powder, a good water purifier contains polyelectrolytes, which constitute active ingredients in water treatment (Muyibi and Evison, 1995). Also, the extract obtained from the leaves of *Moringa* in 80% ethanol contains growth enhancing principles for higher plants (Makkar and Becker, 1996). In view of the fact that *Chlorella* biomass is much sought after for use as health food supplement and for biofuels among other uses, enhanced production using non-toxic media enhancers is desirable. Hence, *Moringa* leaves which are cheap, readily available and affordable are very handy.

The objectives of this study were to investigate the promoting effect of the leaf extracts of this multi-purpose plant (*M. oleifera*) on *C. sorokiniana* and its potential use as nutrient enrichment for culture media in commercial production of *Chlorella*.

MATERIALS AND METHODS

Collection and identification of plant materials

Axenic culture of *C. sorokiniana* was collected from the Department of Microbiology, University of Nigeria, Nsukka. Young leaves of *M. oleifera* were collected from Ajuona Obukpa, in Nsukka Local Government Area of Enugu State, Nigeria. Both plant materials were identified in the Department of Plant Science and Biotechnology of the, University of Nigeria, Nsukka using morphological characteristics and taxonomic keys (Shishira and Krauss, 1963; Hutchinson and Dalziel, 1963; Roloff et al., 2009).

Preparation of plant extract (*Moringa oleifera*)

Drying process

Harvested young fresh leaves of *M. oleifera* were washed and dried

for seven days under the shed, at room temperature, to avoid loss of the active compounds. The dried leaves were milled at the National Centre for Energy Research Development, University of Nigeria with a local hand milling machine and weighed on a Mettler balance. The powdered sample was stored in an air-tight glass bottle for further use.

Extraction processes

Fifty grams of the powdered leaf sample were used for the production of aqueous and ethanolic extracts following the procedure of Handa (2008). Ten grams of each of the dried extracts were dissolved in one litre of distilled water for use in the experiment (10 mg of aqueous extract in 1 ml solution).

Sub-culturing of *C. sorokiniana* cells

The axenic culture from the Department of Microbiology was sub-cultured in a modified Basal Medium, following the procedures of Ogbonna et al. (1997). Two litres of *Chlorella* medium were produced by sub-culturing at the ratio of 1:5 inoculum to the growth medium in glass jars plugged with non-absorbent cotton wool. The jars were left on a sterilized work bench in a screen house in the Botanic Garden of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, for seven days. Each of the bottles containing the organisms was agitated at least three times every day to avoid clumping of the cells (Ukeles, 1973; Ogbonna et al., 1997).

Experimental design

Completely Randomized Design (CRD) of two different plant extracts (aqueous and ethanolic extracts), a microalga (*C. sorokiniana*) and six concentrations including a control was used. The concentrations were replicated three times.

Application of *M. oleifera* leaf extract

Two hundred millilitre aliquots of basal medium prepared following the methods of Ogbonna et al. (1997) were placed in 33 transparent glass jars and 40 ml aliquots of *Chlorella* culture were added. The setup was allowed to stand on a sterilized bench in the screen house for two weeks, and shaken three times daily to prevent clumping of cells.

The *C. sorokiniana* cultures were treated with aqueous and ethanolic leaf extracts of *M. oleifera* after two weeks of culture. Five levels of concentrations of the diluted aqueous and ethanolic extracts and a control were set up with 200 ml of *Chlorella* culture as follows: Level 1: 2 ml extract + 200 ml *Chlorella* culture = 100 mg extract/Litre = 10%; Level 2: 4 ml extract + 200 ml *Chlorella* culture = 200 mg extract/Litre = 20%; Level 3: 6 ml extract + 200 ml *Chlorella* culture = 300 mg extract/Litre = 30%; Level 4: 8 ml extract + 200 ml *Chlorella* culture = 400 mg extract/Litre = 40%; Level 5: 10 ml extract + 200 ml *Chlorella* culture = 500 mg extract /Litre = 50%; Control: 200 ml *Chlorella* culture without extract = 0%.

Estimation of the population growth

Growth was monitored over a period of ten days. The cell population was determined at two-day intervals by counting method using 0.1 mm deep hemacytometer with improved Neubauer ruling (Guillard, 1973). The culture population density per millilitre of culture sample was calculated as follows:

Table 1. Analysis of variance (ANOVA) of the effect of aqueous and ethanolic leaf extracts of *Moringa oleifera* on *Chlorella sorokiniana* biomass.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Extract	1	62720.0	62720.0	497.34*	<.001
Concentration	5	468244.4	93648.9	742.59*	<.001
Day	4	1747930.0	436982.5	3465.06*	<.001
Extract × Concentration	5	13566.7	2713.3	21.52*	<.001
Extract × Day	4	9474.4	2368.6	18.78*	<.001
Concentration × Day	20	59850.0	2992.5	23.73*	<.001
Extract × Concentration × Day	20	4372.2	218.6	1.73*	0.037
Residual	120	15133.3	126.1		
Total	179	2381291.1			

Grand mean 195.22. *Significant difference at $P < 0.05$. d. f.= degrees of freedom, s. s.= sum of squares m. s. mean square, v. r.= variance ratio, F pr.= Probability > Frequency.

Table 2. Effect of aqueous and ethanolic extracts of *Moringa oleifera* on the biomass of *Chlorella sorokiniana*.

Extract	Biomass ($\times 10^4$ individuals per millilitre)		% increase over control
	Population (Mean \pm SE)		
Aqueous	176.56 \pm 10.96 ^b		61.49
Ethanolic	213.8 \pm 13.0 ^c		95.55
Control	109.33 \pm 18.33 ^a		
LSD _{0.05} = 3.315			
% difference, aqueous against leaf extract			21.09

Different letters indicate significant difference at $P < 0.05$.

$$d = (10^4 \times Q) f$$

Where 'Q' is the average number of cell in each 1 mm square; 'f' is the dilution factor; and 'd' is the culture density.

Statistical analysis

The results of the culture population growth were analysed using GENSTAT statistical package. Analysis of Variance (ANOVA) was used to test for significance at $P < 0.05$ while LSD was used to compare the means of the treatment groups.

RESULTS

Analysis of variance (ANOVA) on the effects of aqueous and ethanolic extracts on *Chlorella* biomass revealed that there were significant differences ($P < 0.05$) in all the main effects and all the interactive effects of the extracts (Table 1). There was a significant difference ($P < 0.05$) between the effect of the aqueous and ethanolic leaf extracts of *M. oleifera* on the population of *Chlorella* (Table 2). The aqueous and ethanolic extracts had mean populations of 176.56 ± 10.96 and 213.8 ± 13.0 ($\times 10^4$ individuals/mL) respectively. These represent 61.49 and 95.55% increases over the control respectively. Moreover, the ethanolic extract had 27.09% more biomass than the

aqueous extract and it exhibited higher population growth than the aqueous extract. Table 3 shows a significant difference in the interaction between the aqueous and ethanolic leaf extracts and levels of concentrations at $P < 0.05$. Significant differences were observed in the interaction between the aqueous and ethanolic extracts and day in the population growth at $P < 0.05$ (Table 4). There were significant differences in the interactions between the leaf extracts, levels of concentration and day of the population growth of *Chlorella* ($P < 0.05$) except on days 8 and 10 for both extracts (Table 5). Within the first four days, the population growth increased significantly in both leaf extracts irrespective of level of concentration but dropped significantly on day 10 at 20, 30 and 40% levels of concentrations. It remained the same at 10% and increased at 50% level of concentration for the aqueous extract. It significantly dropped at 50%, remained the same at 10 and 30%, and increased at 20 and 40% for the ethanolic extract.

DISCUSSION

The *Moringa* leaf extracts used in this study promoted the growth of *Chlorella*. The aqueous and ethanolic leaf extracts of *M. oleifera* led to a significant increase in the population

Table 3. Interactive effect of aqueous and ethanolic extracts of *Moringa oleifera* and levels of concentration on *Chlorella* biomass.

Levels of concentration (%)	Population ($\times 10^4$ individuals per millilitre)	
	Aqueous extract	Ethanolic extract
0.00	109.33 \pm 18.83	109.33 \pm 18.83
10.00	141.33 \pm 20.07	178.67 \pm 23.50*
20.00	163.33 \pm 20.89	209.33 \pm 26.70*
30.00	186.00 \pm 22.41	238.00 \pm 30.40*
40.00	217.33 \pm 24.20	265.33 \pm 33.80*
50.00	242.00 \pm 27.50	282.67 \pm 36.60*

LSD_{0.05} 8.119

*Significant difference at P < 0.05

Table 4. Interactive effect of aqueous and ethanolic extracts of *Moringa oleifera* and day on the *Chlorella* biomass.

Day	Population ($\times 10^4$ individuals per millilitre)	
	Aqueous extract	Ethanolic extract
2	66.67 \pm 7.37	84.44 \pm 8.64*
4	92.78 \pm 7.09	115.00 \pm 8.13*
6	157.78 \pm 11.19	201.67 \pm 15.09*
8	283.89 \pm 14.80	333.89 \pm 20.00*
10	281.67 \pm 14.80	334.44 \pm 19.90*

LSD_{0.05} = 7.411

*Significant difference at P < 0.05.

Table 5. Interactive effect of leaf extracts (aqueous and ethanolic) of *Moringa oleifera*, levels of concentration and day on the population of *Chlorella*.

Extract	Concentration (%)	Population ($\times 10^4$ individuals per millilitre)				
		Day 2	Day 4	Day 6	Day 8	Day 10
Aqueous	0.00	23.33 ^a	46.67 ^b	96.67 ^c	190.00 ^d	190.00 ^d
	10.00	43.33 ^e	66.67 ^f	123.33 ^g	236.67 ^h	236.67 ^h
	20.00	56.67 ⁱ	90.00 ^j	133.33 ^k	270.00 ^l	266.67 ^l
	30.00	70.00 ^m	103.33 ⁿ	163.33 ^o	300.00 ^p	293.33 ^p
	40.00	96.67 ^q	123.33 ^r	200.00 ^s	336.67 ^t	330.00 ^t
	50.00	110.00 ^u	126.67 ^v	230.00 ^w	370.00 ^x	373.33 ^x
Ethanolic	0.00	23.33 ^{ab}	46.67 ^{bb}	96.67 ^{cb}	190.00 ^{db}	190.00 ^{db}
	10.00	53.33 ^{eb}	103.33 ^{fb}	170.00 ^{gb}	283.33 ^{hb}	283.33 ^{hb}
	20.00	90.00 ^{ib}	126.67 ^{jb}	180.00 ^{kb}	323.33 ^{lb}	326.67 ^{lb}
	30.00	103.33 ^{mb}	130.00 ^{nb}	223.33 ^{ob}	366.67 ^{pb}	366.67 ^{pb}
	40.00	116.67 ^{qb}	136.67 ^{tb}	263.33 ^{sb}	400.00 ^{tb}	410.00 ^{tb}
	50.00	120.00 ^{ub}	146.67 ^{vb}	276.67 ^{wb}	440.00 ^{xb}	430.00 ^{xb}

LSD_{0.05} = 18.152

Different letters indicate significant difference at P < 0.05. The same letters indicate not significant at P < 0.05.

growth of *Chlorella* when compared with the untreated culture (control). The ethanolic extract exhibited more

pro-motion effect on the population growth than the aqueous extract. The promoter effects also increased as

the level of concentration of the extracts increased with time in days. These findings are in line with the observations of Makkar and Becker (1996) on the use of *M. oleifera* leaf extract as growth enhancing principle in higher plants. However, they contrast previous reports that the seeds of this same plant species have been used locally and industrially in water purification as a natural coagulant (Muyibi and Evison, 1995; Ndabigengesere et al., 1995; Schwarz, 2000; Amagloh and Benang, 2009).

Furthermore, these results support the use of some plant species as enrichment in algae culture media. Nichols (1973) reported that peat moss was used in conjunction with soil in soil water cultures for successful cultivation of Charophyceae or desmids. Most algal culture media can be modified with a variety of components (organic or inorganic) to improve growth population. Such components are called "enrichments".

Although Gibson et al. (1990) and Ferrier et al. (2005) observed in *ex situ* and *in situ* that small quantity of barley straw (*Hordeum vulgare*) promote the growth of certain algal population in water bodies, it has been reported that the presence of large quantity of decomposing barley straw in water can reduce the growth of a range of algal species in the field (Newman and Barrett, 1993; Waybright et al., 2009). In contrast, results from this study did not show a decrease in population of *Chlorella* with increase in concentration of *Moringa* leaf extracts.

The constituent minerals and nutrients reported in the leaf extract of *M. oleifera* include sodium, potassium, calcium, magnesium, zinc, iron, manganese and nutrients such as carbohydrate, protein, fat, crude fibre, moisture and ash (Krishnaiah et al., 2009; Oluduro, 2012; Nweze and Nwafor, 2014). Appreciable amount of nutrients and minerals were undoubtedly the reason for the increase in population growth of *C. sorokiniana* when compared with the untreated culture (control). These minerals (especially, Na, K, Mg, and Ca) constitute the major elements for green algal growth medium as noted by Ogbonna et al. (1997). Nichols (1973) suggested that nutrients such as carbohydrate and ash can also serve as the carbon source for the growth of green algae.

The ethanolic extract had higher growth response on the population of *C. sorokiniana* proving that it is a better solvent for extraction of nutrients from *M. oleifera* leaves than water for enrichment purposes. This is contrary to the observations of Kasolo et al. (2010) and Oluduro (2012) that the aqueous extract had more antimicrobial activities on both Gram -ve and Gram +ve bacteria than the ethanolic.

In all the levels of treatment, the population doubled within six days of treatment. This response could be harnessed to multiply the yield of *Chlorella* during commercial production thereby saving costs and time. *Moringa* is used extensively in food and herbal preparations (Kasolo et al., 2010), hence the results of this study, suggest its use in facilitation of *C. sorokiniana* biomass production in commercial quantities at minimal cost.

Conclusion

The extracts of *M. oleifera* at the levels of concentration used showed appreciable promoter activity on the population growth of *C. sorokiniana* when compared with the untreated samples (control). The phytochemical, mineral and proximate composition of *M. oleifera* may be responsible for the promotion effects and variations in the effectiveness of this plant leaf extracts. Further studies need to be conducted to determine the suitability of the extracts as natural enrichments in commercial production of *Chlorella* and other micro-algae used for food supplements and source of biofuel.

Conflict of Interests

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

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Full Length Research Paper

Phytoextraction potential of cadmium and lead contamination using *Melia azedarach* and *Populus alba* seedlings

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Vegetative growth, biomass, chemical content and uptake of cadmium (Cd) and lead (Pb) in *Melia azedarach* L. (chinaberry) and *Populus alba* L. (white poplar) seedlings were investigated using a 2-year pot experiment. The results indicated that *P. alba* and *M. azedarach* are tolerant to contaminated soil by Cd or Pb without any toxicity symptoms. Vegetative growth and chemical properties of *M. azedarach* are negatively affected by Cd more than Pb whereas, biomasses are negatively affected by Pb little than Cd. Likewise, vegetative growth and chemical properties are negatively affected by Cd more than Pb however, biomasses are negatively affected by Cd and Pb with the same significant level. Both species accumulate more concentrations of Cd and Pb in their roots than in leaves and stem. As a result, *P. alba* and *M. azedarach* are considered suitable phytoremediators for contaminated soils by Cd or Pb.

Key words: Phytoremediation, contamination, heavy metals, cadmium, lead, *Populus alba*, *Melia azedarach*.

INTRODUCTION

Pollution of soil and agricultural land is a complex and serious phenomenon that in recent decades has increased its negative effects on the environment. Transfer of toxic elements to human food chain is a concrete danger that has to be faced, taking into account the possibility for plants to accumulate and translocate contaminants to edible and harvested parts (Puschenreiter et al., 2005). Elevated Cd levels in agricultural soils are becoming a major environmental problem due to the high toxicity of Cd and its mobility from soil to plants and therefore into the food chain (Zhu et al., 2012). Lead accumulation in

plant tissue impairs various morphological, physiological, and biochemical functions in plants, either directly or indirectly, and induces a range of deleterious effects. It causes phytotoxicity by changing cell membrane permeability, by reacting with active groups of different enzymes involved in plant metabolism (Pourrut et al., 2011). Heavy metals cannot be metabolized, therefore the only possible strategy to apply is their extraction from contaminated soil and transfer to the smaller volume of harvestable plants for their disposal (Padmavathamma and Li, 2007) biomass can also be used in producing

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Table 1. Physical and chemical analysis of the used soil.

Parameter practical size distribution	Mean	Parameter soluble anions (meq/L)	Mean
Sand (%)	70.00	CO ₃ ²⁻	0
Silt (%)	20.00	HCO ₃ ⁻	2.60
Clay (%)	10.00	Cl ⁻	20.05
Soil texture	Sandy loam	SO ₄ ²⁻	8.62
pH	8.22	Available N (ppm)	2.17
E.C (ds/m)	3.38	Available P (ppm)	3.52
CaCO ₃ (%)	33.36	Available K (ppm)	2.15
Organic matter (%)	0.28		
Soluble Cations (meq/L)		Total heavy metals (ppm)	
Ca ⁺⁺	10.40	Cd	0
Mg ⁺⁺	1.71	Pb	0
Na ⁺	22.27	Fe	4.15
K ⁺	44.00		

energy and, if economically profitable, metals can be eventually recovered (Zacchini et al., 2009). However, on a large scale, metal uptake by trees can be more effective, mainly because of a deeper root system and a greater yield of biomass (Fischerová et al., 2006). High productivity and elevated uptake and translocation of pollutants to the harvestable biomass are the basis for efficient *in situ* restoration by means of vascular plants (Chaney et al., 2007). There is an active effort to develop new, more cost-effective methods to remediate contamination of polluted soils, hence attention is now focusing on innovative biological technologies such as phytoremediation, based on the use of plants to extract, sequester and/or detoxify pollutants (Salt et al., 1993). Many woody species are now considered of interest to this aim where they are fast growing, have deep roots, produce abundant biomass, are easy to harvest, and several species revealed some capacity to tolerate and accumulate heavy metals. Chinaberry trees have been recommended for planting at landfills in developing countries to offset environmental problems caused by landfills (Kim and Lee, 2005). Poplars are particularly suitable for remediation purposes (Schnoor, 2000) also, salicaceae family are reported to grow even in severe soil conditions and to accumulate heavy metals (Berndes et al., 2004). Many studies have thus been focused on the use of willows and poplars in phytoextraction (Jensen et al., 2009). The objective of this study is exploring the effects of different levels of Cd and Pb on vegetative growth, biomass and mineral content of *Populus alba* and *Melia azedarach* seedlings to evaluate the suitability of both tree species in phytoremediation.

MATERIALS AND METHODS

Pot experiment was carried out at the Nursery of Timber Trees Research Department of Sabaheia, Horticultural Research Station

at Alexandria, Egypt. The study persisted from 22th April 2010 to 1st November 2012 to investigate the effect of Cd and Pb heavy metals on the vegetative growth, biomass and chemical composition of *P. alba*, L. and *M. azedarach*. L. transplants after two growing seasons (from 22th April 2010 to 1st November 2012) as well as, the effect of these tree species on soil properties at the end of plantation period.

Tree species

One year- old *M. azedarach* L. transplants of averaged 90 cm in height and 5 mm in diameter (from the soil surface) as well as, three months old *P. alba* L. transplants of averaged of 35 cm in height and 4 mm in diameter (from the soil surface) were used in this study. All transplants were homogenous and brought from the nursery of Timber Trees Research Department of Sabaheia, Horticultural Research Station.

Pollutant treatments

The pollutants were cadmium at the rates of 10, 20, 40 (as cadmium chloride CdCl₂.H₂O) and lead at the rate of 200, 400, 800 ppm (as lead acetate trihydrate Pb(C₂H₃O₂)₂.3H₂O) in addition to non-pollutant treatment as control. The seedlings were transplanted on April 2010 in polyethylene bags (75 cm in depth and 52.5 cm in diameter), filled with 25 Kg of sandy loam soil which their physical and chemical properties are showed in Table 1 then, irrigated with pollutant solution to field capacity. After 2 weeks, each bag was planted with one seedling and irrigated with tap water to field capacity.

Experimental design

The experiment was laid out in complete randomized design (CRD) as described by Snedecor and Cochran (1989) that consisted of seven treatments replicated three times (21 seedlings per species). The treatments were conducted as follows: Control (without pollutants), Cd-10 ppm, Cd-20 ppm, Cd-40 ppm, Pb-200 ppm, Pb-400 ppm, Pb-800 ppm. Data generated from the experiment were analyzed using one-way ANOVA tests with Duncan's multiple range tests to separate means and data were processed by using SAS procedures.

Table 2. Response of vegetative growth of *M. azedarach* to different levels of cadmium and lead pollutants after two consecutive seasons

Treatment	Height increment (%)	Diameter increment (%)	Branch number (branch)	Leaf area (cm ²)	Length of the longest root (cm)	Green colour intensity (SPAD)
Control	178.03 ^a	252.72 ^a	8.00 ^a	42.67 ^a	94.70 ^a	53.57 ^a
Cd -10	165.60 ^{bc}	250.17 ^{ab}	4.33 ^{bc}	31.20 ^{bc}	92.68 ^{ab}	51.25 ^a
Cd -20	157.92 ^{cd}	245.28 ^{ab}	4.00 ^{bcd}	29.63 ^{bc}	84.00 ^{abc}	43.04 ^c
Cd -40	152.19 ^d	234.61 ^c	3.00 ^d	25.10 ^c	73.33 ^c	42.61 ^c
Pb -200	174.05 ^{ab}	249.14 ^{ab}	5.00 ^b	34.87 ^{ab}	94.02 ^{ab}	47.48 ^b
Pb -400	159.10 ^{cd}	249.30 ^{ab}	4.33 ^{bc}	26.64 ^{bc}	88.68 ^{ab}	45.32 ^{bc}
Pb -800	154.75 ^d	243.51 ^b	3.33 ^{cd}	22.34 ^c	79.68 ^{bc}	44.13 ^{bc}

Means followed by a similar letter within a column are not significantly different at the probability level of 0.05 using Duncan's Multiple Range Test.

Measurements

At first at November 2012 for each treatment of the two species, the total heights were measured from ground level to the seedling apex to the nearest 0.5 cm. Also, stem diameter was measured at ground level to the nearest 1.0 mm. The measurements were done to calculate the increment of height and diameter growth (%). Number of the branches per seedling was counted for each treatment. The three seedlings from each treatment were harvested, and then 15 leaves from each were harvested from different locations along the seedling level (top, middle and bottom). As well leaf area in cm² was measured by using Auto Cad software. A SPAD-502 chlorophyll meter (based on light transmittance through leaves) was used as a non-destructive tool for estimating leaf chlorophyll (Markwell et al., 1995). Two readings per leaf were taken midway between the leaf mid vein and margin and then averaged. On the other hand, a half gram of the ground material of different plant parts was digested by sulfuric acid (H₂SO₄) and hydrogen peroxide (H₂O₂) mixture on hot plate until a clear digest was obtained. The solution was left to cool then, filtered and diluted to 50 ml with distilled water (Evenhuis and DeWaard, 1980). The digested samples were prepared for measuring nitrogen, phosphorus, potassium, cadmium and lead. Nitrogen and phosphorus were measured colorimetrically as determined by Evenhuis (1976) and Murphy and Riley (1962). Potassium was measured using a flame photometer (Page et al., 1982). Cadmium and lead was measured by using Perkin Elmer, 3300 Atomic Absorption Spectrophotometer. Contents of cadmium and lead were expressed as mg/kg dry weight then uptake (expressed as mg) was calculated as follows: Cd or Pb content X dry weight (leaves, stem, roots) / 1000. Total Cd or Pb uptake = leaves uptake + stem uptake + roots uptake. Available N of the soil was determined using Kjeldahl method (Bremner and Mnlvaney, 1982) and available P was determined according to Olsen and Sommers (1982). Soil micronutrients were extracted by 0.05 M DTPA solution (Lindsay and Norvell, 1978). Also, the concentrations of Cd and Pb were quantified through Atomic absorption spectrophotometer (AAS).

RESULTS AND DISCUSSION

Vegetative growth

Table 2 reveals that both stem height increment and stem diameter increment (%) of *M. azedarach* were affected by all rates of Pb and Cd, therefore, the highest rates of Cd (40 ppm) and Pb (800 ppm) significantly decreased both parameters. On the other hand, the number of branches

was extremely affected by contamination of Cd and Pb, therefore, after the two growing seasons, the decreases in numbers of branches were maximized up to 62.5 and 58.4% for Cd 40 ppm and Pb 800 ppm, respectively. Likewise, the leaf area responded to different rates of Cd and Pb where Cd 40 ppm and Pb 800 ppm decreased the leaf area by 41.2 and 47.7%, respectively. Also, the length of the longest root in the soils contaminated by Cd 40 and Pb 800 ppm were decreased by 22.6 and 15.9%, respectively; less than the non-contaminated (Table 2). However, both Cd 40 and 20 ppm declined the green colour intensity (GCI) by 20.5 and 19.7%, respectively; less than non-contaminated leaves. Likewise, Pb 800 and 400 ppm declined (GCI) of *M. azedarach* leaves by 17.6 and 15.4%, respectively, less than the non-contaminated. Table 3 shows that the high rates of cadmium (40 ppm) and lead (800 ppm) slightly decreased both stem height increment and stem diameter increment (%) compared to the control. On the other hand, branch number of white poplar was varied significantly by increasing the rates of Cd and Pb therefore, Cd 40 ppm and Pb 800 ppm were intensely minimized the branch numbers by 49.2 and 36.1%, respectively, fewer than non-contaminated seedlings. Also, leaf area of *P. alba* seedlings that planted in contaminated soil with Pb 800 ppm and Cd 40 ppm reduced the leaf area by 34.1 and 32.9%, respectively, comparing to non-contaminated seedlings. Moreover, the seedlings in contaminated soil with Cd 40 ppm and Pb 800 ppm reduced the length of the longest root by 37.4 and 30.3%, respectively, comparing with non-contaminated seedlings. The data in Table 3 presented that the GCI were declined by 16.7 then 16.0% less than control when contaminated by Cd 40 and Pb 800 ppm, respectively.

These results are in agreement with those of Cosio et al. (2006) on *Salix viminalis*. Pb is considered as a general protoplasmic poison, which is accumulative, slow acting and subtle. Also, Kabata-Pendias and Pendias (1992) mentioned that when Pb is presented in soluble forms in nutrients solutions, plant roots are able to take up great amounts of this metal which leads to an inhibitory effect on plant metabolism. As well as, Hall

Table 3. Response of vegetative growth of *Populus alba* to different levels of cadmium and lead pollutants after two consecutive seasons.

Treatment	Height Increment (%)	Diameter increment (%)	Branch number (branch)	Leaf area (cm ²)	Length of the longest root (cm)	Green colour Intensity (SPAD)
Control	535.30 ^a	247.10 ^a	18.3 ^a	11.76a	74.60 ^a	52.00 ^a
Cd -10	532.22 ^a	244.37 ^{bc}	15.7 ^{bc}	11.01 ^{ab}	68.70 ^{ab}	50.57 ^b
Cd -20	529.34 ^a	241.18 ^d	12.0 ^d	9.65 ^c	65.36 ^b	48.36 ^c
Cd -40	500.93 ^b	200.97 ^f	9.3 ^e	7.89 ^d	46.67 ^c	41.76 ^e
Pb -200	532.93 ^a	245.11 ^{ab}	17.7 ^{ab}	10.29 ^{bc}	71.67 ^{ab}	50.34 ^b
Pb -400	530.69 ^a	242.52 ^{cd}	15.0 ^c	9.36 ^c	64.00 ^b	48.72 ^c
Pb -800	505.27 ^b	222.22 ^e	11.7 ^d	7.75 ^d	52.00 ^c	43.67 ^d

Means followed by a similar letter within a column are not significantly different at the probability level of 0.05 using Duncan's Multiple Range Test.

Table 4. Response of biomasses of *Melia azedarach* and *Populus alba* to different levels of cadmium and lead pollutants after two consecutive seasons.

Treatment	<i>Melia azedarach</i>			<i>Populus alba</i>		
	Leaves dry weight (g plant ⁻¹)	Stem dry weight (g plant ⁻¹)	Roots dry weight (g plant ⁻¹)	Leaves dry weight (g plant ⁻¹)	Stem dry weight (g plant ⁻¹)	Roots dry weight (g plant ⁻¹)
Control	18.39 ^a	94.27 ^a	57.28 ^a	15.66 ^a	58.32 ^a	55.21 ^a
Cd -10	14.41 ^b	93.83 ^a	56.21 ^{ab}	12.27 ^b	55.90 ^{ab}	50.19 ^{abc}
Cd -20	13.61 ^b	87.06 ^{ab}	51.02 ^c	10.96 ^{bc}	53.07 ^c	48.89 ^{abc}
Cd -40	10.47 ^c	77.64 ^{bc}	43.38 ^d	9.96 ^c	47.22 ^d	43.76 ^c
Pb -200	14.43 ^b	92.67 ^a	54.70 ^{ab}	15.90 ^a	56.73 ^{ab}	50.41 ^{ab}
Pb -400	13.44 ^b	88.07 ^{ab}	52.80 ^{bc}	15.15 ^a	55.34 ^{bc}	48.65 ^{bc}
Pb -800	11.72 ^{bc}	73.44 ^c	45.17 ^d	10.04 ^c	49.14 ^d	36.75 ^d

Means followed by a similar letter within a column are not significantly different at the probability level 0.05 using Duncan's Multiple Range Test.

(2002) indicated that Cd and Pb can result in growth inhibition and toxicity symptoms. Bindhu and Bera (2001) studied the effect of different concentrations of CdSO₄ on leaf area in mungbean seedlings. Leaf area decreased with an increase in the concentration of CdSO₄ in comparison to untreated control. Total chlorophyll decreased thereafter with an increase in the concentration of Cd²⁺. The toxic effects of cadmium on the photosynthetic system cause several structural and functional disorders. The main targets are the photosynthetic pigments biosynthesis pathways though cadmium reduces chlorophyll production by the inhibition of proto-chlorophyllide reductase. Also, it can interfere with the photosynthetic pigments by substituting Mg²⁺ ions with Cd²⁺ ions in chlorophyll molecules. These substituted molecules have much lower fluorescence quantum yields compared to magnesium chlorophylls. These two toxic effects reduce the production of chlorophyll and consequently photosynthesis, which can then lead to senescence and cell death (Santos et al., 2010). Also, positive correlations between the photosynthetic rate and N content of the plant have been reported by Keulen and Stol (1991) and

Makino et al. (1994). Lead accumulation in plant tissue is changing cell membrane permeability, by reacting with active groups of different enzymes involved in plant metabolism and by reacting with the phosphate groups of ADP or ATP, and by replacing essential ions. Lead toxicity causes inhibition of ATP production, lipid peroxidation, and DNA damage by over production of ROS. In addition, lead strongly inhibits root elongation, seedling development, plant growth, transpiration and chlorophyll production. The negative effects that lead has on plant vegetative growth mainly result from the following factors: distortion of chloroplast ultrastructure, obstructed electron transport, inhibition of Calvin cycle enzymes, impaired uptake of essential elements, such as Mg and Fe, and induced deficiency of CO₂ resulting from stomatal closure (Pourrut et al., 2011).

Biomass

Table 4 presented that the higher rate of cadmium (40 ppm) and lead (800 ppm) were the most harmful

Table 5. Mean content and uptake of cadmium in the leaves, stem and roots and total uptake of *Melia azedarach* and *Populus alba* responded to different levels of cadmium pollutant at the end of second season.

Treatment	Content (mg/kg)			Uptake (mg)			Total uptake (mg/plant)
	Leaves	Stem	Roots	Leaves	Stem	Roots	
<i>Melia azedarach</i>							
Control	-	-	-	-	-	-	-
Cd -10	2	3.6	13.5	0.03	0.34	0.76	1.13
Cd -20	2.5	5.2	16.5	0.22	0.45	0.84	1.51
Cd -40	2.5	5.9	17.5	0.19	0.46	0.76	1.41
<i>Populus alba</i>							
Control	-	-	-	-	-	-	-
Cd -10	4.5	3.5	8.5	0.06	0.19	0.43	0.68
Cd -20	7.5	4	17	0.08	0.21	0.83	1.12
Cd -40	14.5	11.5	17.5	0.14	0.54	0.77	1.45

Each value represented the average of three replicates.

treatments that decreased the leaves dry weight (LDW) of chinaberry seedlings by 43.1 and 36.3%, respectively, less than the non-contaminated. Also, roots dry weight (RDW) had a same manner, therefore, it was significantly shorter by 24.3 and 21.1%, respectively, less than non-contaminated seedlings. On the other hand, Pb 800 and Cd 40 ppm decreased the stem dry weight (SDW) of Chinaberry by 22.1 and 17.6%, respectively, less than non-contaminated seedlings. Table 4 shown that Cd was more negatively affect leaves and stem biomass of white poplar seedlings than Pb though, both Cd 40 and Pb 800 ppm decreased (LDW) by 36.4 and 35.9%, and (SDW) by 19.0 and 15.7%, respectively, less than non-contaminated seedlings. In contrast, the biomass of the roots was negatively affected by Pb more than Cd contamination, therefore, Pb 800 followed by Cd 40 ppm significantly decreased (RDW) of white poplar by 33.4 and 20.7%, respectively, less than non-contaminated seedlings. The above mentioned results are in parallel with Chiraz et al. (2004) that they observed inhibition of photosynthesis, and as a consequence, a decrease in dry weights. Also, the roots from Cd-stressed plants were shorter than those from controls. The decrease in biomass as a result of contamination by Cd and Pb may be due to the decrease in photosynthesis, carbohydrates metabolism as well as, production of reactive oxygen, therefore, Santos et al. (2010) concluded that the effects of cadmium in the carbohydrate metabolism are mostly due to the inhibition of enzymes such as RuBisCO. Also, the exposure of plants to metals such as cadmium can also stimulate the production of reactive oxygen species that cause the oxidation of proteins, lipids and nucleic acids, membrane damage, mutagenesis and the inactivation of enzymes.

Mineral content

Table 5 indicated that cadmium content and uptake of stem and roots of *M. azedarach* increased with

increasing the soil contamination level from 10 to 40 ppm therefore, Cd contents of leaves, stem and roots rose up to 25.0, 63.9 and 29.6%, respectively, also, Cd uptake of stem and roots rose up to 35.3 and 10.5%, respectively, whilst, the uptake of leaves of Cd-40 treatment rose up to 533.0% more than Cd-10 treatment. The highest content and uptake of Cd was recorded for roots, stem then leaves in decreasing order. Likewise, content and uptake of leaves, stem and roots increased by about 1.0 to 1.6-fold when contamination level in the soil increased from Pb 800 to Pb 200 ppm and the most content and uptake recorded for roots followed by stem. In addition, the total uptake of Pb increased by 20.3% when contamination level of Pb increased from 200 to 800 ppm (Table 6). Also, Table 5 demonstrated that Cd content of leaves and stem of *P. alba* contaminated by Cd 40 rose approximately 3-fold more than Cd 10 ppm and Cd uptake rose 2.3 to 2.8-fold more than Cd 10 ppm whilst, the increase in content and uptake of roots were slight. The highest uptake of Cd was recorded for roots, stem then leaves in decreasing order. The increase of Pb uptake for leaves, stem and roots ranged 1.3 to 1.5 fold when the contamination level in the soil increased from Pb 200 to Pb 800 ppm (Table 6).

These results were matched with Nylund (2003) that the uptake of Cd in tree seedlings was proportional to the concentration of Cd in soil. Also, Zhivotovskya (2011) on various tree species determined that Cd and Pb were mainly accumulated in roots higher than leaves and stem. Plant roots are able to release into the rhizosphere chelating agents with binding ability for metals (Salt et al., 1993). These metal chelators or other molecules within plant cells that have a high affinity for metals can help in the metal sequestering (Fulekar et al., 2009). Consequently, most of the Cd uptake occurs in the epidermis of the root tips (Landberg and Greger, 1996). Root tips lack the casparian band, and Cd is therefore transported apoplastically through cell walls directly to the xylem.

Table 6. Mean content and uptake of lead in the leaves, stem and roots and total uptake of *Melia azedarach* and *Populus alba* responded to different levels of lead pollutant at the end of second season.

Treatment	Content (mg/kg)			Uptake (mg)			Total uptake (mg/plant)
	Leaves	Stem	Roots	Leaves	Stem	Roots	
<i>Melia azedarach</i>							
Control	-	-	-	-	-	-	-
Pb -200	12	10.3	36.2	0.17	0.95	1.98	3.1
Pb -400	12.1	11.4	45.5	0.16	1	2.4	3.56
Pb -800	17	16.3	51.5	0.2	1.2	2.33	3.73
<i>Populus alba</i>							
Control	-	-	-	-	-	-	-
Pb -200	25	21.6	48.6	0.4	1.22	2.45	4.07
Pb -400	40.6	30.2	41.5	0.62	1.67	2.09	4.38
Pb -800	60.8	31.1	68.5	0.61	1.53	2.52	4.66

Each value represented the average of three replicates.

Table 7. Mean soil Extractable Cd and Pb as affected by Cd and Pb contamination at the end of second season.

DTPA extractable	Control	Cd 10 ppm	Cd 20 ppm	Cd 40 ppm	Pb 200 ppm	Pb 400 ppm	Pb 800 ppm
<i>Melia azedarach</i>							
Cd	0.00	03.50	11.60	13.90	0.00	0.00	0.00
Pb	0.00	0.00	0.00	0.00	121.14	217.50	367.18
<i>Populus alba</i>							
Cd	0.00	1.30	1.80	2.45	0.00	0.00	0.00
Pb	0.00	0.00	0.00	0.00	114.50	200.17	247.50

Each value represented the average of three replicates.

Cations in the xylem move upwards in the negative walls of the xylem, but most (70 to 90%) remain in the root tissue. The reason for this may possibly that Cd is adsorbed to negative charges on cell walls and macromolecules in cells, or is taken up by the root cell and accumulates in the cytoplasm and vacuoles. Also, Pourrut et al. (2011) mentioned that under lead stress, plants possess several defense strategies as reduced uptake into the cell, sequestration of lead into vacuoles by the formation of complexes and binding of lead. In addition, activation of various antioxidants to combat increased production of lead-induced ROS constitutes a secondary defense system.

Soil extractable

Table 7 demonstrated that, extractable Cd and Pb were increased with increasing the contamination rate of each pollutant from lower to higher rates for both tree species.

Recommendation

M. azedarach and *P. alba* could be employed in phytoremediation of soils contaminated with cadmium (up to 40

ppm) or lead (up to 800 ppm) whereas, they grow reasonably in these soils without any toxicity symptoms. In addition, autumn litter fall of both species do not create a risk of cadmium and lead input into the soil.

Conflict of Interests

Authors have not declared any conflict(s) of interests.

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Full Length Research Paper

Karyotype evolution and species differentiation in the genus *Rattus* of Manipur, India

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Rattus is the most studied genus all over the world but species of the genus are not thoroughly reported from Manipur. The present paper deals with the morphometric, cytotaxonomic and phylogenetic studies of Manipur, India. The different species of *Rattus* namely *Rattus rattus*, *Rattus brunneusculus*, *Rattus tanezumi* and *Rattus nitidus* were studied. *Berylmys manipulus* and *Berylmys mackenziei* were taken from the genus *Berylmys*. The studies of the species reveal that the polymorphic chromosomes in the genus *Rattus* were pair numbers that is, 1, 9, 10 and 13. The change of acrocentrics which are regarded as primitive/ancestral types of chromosomes into either subtelocentric or small metacentrics leads to speciation or simply new species particularly in Manipur. It is generally accepted cytologically that $2n=40$ (*Berylmys manipulus*, *B. mackenziei*, *R. kandianus* etc) are derived from the $2n=42$ through centric fusion/Robertsonian fusion, but the soft palate studies shows affinity of the species that is, *B. manipulus* and *B. mackenziei* from Manipur towards *Niviventer*. The present study is the first step towards the understanding of the relationship between these two genera-*Rattus* and *Berylmys*.

Key words: *Rattus*, *Berylmys*, Manipur.

INTRODUCTION

The genus *Rattus* has been defined as a widely distributed and taxonomically mixed group including many species and subspecies throughout the World (Ellerman and Morrison-Scott, 1951; Vinogradov and Argyropulo, 1941; Corbet, 1978; Harrison and Bates, 1991). Out of a total of 4,629 species of mammals known in the world, 372 species occur in the Indian union (Corbet and Hill, 1992). Of these, 69 species are reported

in Manipur (Mandal et al., 2005). Mandal et al. (2005) reported five species and seven subspecies: *Rattus rattus brunneusculus* (Hodgson), *Rattus rattus bullocki* Roonwal, *Rattus rattus tistae* Hinton, *Rattus nitidus obsoletus* Hinton, *R. mackenziei* (Thomas) and *Rattus manipulus manipulus* (Thomas). The latter two taxa were then included in the genus *Berylmys* as *Berylmys mackenziei* (Thomas) and *Berylmys manipulus*

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Table 1. The different rat specimens collected from the different parts of Manipur.

S/N	Species	Location	Localities	Number of Specimens	Sex	
					Males	Females
1	<i>Berylmys mackenziei</i>	Senapati District	Kharam Waiphei	29	12	17
2	<i>Berylmys manipulus</i>	Bishnupur, Senapati District	Keonou, Kharam waiphei	15	8	7
3	<i>Rattus rattus</i>	Imphal West, Senapati District, Thoubal District	Langol, Imphal, Canchipur, Kharam Waiphei, Lilong,	22	16	6
4	<i>R. r. brunneusculus</i>	Imphal East District	Singjamei Kshetri Leikai	4	0	4
5	<i>R. nitidus</i>	Senapati District,	Mao	4	1	3
6	<i>Rattus tanezumi</i>	Loktak Lake Bishnupur District	Karang	18	6	12
7	<i>Rattus norvegicus</i>	Imphal West District	Keishampat, iroisemba, Thangmeiband	25	12	13
Total	7 species	5 district	14 localities	107	55	52

(Thomas), respectively (Agrawal, 2000). Manipur is one of the passage to the South East Asia from Indian continent or *vice-versa*. *Rattus rattus* originated from South East Asia, whereas the India Continent is the old resident of the species (Suzuki et al., 2012). It is pertinent to study the different species of the genus *Rattus* and some species of the *Berylmys*. The present study is the first step towards understanding the chromosomal profiles of some species of the *Rattus* and to investigate some of the relationship with *Berylmys* particularly in the context of Manipur

MATERIALS AND METHODS

A total of 107 (55 males, 52 females) specimens of the genus *Rattus* were caught from 14 localities in five districts of Manipur using live traps during June-October, 2012 (Table 1). Before the work began, each specimen was given an accession code. The wet specimens, skulls and skins were deposited in the University Museum of Central Agricultural University, Imphal. The morphological studies like fur colours, Head-body Length, Tail length, length of hind foot etc. were carried out according to Agrawal (2000), Aplin et al. (2003) and Alfred (2005). The skulls were set (Herbreteau et al., 2011) to study the cranial features according to Agrawal (2000). Chromosomes were collected from the somatic cells from bone marrow cells of femur after treating the rats with colchicine for 2 h.

The cells were then treated with KCl for 45 min, and fixed in acetic acid and methanol in the ratio 1:3 by volume. Finally the cell suspension was spread on slides pre-chilled in methanol and flame dried.

The chromosomes were stained using 4% Giemsa solution. C-banding was done according to Sumner (1972) with some modification. Chromosome number and morphology were recorded from 50 metaphases per specimen directly under a 100X and photographs of selected cells were taken by camera attached in Olympus BX-41. The sorting types of chromosomes and karyotypes were done according to Yosida (1983).

Ethical approval was obtained from the Institutional Ethics Committee (IEC) of the Central Agricultural University whose protocols and guidelines about the using of living animals in science were strictly followed.

RESULTS

***Rattus rattus* (Linnaeus, 1758):** 1758. *Mus rattus* Linnaeus, Syst.nat., 10th ed., 1: 61 (Uppsala, Sweden). (Figure 1a to c)

General characters

Medium-sized rat, with a completely uncoloured tail, generally longer than head and body length. Fur short and harsh, with many soft flattened spines which were from the plains and soft without spines in winter from mountain habitats. Dorsum are in various shades of brown or of two colours that is, (i) greyish, tipped with buff (indoor forms) and (ii) white or creamish, with a medial streak of grey or irregular grey patches (outdoor forms). Mammary is generally 5 pairs, if 6th pair is present, then postaxial pairs will be close together (less than 10 mm apart).

Occipitonasal length of skull ranges from 36.9 to 46.5 mm; palate always more than one-half of occipitonasal length; extending posteriorly behind third upper molars; nasals relatively short, less than 38% of occipitonasal length and not extending behind ascending process of premaxillary tooth. the teeth row ranges from 5.5 to 7.5 mm and forms one of the main characters in the classification of species into subspecies through Short-toothed forms (less than 6.6 mm in length) and long-toothed forms (more than 6.6 mm in length).

***Rattus rattus* from Kharam Waiphei, Senapati Districts and Thoubal District** (24° 71.000", E 93° 92.550")

Morphological features of the rats from the Kharam Waiphei, Senapati Districts and Thoubal were agouti dorsal but the ventral fur is light greyish (Figure 1c).



Figure 1. *Rattus rattus* (a-e), *Rattus rattus alexandrinus* upper surface-a and lower surface-b, venture-c, *R. rattus alexandrinus* upper surface-f and lower surface-e, venture-d; venture-g (arrowed T- shaped mark), upper surface-a and lower surface-b -h, i-pes of *R. tanezumi*, j-pes and manus-k of *R. nitidus*; l-pes of *B. manipulus*, soft palates of m- *Rattus rattus alexandrinus*, n- *R. rattus alexandrinus*, o- *R. tanezumi*, p- *R. nitidus*, q- *B. manipulus*, tail-r, venter-s of *B. manipulus*.

Cytology

The $2n=42$, karyotype was found in ten individuals (8 females and 2 males) collected from Senapati and Thoubal District. It consisted of thirteen acrocentric pairs (#1 to 13), seven pairs of small metacentric pairs (#14 to 20) and acrocentric X and Y (Figure 2). The X chromosome is comparable in size to the autosomal pair 6. The Y chromosome is the smallest acrocentric chromosome. This type is here considered as ancestral or primitive type

***Rattus rattus* from Keishampat (N 24° 47.806", E 93° 55.926") and Langol, Imphal West (N 24° 82.798", E 93° 89.965")** (Figure 1d to f).

Morphological features of the rats from the Imphal West

were agouti dorsal and venter yellowish white fur (Figure 1d).

Cytology

The $2n = 42$ karyotype was found in three individuals (1 females and 2 males) collected from Imphal West district (Langol Locality). Their karyotype consists of eleven acrocentrics pairs (#1 to 9, 11 and 12); pair 10 was homomorphic subtelocentric pair, one heteromorphic pair to subtelocentric/acrocentrics (#13), seven pairs of small metacentric (#14 to 20) elements, and the two acrocentrics X and Y chromosomes. The rats from Keishampat (one male) showed polymorphic in pair #1 due to acrocentric and subtelocentric and rest of the karyotype was quite similar to Langol type. The X

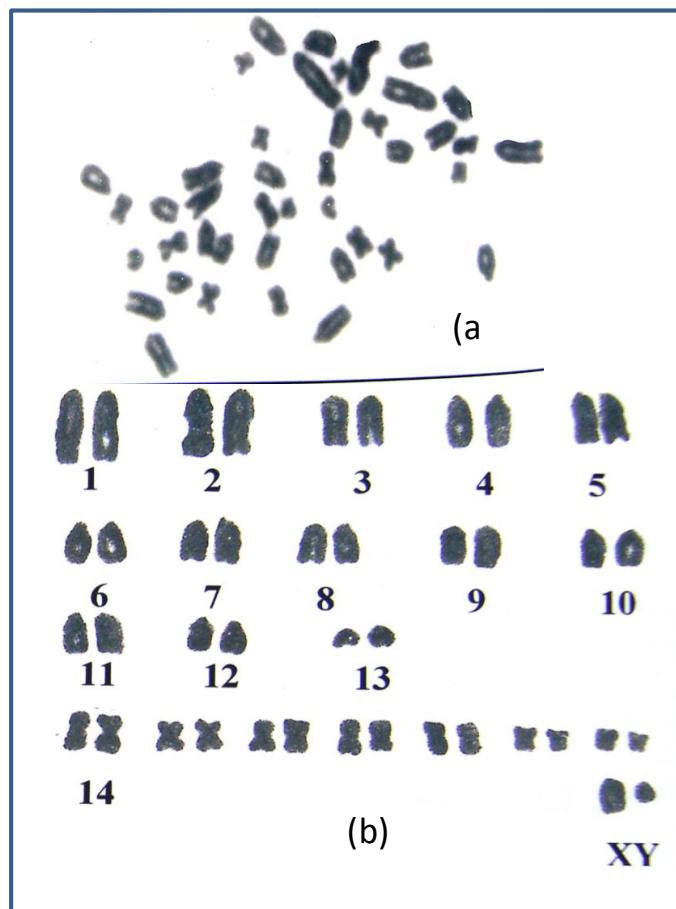


Figure 2. Metaphase a and karyotype b of *Rattus rattus* from Kharam Waiphei.

chromosome is comparable in size to the 12th autosomal pair while the Y is the shortest element of the male genome (Figure 3).

***Rattus tanezumi* Temminck, 1844 from Karang of Loktak Lake (N 24° 54.770" E 93° 83.300"), Bishnupur District**

The rats from the Karang (PBG 345) were identified as *Rattus tanezumi* with the help of mitochondrial DNA (COI). The *Rattus tanezumi* have a unique feature which is the presence of a smoky T-shaped around throat and as on upper parts of manus (Figure 1g to i)

Cytology

2n=42, without exception the karyotype consisted of thirteen acrocentrics pairs (#1 to 13), seven pairs of small metacentric pairs (#14 to 20) and acrocentrics XY, and sex chromosomes were acrocentrics. The X chromosome

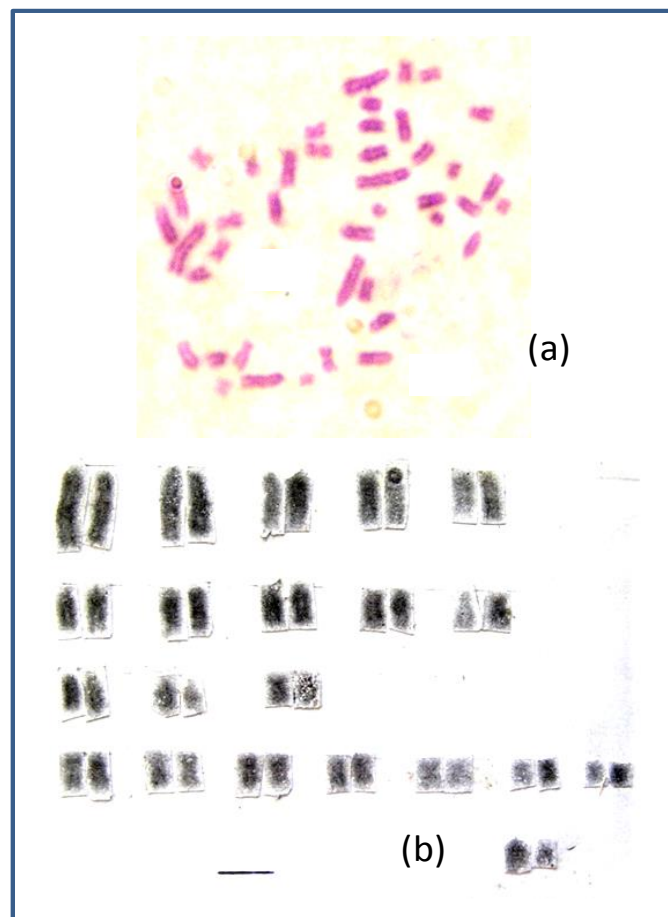


Figure 3. Metaphase a and karyotype b of *Rattus rattus* from Keishampat.

was 10th of autosomal chromosome in length and Y chromosome is the shortest in the genome (Figure 4). Chromosome numbers 15, 19 and 20 were heteromorphic due to metacentric and submetacentrics homologues.

***Rattus rattus brunneusculus* (Hodgson, 1845) from Singjamei, Imphal East (N 24° 49.196", E 93° 56.564")**

Diploid count 42 consists of chromosome numbers 1 and 9 being submetacentrics, # 2-5, # 7 to 13 being acrocentric, the X chromosome is acrocentric which is comparable to sixth autosomal pair. The Y chromosome is the smallest acrocentric chromosomes (Figure 5).

***Rattus norvegicus* (Berkenhout, 1769) from Keishampat, Imphal West 1769.**

Mus norvegicus Berkenhout, *Outlines Nat. Hist. Gt. Britain and Ireland*, 1; 5(Great Britain).

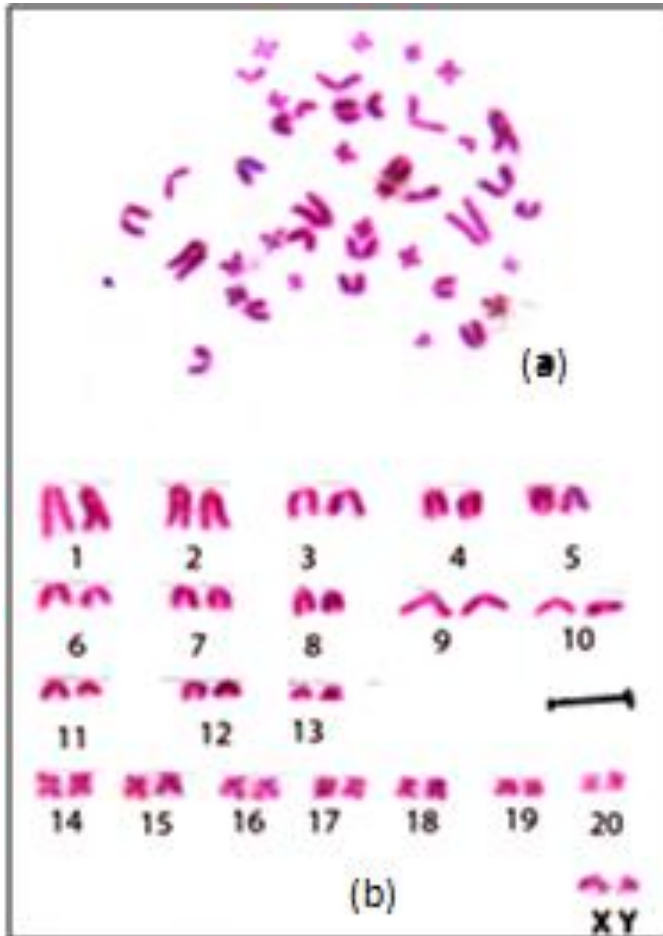


Figure 4. Metaphase a and karyotype b of *Rattus tanezumii*.

General characters

A bandicoot-like large rat, have an obscurely bicoloured tail, shorter than head and body length (80 to 95%) and relatively smaller ears (16 to 23). Fur coarse with spines, Dorsum dark brown and venter grey. Large skull, occipitonasal length 45 to 55 mm; supraorbital ridges powerful, extending backwards fairly straight up to occiput; palate long, more than one half of occipitonasal length (*onl*), extending posteriorly far behind third upper molars; maxillary toothrows less than 15% of *onl* and its width ranges from 2.0 to 2.3 mm; anterior palatal foramina, on average, 17% of *onl* and broad (3.0 to 4.6), ending far ahead of first upper molars; braincase narrow, cranial width less than 28% of *onl*, Mammae 5 or 6 (Agrawal, 2000).

This species is robust and heavily built. The tail is always less than head and body length in adult specimens. The ear is short and when drawn forward, does not reach the eyes. External measurements are given in Table 2. Dorsal fur colour varies slightly from dark brownish to ochre (particularly in old specimens) and the dorsal hair bases are greyish. Tail is slightly is

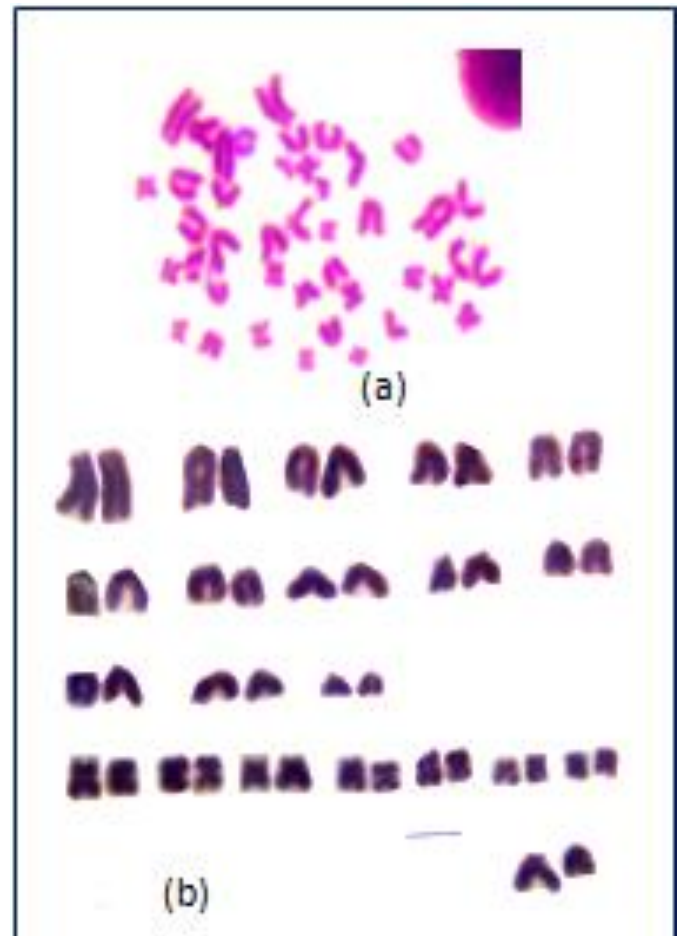


Figure 5. Metaphase a and karyotype b of *Rattus brunneusculus*.

bicoloured and covered with short, sparse, dirty, whitish hairs. The soles of the fore and hind feet are completely naked. The upper surface of both the fore and hind feet are covered with tiny whitish hairs but the base of the hind upper surface has patches of grayish hairs. The hairs on ventral fur are dirty white but bases are greyish. The line of demarcation is quite distinct. The species has six pairs of mammae (2 pairs pectoral, 1 pair abdominal and 3 pairs inguinal).

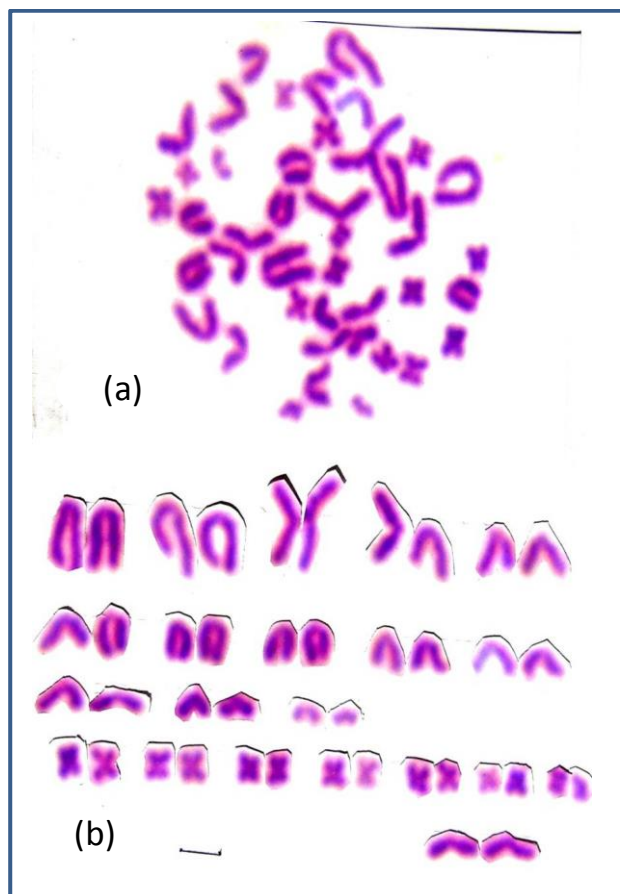
Cytology: The rats from Imphal West district with the parallel supra-orbital ridge are to be *Rattus norvegicus* (Berkenhout). The species has $2n=42$ with exception to karyotype thirteen acrocentrics pairs (#1 to 13), seven small metacentric pairs (14 to 20) and acrocentrics sex chromosomes. The X chromosome was 11th of autosomal chromosome in length and Y chromosome is the shortest in the male genome (Figure 6).

***Rattus nitidus* (Hodgson, 1845) from Mao (25° 43.520'' E 94° 06.820''), Senapati District, 1845**

Mus nitidus Hodgson, *Ann.Mag.nat.Hist.*, 15,267

Table 2. The morphometrics and crannial measurement of rat species of Manipur

Measurement	<i>B. manipulus</i>	<i>B. mackenziei</i>	<i>R. rattus</i>	<i>R. tanezumi</i>	<i>R. nitidus</i>	<i>R. norvegicus</i>
Length of head & body length (mm)	165		162	150.3	146	215
Length of tail (mm)			190	168.3	157	195
Length of hind foot (mm)	31		35	32.8	36	43
Length of ear (mm)	24		22	20.7	22	21
Length of Occipitonasal (mm)	40.11	46.79	41.77	41.4	41.63	48.83
Condylbasal (mm)	39.73	45.61	38.82	39.3	37.93	46.09
Height of rostrum (mm)	9.67	10.42	9.7	9.1	9.13	10.43
Tympanic bulla (mm)	5.99	6.44	6.91	8.1	6.04	5.67
Length of diastema (mm)	13.94	14.11	11.31	11.1	9.82	14.65
Length of molar (mm)	6.07	7.4	6.49	6.9	6.93	6.23
Length of rostrum (mm)	13.12	15.35	12.41	14.4	13.84	16.42
Length of palate (mm)	22.38	24.51	21.92	21.9	20.2	26.56
Anterior palatal foramen (mm)	7.26	8.69	7.8	7.5	5.86	8.28
Length of nasal (mm)	16.67	17.67	15.31	15.5	17.43	18.42
Nasal width(mm)	3.81	4.77	3.84	4.4	4.2	5.66
Frontal width(mm)	6.5	6.92	5.93	6.1	6.22	7.26
Length of orbit (mm)	12.43	16	14.43	14.4	14.01	17.21
Greatest zygomatic width(mm)	20.69	23.59	19.25	20.7	20.18	23.41
Cranial width (mm)	15.97	18.06	15.92	15.6	16.77	16.72

**Figure 6.** Metaphase a and karyotype b of *Rattus norvegicus*.

General character

In this species the fur is soft and quite thick, particularly in the typical race. The tail is nearly naked, with signs of growth of hairs throughout its length. Hind foot with five toes, all clawed; the hallux shorter than the fifth, which is shorter than the central three; apparently six plantar pads. Tail little longer than head and body as a rule (107% average for typical race, Hinton; 99% average for *obsoletus*). Mammary 12, as a rule.

Colour: The back with is usually dark brown, occasionally with a darker mid-dorsal patch or line. Feet usually yellowish or whitish, rarely dark. Tail normally dark as a whole (Ellerman, 1961).

The rats from the Mao (PBG 344) are identified as *Rattus nitidus* on the basis of mitochondrial DNA (COI) sequences. The sequence is submitted in the NCBI Gene Bank (NCBI accession number was JQ918374).

Cytology: The $2n$ of *Rattus nitidus* is 42. The karyotypes comprising two pairs of subtelocentric chromosomes (#1 and 9), 10 pairs of acrocentric chromosomes (#2 to 8, 10 to 12), chromosome pair 13 is metacentric, seven pairs of small metacentric chromosomes (#14 to 20). The X subtelocentrics which are 8th of autosomal chromosome in length (Figure 7) in which the q-arms are always heterochromatic (Figure 10d).

Berylmys 1947. *Berylmys* Ellerman, *Proc.zool.Soc.Lond.*, **117** : 261,267.

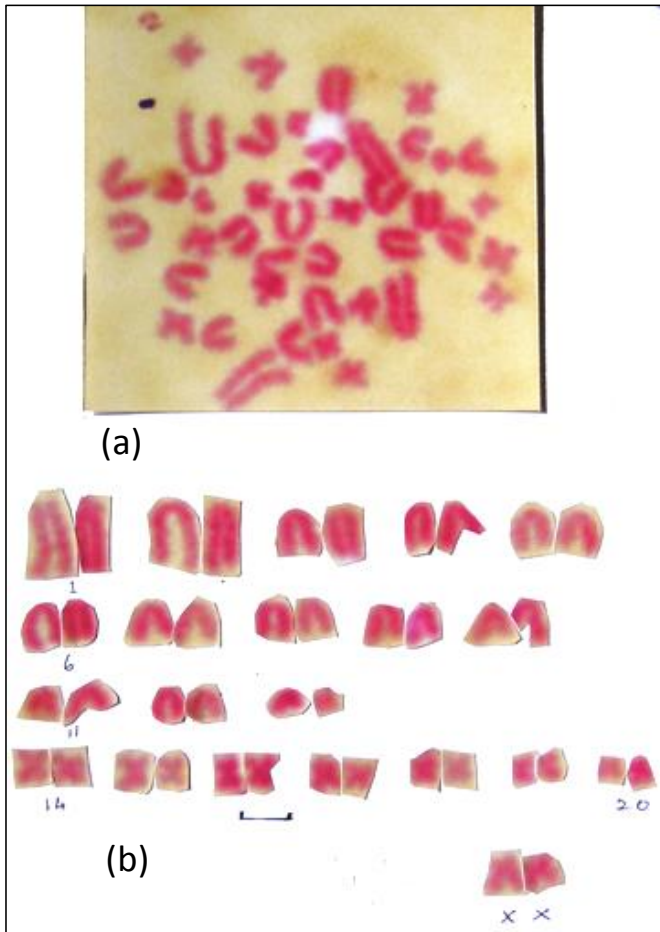


Figure 7. Metaphase a and karyotype b of *Rattus nitidus*.

Type species : *Epimys manipulus* Thomas.

Genus *Berylmys* is characterized by dense, crisp, iron grey pelage, palate long but not extending posteriorly beyond third upper molars, diastema more than 28% of occipitonasal length, bulla, in Indian species, about 15% of occipitonasal length and lower incisor root forming a prominent knob on outer side of lower jaw.

This genus is represented by four species, *Berylmys manipulus*, *B. bowersii*, *B. mackenziei* and *B. berdmorei*.

Key to Indian species of the Genus Berylmys

1. Size small, occipitonasal length of skull less than 41 mm; diastema long, more than 33% of *onl*; upper surface of hind-foot including toes white.....**B. manipulus**
Skull large, occipitonasal length of skull more than 41 mm; diastema short, less than 33% on *onl*; upper surface of hind-foot brown, only toes white.....2
2. Occipitonasal length of skull is more than 52 mm, maxillary tooththrows more than 9.6 mm in length; mammae 4 pairs.....**B.**

bowersii

Occipitonasal length of skull is less 52 mm; maxillary tooththrows less than 9.6 mm in length; mammae 5 pairs.....**B. mackenziei**

***Berylmys manipulus* (Thomas, 1916) from Keinou, (N 24° 64.260", E 93° 78.000") Senapati District**

***Berylmys manipulus* (Thomas)**

1916. *Epimys manipulus* Thomas, *J. Bombay nat. Hist. Soc.*, **24**: 413 (Kabow Valley, Kindat, Myanmar).

1948. *Rattus manipulus kekrimus* Roonwal, *J. Bombay nat. Hist. Soc.*, **14**: 386 (Kekrima, Nagaland, India).

General characters: It is smallest of the Indian species of the genus *Berylmys*, having the tail equal to or longer than head and body length, body having dense, crisp, iron grey pelage above, and white below; distal one-third of tail white, rest brown; hind foot including the toes white (Figure 1 l, r and s).

Skull small, occipitonasal length less than 41 mm, having lengthened diastema (more than 33% on *onl*), smaller bulla (about 15% of *onl*) and smaller and narrower maxillary tooththrows (length 5.5 to 6.1 mm and width 1.8 to 2.0 mm).

The species *Berylmys berdmorei* with which *B. manipulus* was originally confused, differs from it by the length of tail which is smaller than head and body and its distal half brown instead of white, and large inflated bulla (more than 7mm or more than 18% of *onl*). Total length of the tail is 14.5 and white part is 9 cm and % of the white is 62.06%.

This rat is initially named as *Rattus manipulus manipulus* (Thomas, 1916) but according Musser and Newcomb (1983), it had changed to *Berylmys manipulus* (Thomas, 1916).

Cytology: The diploid count is 40 consisting of three subtelocentric pairs (# 1, 2, and 3), 8 acrocentric pairs (# 4-10, and 13) and one big metacentric pair (#11) that is formed by the Robertsonian fusion of chromosome numbers 11 and 12 of the ancestral Asian black rat *Rattus rattus*, 7 small metacentric pairs (# 14-20), the X chromosome is acrocentric which is 9th in length of the autosomes and Y chromosome is acrocentrics comparable to 13th of the autosomes (Figure 8).

***Berylmys mackenziei* (Thomas, 1916) from Kharam, Senapati District (N 24°81.880", E 93°49.930")**

1916. *Epimys mackenziei* Thomas, *J. Bombay nat. Hist. Soc.*, **24**: 410 (Haingyan ca. 80 km w. Kindat, Chin Hills, Myanmar).

Diagnostic characters: Smaller than *B. bowersii* in size (Figure 1a). This is reflected in length of head and body,

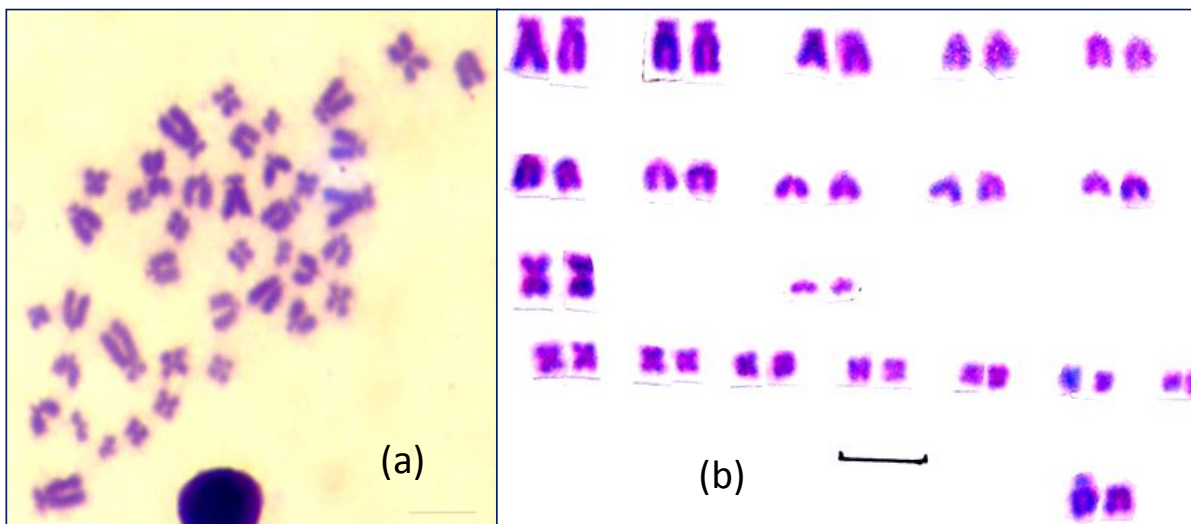


Figure 8. Metaphase a and karyotype b of *Berylmys manipulus*.

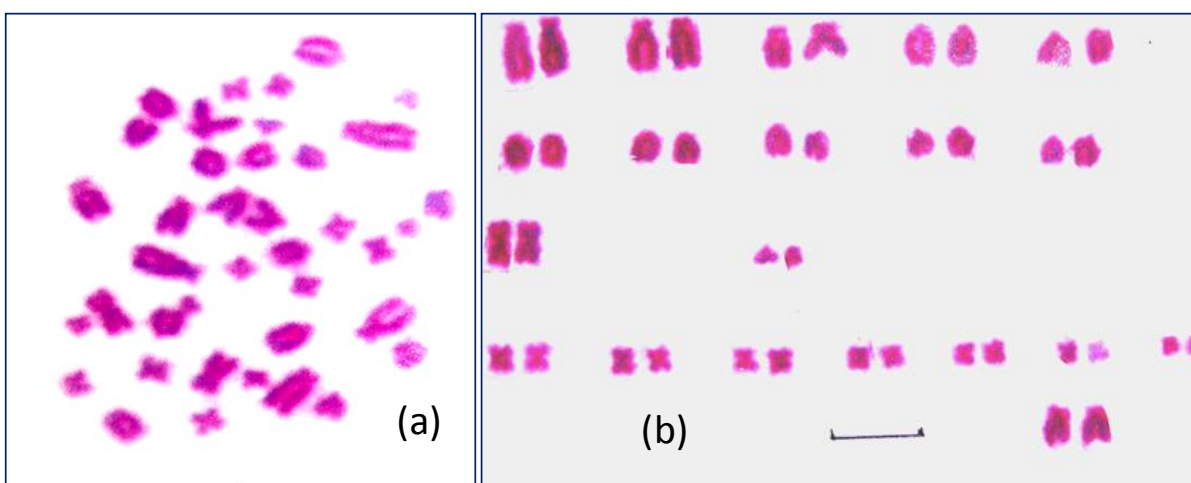


Figure 9. Metaphase a and karyotype b of *Berylmys mackenziei*.

hindfoot, occipitonasal and maxillary toothrows; bulla less than 15% of *onl*; diastema less than 31% of *onl* as in *bowersii* but shorter than *manipulus*. Body colour iron grey above, white below; distal one-third to one-half of tail white; hind foot brown, toes white. Mammae 5 pairs.

Cytology: This rat is initially named as *Rattus mackenziei* (Thomas, 1916) but according Musser and Newcomb (1983), it had changed to *Berylmys manipulus* (Thomas, 1916). The diploid count is 40 consisting of three subtelocentric pairs (# 1, 2, and 3), eight acrocentric pairs (# 4 to 10, and 13) and one big metacentric pair (#11) that is formed by the Robertsonian fusion of chromosome numbers 11 and 12 of the ancestral Asian black *Rattus rattus*, 7 small metacentric

pairs (# 14 to 20), the X chromosome is subtelocentric which is 6th in length of the autosomes and Y chromosome is acrocentric comparable to 9th of the autosomes (Figure 9).

The heterochromatic region as evidence from the C-banded pattern points the difference between *B. manipulus* and *B. mackenziei* (Figure 10a and b).

Soft palate of the species in the study: The soft palate photographs of different species were compared and it can be shown that the *Rattus rattus*, *R. tanezumi*, and *R. nitidus* have three chevron-shaped ridges while the *B. manipulus* has only four tapering ridges arising from the base of the molar teeth (Figure 1m to q).

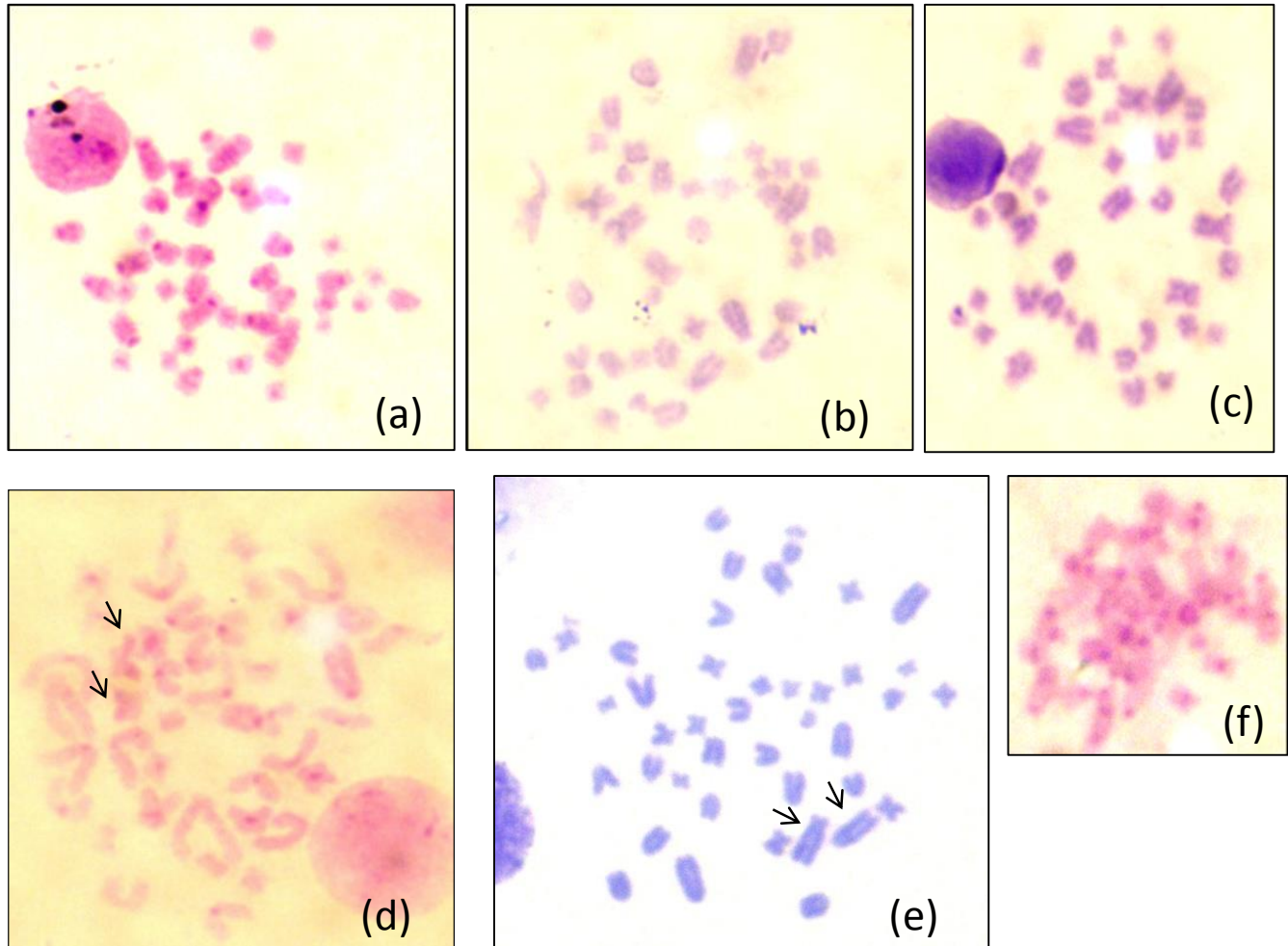


Figure 10. a-C-banded metaphase plate of *B. manipulus*, b-C-banded metaphase plate of *B. mackenziei*, c-G-banded metaphase plate of *B. manipulus*, d-C-banded metaphase plate of *R. nitidus* (heteochromatic q- arm of sutelocentric XX, e-heteromorphic #1 in *R. rattus* (arrowed, and f-small centromeric banding in *R. rattus*).

DISCUSSION

Ellerman and Morrison-scout (1951) classified *R. rattus* with black coat colour inhabiting the northern part of the world and *R. rattus alexandrinus* (Geof) with agouti coat colour inhabiting the southern part. Specimens of *Rattus rattus* species collected from hills have agouti as well as black colour while those from valley areas have agouti coat colour agreeing with the above two coat colours of this species. Tolga et al. (1998) reported four main colorations in this species. The first type, has a dark, slate blackish dorsal fur and greyish ventral fur, and is also referred to as *R. rattus rattus*. The second type, has dark brownish dorsal fur with yellowish white ventral fur, and is known as *R. rattus elaxandrinus*. The third type is dorsally the same as in the second type, but the ventral fur is light greyish and the upper sides of the feet are pale brownish, and it is referred to as *R. r. alexandrinus*. In these three types, the hair bases of dorsal and ventral

furs are greyish. The fourth type, which is dorsally similar to the second and the third types, has ventral fur that is pure white, and is referred to as *R. rattus frugivorus*. In the first type, the tail and the ear are uniformly covered with short, sparse blackish hairs, the dorsal colour grows paler toward the flanks, but the line of demarcation along the flanks is not very distinct. If available published data are for sure, the present specimen from the Senapati District might be *R. r. alexandrines* while the rats from Thoubal and Imphal West might be *R. rattus elaxandrinus*. The morphological features frequently lead to misidentification in the cryptic species and so the specimen in the present study should be re-examined in future to confirm the true identity through sequencing the mitochondrial COI.

The chromosome compliment of the *Rattus rattus* in the present study shows the polymorphic chromosome numbers of 1 (heteromorphic in Imphal West, homomorphic in the Imphal East), 9 (homomorphic in *Rattus brunneuculus*)

and 13 (heteromorphic in *Rattus brunneusculus*) are polymorphic in Manipur. Another polymorphic chromosome pair is number 10 (Imphal West) which is found in *Rattus bullocki*. Hence all the polymorphic chromosomes in the present study are one of the polymorphic chromosomes of the *Rattus rattus* as reported by Yoshida (1983) except pair number #10. Depending upon the length of the acrocentric and subtelocentrics in the study, it can be referred that the subtelocentrics were derived from the acrocentric counterparts through the pericentric inversion as reported by Yosida (1983) and Pages et al. (2011).

Gadi and Sharma (1983) reported $2n=42$ for *Rattus rattus*, consisting of 7 small metacentric, 3 subtelocentric, 10 telocentric pairs and telocentric X and Y chromosomes which is agree with the present work except for the number of the subtelocentric. The number of the subtelocentrics may vary due to the pericentric inversion. Wilson and Reeder (2005) synonymised *Rattus brunneusculus* (Hogson, 1945) with *Rattus tanezumi*. In Indian context too *Rattus brunneusculus* was reported from Manipur instead of the *Rattus tanezumi* (Alfred, 2005) and Ellerman (1961) from The Loktak Lake. Alfred reports that the *brunneusculus* had longer tail, being 123-131% of head and body length but in our study it is 90%. Again Ellerman (1961) synonymised it with *sikkimensis* and argued that the typical *brunneusculus* has the underparts yellowish, not very typical for a wild race. Besides this the characteristic feature of both *brunneusculus* and *sikkimensis* is "the bullae are usually less than 17% of the occipitonasal length" but in our study it is 19.51%. Therefore, it is yet to decide the true taxonomic position of *Rattus tanezumi* particularly in Indian context. A further study in involving the three species through morphology, cytology and COI sequence are much to resolve the taxonomic conflicts of these three species in future. Here in the present study the *Rattus brunneusculus* is quite different from the *tanezumi* in both morphological and cytological point of view. In *tanezumi* the chromosomes are telocentric in all the autosomal as well as the sex chromosomes bit in the *brunneusculus* the chromosome numbers 1 and 9 were homomorphic subtelocentrics but sex chromosomes are similar. So much work should be done to discriminate these two taxa. Out of the five types of the karyotypes recognised by Yosida (1980), the Manipuri *Rattus rattus* should be regarded as Japanese types- ($2n=42$, with low C-banding; *R. tanezumi*). This shows that the heterochromatin diminution is an effective mechanism in the evolution of the karyotypes that led to obvious differentiation among the species and in the speciation process.

Rattus nitidus is indigenous to mainland Southeast Asia and occurs in south China (including Hainan Island), Vietnam, Laos, northern Thailand, Burma, Bangladesh, Nepal, Bhutan, and Northern India; it is also found on the islands of central Sulawesi, Luzon island of the Philippines,

Pulau Seram in the Molluccas, the Vogelkop Peninsula of the Province of Papua, and the Palau Islands, probably due to human-mediated introductions (Aplin et al., 2003; Musser and Carleton, 2005). Li et al. (2008) and Gadi and Sharma (1983) reported the $2n=42$ of *Rattus nitidus* comprising of eight metacentric pairs, two subtelocentric pairs, and 10 acrocentric pairs as autosome compliments and acrocentrics XX from China. In the present study also the karyotype is consisting of ten acrocentric pairs (#2-8, 10-12), two subtelocentric pairs (#1, and 9), eight pairs of small metacentric pairs (# 13, 14-20) and acrocentric X and Y. Hence the Manipur *Rattus nitidus* is quite similar to the Chinese species in the autosomal compliments but the heterochromatic q-arms in the X-chromosomes are yet to be reported from the other part of the world.

According to Yosida (1983) the Asian black rat with 42 chromosomes is the ancestor of the Ceylonese black rat with 40 chromosomes and the Oceanian black rat with 38 chromosomes. He proposed that 40 chromosomes (*Rattus rattus kandianus* in the Sri Lanka, *Berylmys manipulus* and *Berylmys mackenziei* of present studies) and 38 chromosome karyotypes evolved from 42 chromosome karyotype through a first Robertsonian fusion of acrocentric pairs 11 and 12 and a second Robertsonian fusion between acrocentric pairs 4 and 7. It is considered that the progenitors of the members of the genus *Rattus* originally had acrocentric karyotype and the subtelocentric and metacentric members were derived by pericentric inversion of the acrocentric chromosomes (Yosida, 1983). Manipur is one of gateways of South Asian to the Indian continent, the ancestral *Rattus rattus* might have passed through, hybridized here and result into diversity of Black rats. The first Robertsonian fusion might had formed here in the Indo-Burma areas and travel through up to Western India and might have occurred there the second Robertsonian fusion and formed the rats with $2n=38$. But the comparative studies of the softpalate shows other facts; these structures having three chevron-shaped ridges are present in the different species of the genus *Rattus* but the fingers like soft palate ridges of the *Berylmys* are not at all related to the genus *Rattus*. The features are quite common in the genus *Niviventer* and morphology too except for the pure white tail tips. The only karyotypic similarity between *Rattus* and *Berylmys* is the 14 metacentric chromosomes and differ in the number of the subtelocentrics. Considering the differences in autosomal chromosomes and soft palate, there is not much identical in these two genera, but much works should take up involving the two genera in future.

Phylogeny of the present study begins as *Rattus sabanus* an ancestral karyotype and divergent karyotypes were obtained due to per-centric inversion of acrocentric chromosomes to subtelocentrics and much deviated karyotypes were seen among the different species of the genus *Rattus* in Manipur (Figure 11). Here

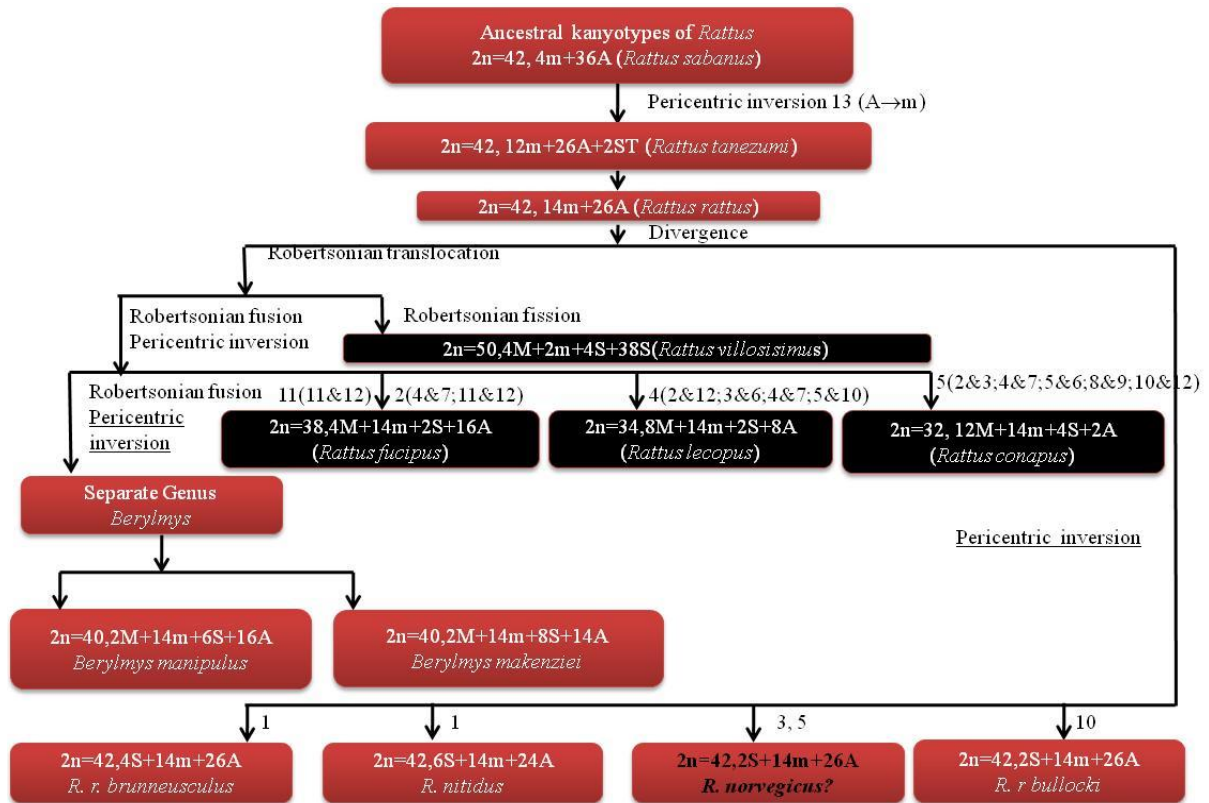


Figure 11. Phylogeny of the genus *Rattus* (without sex chromosomes), involving pericentric inversion and Robertsonian fusion S=subtelocentric, M=large metacentric, m=small metacentric, A=acrocentric, numeral represents the chromosome numbers, black ones are of other authors.

the number of the meta-centric is the reference points to decide the primitiveness of the species. In *Rattus tanezumi* the number of metacentric chromosomes is 12 and rest of the *Rattus* species are 14 in number. The number of subtelocentrics not considered in the study.

The summary of the present study can be as follows:

1. The autosomal chromosome compliments of different species of the genus *Rattus* quite agree with the published data.
2. The chromosomes compliments of different species of the genus *Rattus* are one of the polymorphic forms of the *Rattus rattus*.
3. The studies of the species reveal that the polymorphic chromosomes in the genus *Rattus* were pair numbers 1, 9, 10 and 13.
4. The cytological studies of *Berylmys* species ($2n=40$) as reported by other authors need to be re-evaluated particularly in the Manipur rats.
5. The Manipur rats are tanezumi type ($2n=42$) not the *Rattus* type ($2n=38$) in chromosomal basis.
6. Change in single chromosome leads to differentiation of new species.
7. The biometrics such as morphotaxonomy, cytotaxonomy and molecular techniques are required

for identification of taxon.

Conflict of Interests

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

ACKNOWLEDGEMENTS

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Full Length Research Paper

Identifying salt stress-responsive transcripts from Roselle (*Hibiscus sabdariffa* L.) roots by differential display

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No previous study has been reported on the salt-modulated gene(s) of roselle (*Hibiscus sabdariffa* L.). Identifying the potentially novel transcripts responsible for salt stress tolerance in roselle will increase knowledge of the molecular mechanism underlying salt stress responses. In this study, differential display reverse transcriptase PCR (DDRT-PCR) was used to compare the overall differences in gene expression between salt-stressed and control plants. A total of 81 primer combinations were used and false positive clones were rejected during a screening and quality control assay. The remaining nine cDNA transcript fragments were extracted from the gel, reamplified, cloned and sequenced. A homology search revealed that four transcripts showed significant homology with known genes. Out of five transcripts, real-time PCR demonstrated that four exhibited high expression in salt-stressed root tissues relative to the control and one transcript was down-regulated. These transcripts may be useful for improving tolerance in salt stress-sensitive plants.

Key words: Roselle, *Hibiscus Sabdariffa* L., differential display, salt-stress, differentially expressed transcripts, signal transduction.

INTRODUCTION

Abiotic stresses such as high salinity, drought, extreme temperature and flooding are the main causes of crop loss worldwide, causing a reduction of more than 50% in

the average crop yield, particularly in developing countries (Bray et al., 2000). Because plants are sessile organisms, they are continuously exposed to environ-

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Abbreviations: DDRT-PCR, Differential display reverse transcriptase polymerase chain reaction, ESTs, expressed sequence tags; PCR, polymerase chain reaction.

mental changes. Plants have evolved effective mechanisms to reduce possible damage (Loredana et al., 2011).

Salinity is considered a universal problem that affects approximately 20% of global irrigated cultivated land (Flowers and Yeo, 1995). A survey conducted by FAO indicated that more than 800 million hectares of land are affected by salt worldwide (FAO, 2008). This amount is equal to more than 6% of the world's total land area (Munns and Tester, 2008). Extreme salinity is a critical environmental factor that inimically affects large agricultural land areas. Plant growth, physiological processes and metabolic processes are all affected (Magome et al., 2008; Zhang et al., 2009). High salt levels cause ionic stress in the form of cellular Cl^- accumulation and especially Na^+ ion accumulation. Salt stress also changes the homeostasis of other ions such as Ca^{2+} , K^+ , and NO_3^- (Loredana et al., 2011).

Roselle (*Hibiscus sabdariffa* L.) is an important annual herbaceous shrub belonging to the family Malvaceae that is locally known as "karkade" and grows well in tropical and sub-tropical climates (Cobley and Steele, 1976). Roselle may have been domesticated in western Sudan before 4000 BC (Wilson and Menzel, 1964).

No information is available about possible signal transduction pathways related to abiotic stress in Roselle roots. There are two aspects of salt stress: osmotic stress and ionic stress. As soon as plants are subjected to salt stress, osmotic stress had a major effect on water regulation; continuous ionic stress and osmotic stress reduces plant productivity. To acquire salt tolerance, plants must adapt to both stresses (Ueda et al., 2002).

In this study, differential display reverse transcriptase PCR (DDRT-PCR) was used to identify and isolate salt-induced transcripts from Roselle roots under salt stress. A number of salt stress-responsive transcripts were isolated that had not been previously reported in association with salt (NaCl) stress, providing a preliminary step for identifying and characterizing novel gene(s) with regard to their regulatory elements to provide an understanding of plant adaptations to salt stress conditions.

MATERIALS AND METHODS

Plant material and stress treatment

Roselle (*Hibiscus sabdariffa* L.) seeds were obtained from the Agricultural Research Corporation's Obeid Research Substation in Sudan. Plants were grown in composite soil (peat, sand, soil, 1:1:1) at the Centre of Excellence in Molecular Biology (CEMB) greenhouse at $30^\circ\text{C} \pm 2$ under relative humidity greater than 40%.

A concentrated NaCl treatment was given to the seedlings to their molecular responses to salt stress could be monitored. After 24 h, root samples from treated and control plants were collected, immediately immersed in liquid nitrogen and stored at -80°C for further use (Mohamed et al., 2013). Total RNA was isolated from frozen root samples using a modified CTAB method (Muoki et al., 2012). Agarose gel electrophoresis was used to test the integrity

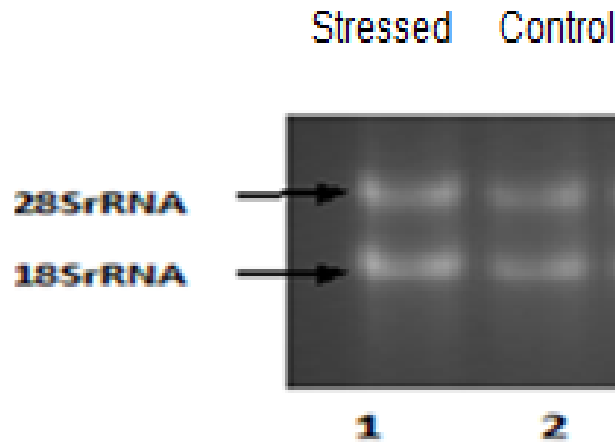


Figure 1. Total RNA extracted from *H. sabdariffa* roots on a 0.8% (w/v) agarose gel. Lane 1, RNA isolated from the control; lane 2, RNA isolated from a salt-stressed plant.

and purity of the total RNA (Figure 1).

Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR)

Total RNA was isolated, and 2 μg of RNA was reverse-transcribed with an anchored oligo-dT primer by using a RevertAid H- First Strand cDNA Synthesis Kit (Fermentas, USA). cDNAs from different samples were stored at -20°C for differential display PCR amplifications.

A total of 9 anchored and 9 arbitrary primers were used (Table 1). A DDRT-PCR reaction was performed in a 25 μL volume containing 0.5 μL of Taq Polymerase (Invitrogen), 1 μL of arbitrary primer, 1 μL of anchored primer, 0.05 mM dNTPs, 2.5 μL of 10 \times PCR buffer, 1 μL of cDNA, and 1 μL of MgCl_2 . The cycling conditions were as follows: an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 42°C for 1.30 min, extension at 72°C for 45 s, and a final elongation step at 72°C for 10 min.

The 10 μL PCR products were separated vertically on denaturing 16% polyacrylamide gels with 5 \times TBE buffer at 180 V for 150 min and stained with silver (Bassam et al., 1991). A 50 bp DNA ladder was used to estimate the transcript sizes. The polyacrylamide gel was silver-stained according to the Bio-Rad silver stain handbook. The gel was placed in fixation solution (40% ethanol and 10% glacial acetic acid v/v) for 20 min. The gel was then washed 3 times with dH_2O and stained with silver staining solution (0.2% AgNO_3) for 20 min. The gel was again washed 3 times with dH_2O for 20 s, and developing fluid (3% NaOH and 0.05% formaldehyde) was applied for 3-5 min. The gel was visualized by GrabIT v2.5 Trans-illuminator software on a gel documenting system (Ultra-Violet Products). Reamplification and confirmation of the differentially expressed transcripts were also completed.

Cloning and sequence analysis of cDNA fragments

Selected amplified cDNA fragments were excised and extracted from the gel using the crush and soak method employed by Maqbool et al. (2008). Twenty-five microliters of PCR product was reamplified using the same set of primers that generated the differential display product. For each reamplified band, a quality control without reverse transcriptase was added to monitor for

Table 1. Random primer sequences for differential display RT-PCR.

Arbitrary primer	Sequences	Anchored primer	Sequence
P1	5'-ATTAACCCTCACTAAATGCTGGGGA-3'	T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
P2	5'-ATTAACCCTCACTAAATCGGTCATAG-3'	T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'	T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'	T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'	T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'	T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'	T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'	T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'	T9	5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

Table 2. Primer sequences for real-time PCR.

Transcript	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon length (bp)
Baha-cemb01	GACTGTGGCGCAAATGAAG	AGATGCTCCCAAATTTCTGC	180
Baha-cemb02	CTCAAGCCATCATCGGTAATCT	GGGACGTGGTCAGAAGTGATAC	169
Baha-cemb03	CTAGAAGATGATGGTCCGGTTG	CGATTGAGTAGGCACCGAAGT	180
Baha-cemb04	AGAAGGGGTACCAGACCTTGAT	GGTGTGAACCAGGGTATGTCT	175
Baha-cemb09	AAAGGACCTACATTTGCAGGA	AGGAATGGCTTCCAACCTGA	164
β- Actin	TGGGGCTACTCTCAAAGGGTTG	TGAGAAATTGCTGAAGCCGAAA	162

potential RNA contamination by residual genomic DNA, which could be amplified during the subsequent PCR. Reamplified PCR products were separated on an agarose gel (Maqbool et al., 2008). Reamplified eluted DNA fragments were cloned into a pCR 2.1 vector with a TA Cloning Kit (Invitrogen, USA), and the vector was transformed into an *E. coli* strain. Plasmid DNA was isolated from a minimum of five clones per transformation (Bimboim and Doly, 1979), and their aliquots were treated with RNAase A (Sigma, Germany). The positive clones containing inserts were confirmed via a restriction digestion of plasmid DNA with EcoR1 (Fermentas, USA). The digested product was run on a 1.5 % agarose gel at 80 V for 90 min and analyzed under UV light.

DNA Sequencing and data analysis

Sequencing reactions were performed with an ABI Prism Dye Terminator Kit and an ABI model 3100 automated DNA sequencer. Transcripts were sequenced on both strands with M13 primers. The nucleotide sequences or the deduced amino acid sequences of each clone were compared by using ESTs and protein sequences from various databases through the basic local alignment search tool (BLAST) (Altschul et al., 1990).

Semi-quantitative RT-PCR analysis

RT-PCR reactions were performed with pairs of specific primers for each transcript that were designed on the basis of the *H. sabdariffa* cDNA sequence (Table 2). β-actin was used as a housekeeping control. The thermal cycling conditions were 3 min of initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 45 s and a final extension at 72°C for 10 min.

Quantitative real-time RT-PCR

Real-time PCR reactions were carried out in an ABI 7500 system (Applied Biosystems, USA) with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, USA). β-actin was used as a housekeeping control to normalize the data. Fifty nanograms of cDNA were used in each reaction. Real-time PCR was programmed for denaturation at 95°C for 3 min and 95°C for 30 s, followed by annealing at 55°C for 30 s, extension at 72°C for 45 s and additional 40 cycles were repeated until the annealing phase. Each reaction was performed in triplicate. The relative gene expression study was conducted using SDS v3.1 software (Applied Biosystems, USA).

RESULTS AND DISCUSSION

Differential display is a molecular biology technique described by Liang and Pardee (1992). It is an efficient method for identifying and isolating differentially expressed genes in specific cells or under altered conditions, and it presents numerous advantages: it is fast and saves time; it produces band patterns in 2 days; it is a simple, well-established and widely accessible technique, making it easily applicable for most investigators; in comparison with prior methods, its sensitivity has increased dramatically, resulting in good detection of low-abundance genes; and both induced and repressed genes can be detected and only a small amount of starting material is needed (Liang and Pardee, 1992).

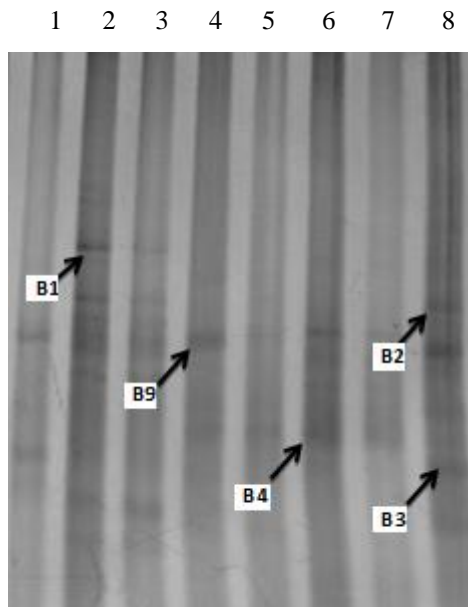


Figure 2. Differential display (DDRT-PCR) from *Hibiscus sabdariffa* roots. Odd-numbered lanes represent the control sample, and even-numbered lanes represent salt-stressed samples. RT-PCR reactions were conducted by using anchored and arbitrary primers (Table 1). cDNA fragments that appeared to be differentially expressed in treated samples are indicated by arrows (lane 2: B9; P8B2-3 417 bp, lane 4: B1; P5T8-a 591bp, lane 6: B2; P10T8 545 bp and B3; 255 bp, lane 8: B4; P5T7-a).

Since 1992, analyzing gene expression has been the primary goal of most molecular biology studies (Liang and Pardee, 1992). In this study, mRNA differential displays and quantitative RT-PCR were used to study Roselle responses to salt stress. The differential display method reveals all aspects of regulation (up and down), as well as the absence/presence of bands to suggest qualitative differences, and it reveals signals with varying intensity, suggesting quantitative differences (Voelckel and Baldwin, 2003).

Nine arbitrary and 9 anchored primer pair combinations were used; 9 transcripts were found, and 4 were repressed by salt stress. The banding pattern of cDNA fragments was amplified by the combination of one primer group, as shown in Table 1. To reduce the false positive rate of the mRNA differential display, each primer pair was used to amplify two different sets of RNA isolated from Roselle plants (Lang et al., 2005).

A GenBank database search revealed that four fragments, with sizes ranging from 200-600 bp (Figure 2), presented significant homologies with known genes (Figure 3). The other cDNA fragments showed no homology to known genes. The expression level of five transcripts was evaluated by real-time RT-PCR. The β -

actin gene was used as a reference gene to normalize expression levels. The results indicate that all transcripts were overexpressed at different levels in salt-stressed roots compared to the control, with the exception of B1, which was down-regulated.

Many salt stress-activated transcripts have been reported in other plant species (Ouyang et al., 2007; Shahid et al., 2012; Wei et al., 2013). The five transcripts exhibiting different expression levels in response to salt stress, transcripts B2 and B3, were similar and showed two-fold higher expression than the control (Figure 4). All of these transcripts were submitted to the NCBI GenBank EST database, and their accession numbers are given in Table 3.

A transcript named Baha-CEMB01 (B1) has high homology with the F-box *Arabidopsis* protein family (Table 3); it has homology to the *Vitis vinifera* cultivar Danuta VINE-1 repeat element gag-pol polyprotein. This transcript was down-regulated under real-time PCR.

A transcript named Baha-CEMB02 (B2) has high homology with the F-box *Arabidopsis* family of proteins (Table 3). The F-box protein family is involved in multiple signaling pathways for regulating root growth; the *F-box protein gene reduces abiotic stress tolerance and promotes root growth in rice* (Yan et al., 2011). The F-box protein family in eukaryotes plays important roles in plant development and abiotic stress responses via the ubiquitin pathway (Bai et al., 1996; Jia et al., 2011).

Transcripts Baha-CEMB 03 (B3) is homologous with a putative serine/threonine protein kinase (*Ipomoea batatas*) (Table 3). *SAPK4* improved germination, growth and development under salt stress in both seedlings and mature plants. *SAPK4*-overexpressing rice accumulated less Na^+ and Cl^- and showed improved photosynthesis in response to salt stress (Diédhiou et al., 2008). In plants, a salt-induced mitogen-activated protein kinase (MAPK) has been identified from alfalfa with a SIMK that is activated by the MAPK kinase SIMKK, and MAPK involvement in osmotic stress signaling has been demonstrated in tobacco and *A. thaliana* (Sanz, 2003; Slocombe et al., 2002).

The Baha-CEMB 04 (B4) transcript has homology with the putative retrotransposon Ty3-gypsy protein subclass (*Oryza sativa* Japonica Group). The transcriptional activation of several well-characterized plant retrotransposons appears to be tightly linked to molecular pathways activated by stress (Grandbastien, 1998). The Baha-CEMB 09 (B9) transcript has no significant homology with any protein.

Transcripts known as Baha-CEMB02 (B2), Baha-CEMB03 (B3) and Baha-CEMB04 (B4) were identified in Roselle (*Hibiscus sabdariffa* var. *sabdariffa* L.), and they shed light on responsive mechanisms to salt acclimation. Identifying these stress-regulated transcripts is an initial step towards cloning and characterizing full-length cDNAs and promoter regions.

This study is the first attempt to investigate the

(a)

Score	Expect	Method	Identities	Positives	Gaps	Frame
115 bits(289)	2e-41	Compositional matrix adjust.	67/151(44%)	96/151(63%)	24/151(15%)	+3
Query 189	KTKSMKVINLLCEFEMKKMKESDLVKEDAELVKIADKVRMLGKKLFNKKIVEKILVTLTP					368
Sbjct 107	+ K+M+V+NL+ EFEMKKM+ESD VK+ A +L+ IA+KVR+L GK+ N+KIV+KILVTLTP					166
Query 369	ENYEPTISSELENSKDLSSMTLEEL*NSFAGFKN*DTHEKREFYRRSissssqigkqskqHL-					545
Sbjct 167	+ YE TISSLENSKDLs+++L EL +S E+R R+ ++ +++ H					220
Query 546	-----YQYGSYPLCPHCKK					587
Sbjct 221	+ G +P CPHC+K					251

(b)

Score	Expect	Method	Identities	Positives	Gaps	Frame
149 bits(375)	2e-39	Compositional matrix adjust.	74/91(81%)	81/91(89%)	0/91(0%)	-1
Query 539	FFALGYLGVKDLLAVERVCRSLRDVVRSDTLLWRSIHIEHSLSRITNDALLKLTNRARG					360
Sbjct 158	FFALGYLGVKDLLAVERVCRSLRD VR D LLWRSIHIE +LSRRI +D LLKLT RA+G					217
Query 359	TLECLSLPGCIKITDDGLRRVFESNPKLTKV					267
Sbjct 218	TLECLSL GC+KITDDGL+RV ESN +LTK+					248

(c)

Score	Expect	Method	Identities	Positives	Gaps	Frame
127 bits(318)	3e-34	Compositional matrix adjust.	61/61(100%)	61/61(100%)	0/61(0%)	+3
Query 12	LKHRPRMSEVLRMLEDDGVPVASQPVETPRSRSCDNTPPQRYSDFIEESSLVMEAMELSGP					191
Sbjct 169	LKHRPRMSEVLRMLEDDGVPVASQPVETPRSRSCDNTPPQRYSDFIEESSLVMEAMELSGP					228
Query 192	R	194				
Sbjct 229	R	229				

(d)

Score	Expect	Method	Identities	Positives	Gaps	Frame
125 bits(315)	2e-32	Compositional matrix adjust.	58/107(54%)	82/107(76%)	0/107(0%)	+2
Query 20	LTADRMIKKGYQTLIATIWNTRGSLSRIEEIPVVEEFSDFVQEELPGLPPDRDVEF*IET					199
Sbjct 45	+ A +++KGY I +A + +T ++E++P+V EF DVF ++LPGLPPDR++EF I+					104
Query 200	YPGSTPISMAYRIAPKEYKELKVQL*ELLDQGFIRPSTSPWGA AVL					340
Sbjct 105	PG+ PIS+ YR+AP E KELKVQL EL+D+GFIRPS SPWGA +L					151

Figure 3. Amino acid sequence homology of 4 clones with known genes. The deduced amino acid sequences were aligned using NCBI BLAST pairwise alignment algorithm programs (<http://www.ncbi.nlm.nih.gov/BLAST/>). **(a)** Transcript Baha-cemb01 showing homology with gag-pol polyprotein [*Vitis vinifera*] Sequence ID: gb|AAF20282.1|Length: 581. **(b)** Transcript Baha-cemb02 showing homology with Ribonuclease inhibitor, putative isoform 2 (*Theobroma cacao*) Sequence ID: gb|EOY02666.1|. **(c)** Transcript Baha-cemb03 showing homology with Myeloblastosis protein (*Hibiscus sabdariffa*) Sequence ID: gb|AGC92177.1|. **(d)** Transcript Baha-cemb04 showing homology with Retrotransposon protein [*Theobroma cacao*] Sequence ID: gb|EOX94090.1|

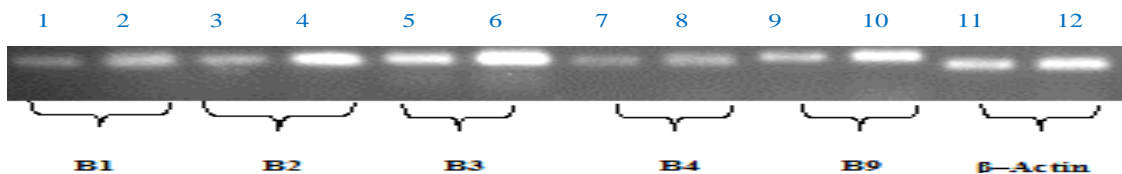
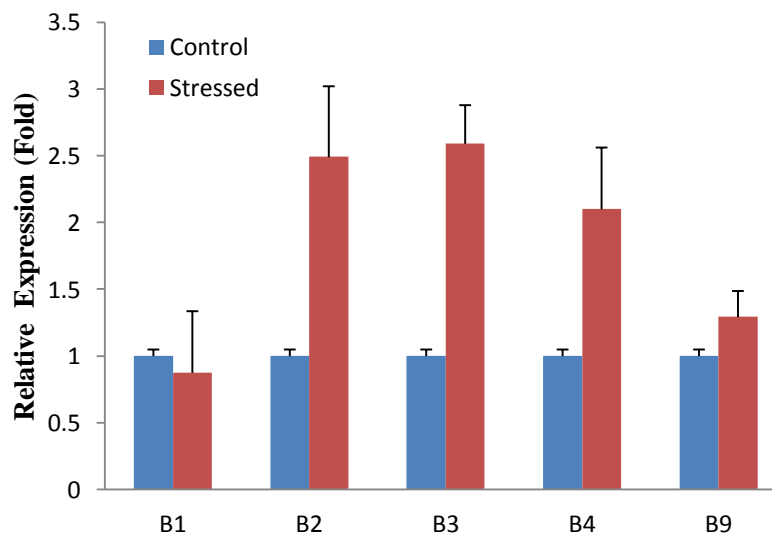


Figure 4. Reverse transcriptase PCR expression analysis of Roselle transcripts in salt-stressed and control roots. Odd numbers indicate control samples, and even numbers indicate salt-stressed samples. The β -actin gene was used as a housekeeping control; products for each sample were separated on a 1.5% (w/v) agarose gel.

Table 3. Clone identification and homologies with known genes.

EST	Gene bank accession#	Length (bp)	Homology
Baha-cemb01	JK757799.1	591	<i>Vitisvinifera</i> cultivar Danuta VINE-1 repeat element gag-pol polyprotein gene, Sequence ID: gb AF116598.1
Baha-CEMB02	JK757800.1	545	<i>Glycine max</i> F-box protein SKIP14-like (LOC100807460), mRNA Sequence ID: ref XM_003532664.1
Baha-CEMB03	JK757801.1	255	Myeloblastosis protein [<i>Hibiscus sabdariffa</i>] Sequence ID: gb AGC92177.1
Baha-CEMB04	JK757802.1	340	<i>Gossypiumhirsutum</i> retrotransposon putative copia, transposon GORGE3-like, Sequence ID: gb EF457753.1
Baha-cemb09	JZ152799.1	417	No significant similarity found

**Figure 5.** Relative expressions of root sample transcripts under control and salt-stressed conditions. β -actin was used as an internal control (housekeeping gene), and gene expression was indicated as a fold increase relative to the same transcript under control conditions.

expression levels of roselle transcripts under salt stress by using quantitative real-time PCR. The β -actin gene (DQ866836.1) of Kenaf (*Hibiscus cannabinus*) actin was used as an internal control. Selected transcripts were overexpressed in the Roselle roots under salt-stressed conditions relative to the control roots (Figures 4 and 5). Transcript B1 was down-regulated, whereas transcripts (B2 and B3) were 1.5-fold higher than those of the control. B4 expression was one-fold higher, and B9 exhibited lower expression. All of these transcripts were submitted to the NCBI GenBank database (Table 3). The regulation of gene expression is one key plant phenomenon used to respond and adapt to salt stress conditions (Jamil et al., 2011).

This study was the first to detect differentially expressed transcripts by applying the differentially expressed mRNA technique to Roselle plants under salt

stress and normal conditions. The three identified cDNA transcripts exhibited a higher level of expression under salt stress. These results indicate that *Hibiscus sabdariffa* variety bulk AIRahad is competent enough to up-regulate some specific genes during salt stress, and these findings are of immense importance for selecting Roselle lines with salt stress-tolerant characteristics.

Conflict of Interests

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

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Full Length Research Paper

Quantification of the gene expression of bell peppers (*Capsicum annuum*) ripening gene(s) using real-time PCR

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Fruits can be divided into two groups according to the regulatory mechanisms underlying their ripening process. The two ripening processes are climacteric and non-climacteric process; bell peppers are part of the non-climacteric plant groups. Bell peppers are members of the *Solanaceae* family. The *Solanaceae* family is best known for its fruits around the world. Today's main focus is targeted to fruit ripening, in an attempt to increase the fruit's shelf life. Many genes have been linked to the maturation of the fruit such as in *Arabidopsis*, the genes found were elongation factor-1 α (LeEF-1A), expansin protein (leEXP1) and ripening inhibitor (RIN). This research focused on discovering similar genes that may play an important role in the ripening of peppers. Real-time PCR was performed on the cDNA of the green bell pepper fruit during its stage of development in order to detect and identify the expression pattern of the gene. Through the comparison of the gene expression found in bell pepper and the pods of *Arabidopsis* as model to dicotyledonous plant, some variations have been detected.

Key words: Bell pepper (*Capsicum annuum*), *Arabidopsis* (*Lycopersicon esculentum*), fruit ripening, expansin gene (EXP1), elongation factor alpha gene (EF-1a), ripening inhibitor (RIN), MADs box, complementary DNA (cDNA), gene expression profiling, real-time PCR.

INTRODUCTION

Pepper (*Capsicum spp.*) is one of the most important cultivated vegetables around the world. It is a member of the *Solanaceae* family that contains almost 2500 species; most of them are edible which gives it a countable economic value (Wien, 1999). One of these species is the bell pepper (*Capsicum annuum*). A group of genes belonging to the pepper plant had been published by Wang and Bosland (2006). Those genes control the qualities, the hereditary foundation of the mutants/lines, activity

mechanisms of genes, gene linkage, molecular markers, and chromosome localization when accessible. The edible part of the pepper plant is the fruit. Therefore, studying its ripening process of fruiting is very important. The biochemical components help in the variability development among species that include: adjustment of colors (of chlorophyll, carotenoid, or flavonoid aggregation), textural alteration through variation of cell turgor and cell wall structure and/or synthesis, alterations of sugars, acids and

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unpredictable profiles that influence nutritious quality, flavor and smell, in addition it improves weaknesses to pathogens. Distinctive types of foods grown from the ground are physiologically characterized by the vicinity (climacteric) or nonattendance (non-climacteric) of expanded breath and amalgamation of the vaporous hormone ethylene at the start of maturation (Lelievre et al., 1997). Therefore, studying this process on a molecular basis may provide better understanding for the fruiting development stages. Constrained data have been published on the likenesses and contrasts between climacteric and non-climacteric fruits on transcriptional and expression levels of the responsible genes. Fruit ripening process is closely similar in both types. The bell pepper is a non-climacteric fruit. The discovery of these genes aided in pointing out the purpose of the maturing components preservation during fruit development. The majority of developmental genes that encode for the ripening process are just regulated in different stages of ripening (Lee et al., 2010). Regardless of the advancement made in Arabidopsis as a model dicot plant, late discoveries demonstrated that flowering and fruit (silique) formation in Arabidopsis are close to those of other plant species (Quinet et al., 2006; Carrari et al., 2007).

The outflow profiles accessed 1100 novel Arabidopsis genes coding for known and putative transcription elements (Tfs) throughout silique improvement through utilizing microarray hybridization. Various leveled bunch investigates uncovered unique expression profiles for the diverse silique developmental stages (De Folter et al., 2004). Many genes discovered that are linked to the fruit ripening in the tomato included the elongation factor-1 alpha (EF-1a), ripening inhibitor (RIN) and expansin proteins (EXP1). These genes are expected to play a major role in most of fruit ripening processes with the majority of the flowering plants (Powell et al., 2003).

MATERIALS AND METHODS

Total RNA extraction

According to Cathala et al. (1983), first mature bell pepper fruit and Arabidopsis samples were homogenized with ice cold extraction buffer [2% hexadecyltrimethyl ammonium bromide (CTAB), 2% polyvinyl pyrrolidone K 30 (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, and 0.5 g/L spermidine] and β -mercaptoethanol. The samples were incubated at -20°C for 30 min then centrifuged at 8000 round per min (rpm) in 4°C to obtain the supernatant of the mixture that contained the nucleic acids (both DNA and RNA). To make sure that all residues were removed from the supernatant except of the nucleic acids, phenol, chloroform and proteinase K were added. The supernatant was shook briefly to allow the extraction through the supernatant and then incubated at room temperature for 30 min. The supernatant was spun at 8500 rpm for 20 min at 4°C and the aqueous layer was removed to other clean tubes by pasture pipette. After that the precipitation buffer (4 M lithium chloride, 100 mM Tris-HCl, 10 mM EDTA) was added and incubated for 3 h at -70°C, where then the supernatant was centrifuged at 12000 rpm for 30 min in 4°C. Finally, the supernatant was discarded and the RNA pellet was dissolved in 100 μ l TE

buffer. The RNA quality was displayed on 1.2% agarose formaldehyde gel electrophoresis at 65 V for 50 min. The rest of the RNA samples were stored at -70°C. In spite of the low content of the mRNA (1% of the total RNA), identification of the transcript of any gene could be done using reverse transcriptase to synthesis a cDNA of the targeted genes. Double stranded cDNAs were synthesized using SuperScript® III Reverse Transcriptase kit (Life technology™). Choosing the correct reverse transcriptase (RT) for cDNA synthesis is critical for obtaining high yields of quality full-length cDNA that accurately represents the input RNA of the bell pepper fruits and Arabidopsis siliques. The primers used for the three ripening-regulated cDNAs were EF-1a with the forward as 'CAGAACGTGAGCGTGGTATCA' and the reverse as 'CAGTTGGGTCCTTCTTGTCAA'; EXP1 with the forward as 'ATGGGTATCATAATTTTCAT' and the reverse as 'AGGTAGAAGATCGATGGTCA'; and RIN gene with the forward as 'GTGGAAATGTTACAACAG' and the reverse as 'TAGGCAATGTATTATTGC'. The cDNA was then amplified exponentially by polymerase chain reaction (PCR).

The amplified transcripts had been cloned using the pGEM®-T vector. A partial-length sequence of the candidate fragments was achieved using an automated DNA sequencer (ABI 377, Perkin Elmer®/ABI, Foster City, CA) using the T7 universal primer. The partial-length sequences were analyzed using the National Center for Biotechnology Information (NCBI), followed by pairwise alignment with the Arabidopsis transcripts using the online CLUSTAL W2 software to compare both sequences: www.ebi.ac.uk/Tools/msa/clustalw2/.

Quantification of the targeted genes using the real-time PCR

Several variables need to be controlled for gene-expression analysis, such as the amount of initial material, enzymatic efficiencies, and the differences between tissues or cells in overall transcriptional activity. For the conduction of the Real-time PCR, this analysis was done using a kinetic PCR instrument (ABI PRISM® 7900 Sequence Detection System "SDS"); The SYBR® Green PCR master Mix (PE Applied Biosystems®, Foster City, CA, USA). The reaction contained cDNA, 10 nM of the specific primers of the investigated genes, SYBR® Green PCR Master Mix, and deionized water. The results were analyzed using ABI PRISM® SDS software (version 2.0), and using the comparative threshold cycle (CT) method ($\Delta\Delta$ CT) for calculations. For data normalization, 18S rRNA gene was used as internal control by the following primers; forward (AGTCATCAGCTCGCGTTGACT) and the reverse 'ACGGGCGGTGTGTACAAAG'. The target amount was normalized to an endogenous reference and relative to a calibrator (fold differences), is given by $2^{-\Delta\Delta$ CT. Using the expression level of target genes in the control tissue as a base line, the expression folds have been detected in the treated ones assuming that both standard and target have same efficiencies (Molestina and Sinai, 2005). Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold in order to convert threshold cycles in copy numbers.

RESULTS

The total RNA of the bell pepper fruits and Arabidopsis siliques were isolated, and visualized on 1.2% formaldehyde agarose gel electrophoresis (Figure 1a). RT-PCR was applied in order to determine expression of the genes under investigation. The RT-PCR products were displayed on 1.2% formaldehyde agarose gel electrophoresis

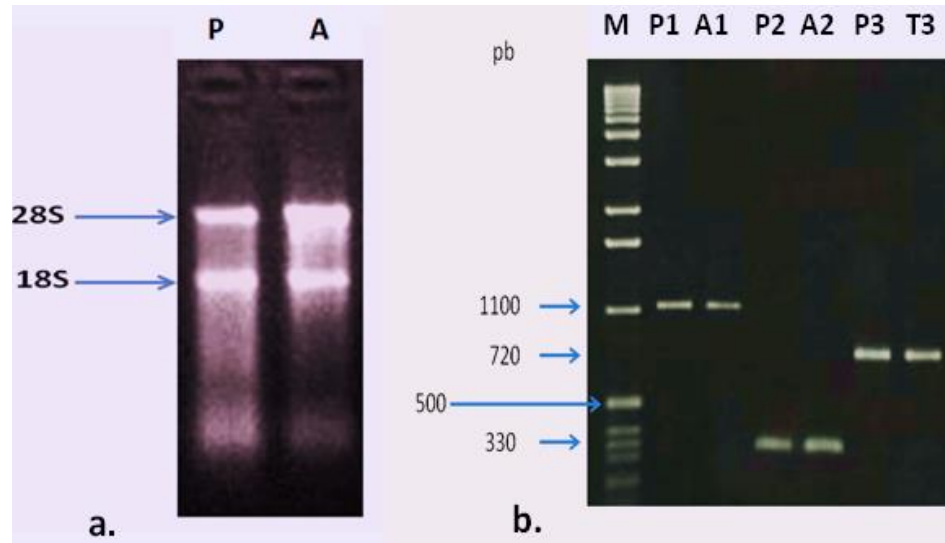


Figure 1. **a.** The 1.2% formaldehyde agarose gel electrophoresis analysis of total RNA of bell pepper and Arabidopsis silique: Lane (P) represents the total RNA of bell pepper and lane (A) represents the total RNA of Arabidopsis. **b.** 1.2% agarose gel electrophoresis analysis for bell pepper fruits and Arabidopsis silique ripening genes (EF-1a, RIN& EXP1). Lane M represents the 100 bp DNA marker which indicates the length of the amplified fragments, lane (P1) shows the expression of EF-1a in bell pepper, lane A1 displays the EF-1a expression in Arabidopsis, lane P2 shows the expression of RIN in bell pepper, lane A2 illustrates the expression of RIN in Arabidopsis, while lane P3 and A3 display the expression of Exp1 in bell pepper and Arabidopsis respectively.

(Figure 1b). The amplification of the three genes fragments were accurate, distinguishable and in the expected size of the designed primers. The elongation factor -1a gene (EF-1a) in both the bell pepper and Arabidopsis siliques were $\approx 1,100$ bp, the RIN fragments ≈ 330 bp, and the EXP1 fragments ≈ 720 bp. RNA with high quality had been obtained (Figure 1a) and underwent RT-PCR which displayed on 1.2% formaldehyde formaldehyde agarose gel. It was clear that the targeted DNA visible fragments were of the size expected on 1.2% agarose gel (Figure 1b). P1 represents the lane of the DNA fragment of the Elongation factor -1a gene (EF-1a) in the bell pepper and A1, that of the Arabidopsis. Both have the exact size of ≈ 1100 bp when juxtaposed with the DNA ladder; P2 lane represents the DNA fragment of the RIN gene which has 330 bp and A2 of the Arabidopsis. In addition to that are P3 A3 lanes: where P3 lane is the DNA fragment of the Expansin gene (EXP1) with a size of roughly 720 bp and A3 of the Arabidopsis.

Partial sequencing of expected differential fragments

Partial-length sequences of each of the positive cDNA clones of the three candidate fragments (EF-1a, RIN and EXP1) had been sequenced using the dideoxynucleotide method (Sanger et al., 1977) as shown in Figure 2. The obtained sequences had been aligned with each other to

detect homology between the genes in both plants (Figures 3, 4 and 5). The alignment results show an 88.59% similarity between the sequences of the cDNA fragment of the bell pepper and the Arabidopsis' EL-1a gene and 100% similarity between the cDNA fragment of the bell pepper and the Arabidopsis' RIN gene. Finally, there was an 82.73% similarity between the cDNA fragment of the bell pepper and the Arabidopsis' EXP1 gene.

Quantification of the genes' expression

In order to evaluate the gene expression fold of the targeted genes, real-time PCR was performed to measure the CT value of each gene in the bell pepper to be compared with the Arabidopsis CT value, in order to measure the expression in both and for the assessment. Slight differences were observed between the CT of both the bell pepper and Arabidopsis; the bell pepper exhibited higher CT value in both the RIN and EXP1 genes along with a higher standard deviation than Arabidopsis. The EF-1 gene exhibited a lower CT value in the bell pepper than the Arabidopsis (Table 1). Gene expression and folds of the genes were measured using RT-qPCR to compare between Arabidopsis and the bell pepper. The Total RNA and rRNA levels are not proper references, because of the observed imbalance between rRNA and mRNA fractions. Accurate normalization of gene-expression

EF-1a of the bell pepper:

```
CAGAACGTGAGCGGTATCACCATTGATATTGCTTTGTGG AAGTTGAGACCACTAAGTACTACTGC ACTGT
TATTGATGCCCCCGCCACAGGGATTCATCAAG AACATGATCACTGGTACCTCTCAGGCTGACTGTGCTG TTC
TCATTATTGACTCCACTACTGGTGGTTTTG AAGCTGGTATCTCCAAAGATGGTCAGACCCGTGAACATGCATTG
CTTGCTTTACCCTTGGTGTCAAGCAAATGATCTGCTGCTGTAACAAGATGGATGTACCACCCCAAGTACTC
CAAGGCTAGGTATGATGAAATCGTGAAGGAAGTTTCTTCTACCTCAAGAAGTTGGTTACACCTGACAA
AATCCCCTTTGTTCCAATCTCTGGTTTTGAAGGAGACAACATGATTGAGAGGTCTACCAACCTCGACTGGTAC
AAGGGACCAACCCCTCTTGAGGCTCTTGACCAGATTAACGAGCCCAAGAGGCCATCAGACAAACCCCTCCGT
CTTCCACTTCAGGATGTTTACAAGATTGGTGGTATTGGAAGTGTCCCTGTTGGTTCGCGTTGAGACTGGTGTGA
TCAAGCCTGGTATGGTTGTGACCTTTGGCCCTACTGGTTGCAACTGAAGTCAAGTCTGTTGAGATGCACCA
CGAAGCTCTCCAGGAGGCCTCCCTGGTGAACATGTTGGGTTCAATGTTAAGAATGTTGCTGTTAAGGATCTT
AAGCGTGTTATGTTGCCTCAAACCTCAAGGATGACCCAGCCAAGGGGGCAGCCAGCTTCACTGCCAGGT
CATCATGAAACCATCTGGCCAGATTGGAATGGATATGCTCCAGTCTTGATTGTCACACTTCCACATTG
CTGTCAAGTTTGTGAGATCTTGACCAAGATTGACAGGCGTTCAGGTAAGGAAGTTGAGAAGGAGCCTAAG
TTCTTGAAAGACGGTATGCTGGTATGGTTAAGATGATCCACCAAGCCCATGGTTGTTGAGACTTTTGTG
AATACCTCCATTGGGTCGTTTTGCTGTGAGGGACATGAGGCAGACTGTTGCTGTTGGTGTGTTCAAGAATGT
TGACAAGAAGGACCAACTG
```

RIN of the bell pepper:

```
GTGGAAATGTTACAACAGTCTCAAAGGCATTGCTAGGTTGAGGATTGGGCAATTGGGCACAAAA
GACTTGGAACAGCTTGAACGTCAATTGGATTCACTATTGAGGCAAATTAGGTCAACAAAGACACAAC
ACATTCTTGATCAACTTGCTGAACTTCAACAAAAGGAACAATCTTACTGAAATGAACAAATCTTTG
AGAATAAAGTTGGAAGAACTTGGTGTACCTTTCAAACATCATGGCATTGTGGTGAGCAAAGTGATC
AATATAGACATGAACAGCCTTCTCATCATGAGGGATTTTTTCAACATGTAATTGCAATAATACATTGC
CTA
```

EXP1 of the bell pepper:

```
ATGGGTATCATAATTTTATCCTTGTCTTCTTTTTGTAGACTCATGTTTCAACATTGTTGAAGGAAGAA
TCCCTGGTGTTTACTCTGGTGGTTTATGGGAAACTGCACATGCTACATTTTACGGCGGAAGTGATGCT
TCTGGAAACATGGGCGGTGCGTGTGGTTATGGAAATTTATACAGCCAAGGATACGGAGTTAACACAG
CAGCACTGAGTACTGCTTTGTTTAAACAATGGATTAAGTTGTGGAGCCTGTTTGAACCTAAATGTACA
AATACTCCTAATTGGAAATGGTGTCTTCTGGAAACCCCTTCCATTTTAAATCACAGCTACCAATTTCTGC
CCACCAAATTACGCGTTGCCAAATGACAATGGTGGCTGGTGTAAACCCTCCTCGCCCTCACTTTGACCT
CGCTATGCTATGTTTCTCAAACCTGCTCAGTACCAGCGTGGCATTGTTCTGTAACCTTATCGCAGGATC
CCATGCCGAAAGCAAGGAGGAATCAGATTTACCATCAATGGATTCCGTTACTTCAACTTAGTGTTGAT
CACGAATGTAGCAGGTGCAGGGGATATTATTAAGGTTTGGGTAAAAGGAACAAAGACAAATTGGAT
TCCATTGAGCCGTAATTGGGGACAAAATTGGCAATCAAATGCGGTTTTAACTGGTCAATCACTCTCTT
TCAGAGTTAAAGCTAGTGACCATCGATCTTCTACCT
```

Figure 2. The cDNA fragments' sequences of the bell pepper fruits.

levels is an absolute prerequisite for reliable results, especially when the biological significance of subtle gene-expression differences is studied. Still, little attention has been paid to the systematic study of normalization procedures and the impact on the conclusions. The expression folds of the candidate genes were calculated and represented in Figure 6. The used formula for copy number detection was:

$$Y \text{ molecules } \mu\text{L}^{-1} = (Xg \mu\text{L}^{-1} \text{ DNA} / [\text{Length of PCR product in base pairs} \times 660]) \times 6.022 \times 10^{23}$$

which is a simple method for determining the variable values in experimental biology (Reed and Muench, 1938).

DISCUSSION

There have been impressive advances in understanding the progressions connected with fruit development at the physiological, biochemical and molecular levels. These advances incorporate revelations on the components and indicate pathways involved and linked with the methodology of products of the fruit maturation. Late research has uncovered that the few genes that control fruit development and ripening have been conserved throughout the course of advancement. The image formulating indicates plainly the conjunction of ethylene-dependent and ethylene-independent pathways in both categories. It has been indicated unmistakably that numerous controllers of fruit development and maturing are normal



Figure 3. The EF-1a alignment between bell pepper and Arabidopsis sequences.

PEPPER	-----GTGGAAATGTTACAACAGTCTCAAAGGCAT	30
Arabidopsis	TACCAAGAGTATTTGAAGCTTAAACAAGAGTGGAAATGTTACAACAGTCTCAAAGGCAT	540

PEPPER	TTGCTAGGTGAGGATTTGGGACAAATTGGGCACAAAAGACTTGAACAGCTTGAACGTCAA	90
Arabidopsis	TTGCTAGGTGAGGATTTGGGACAAATTGGGCACAAAAGACTTGAACAGCTTGAACGTCAA	600

PEPPER	TTGGATTCATCATTGAGGCAAATTAGGTCAACAAAGACACACACATTCTTGATCAACTT	150
Arabidopsis	TTGGATTCATCATTGAGGCAAATTAGGTCAACAAAGACACACACATTCTTGATCAACTT	660

PEPPER	GCTGAACTTCAACAAAAGGAACAATCTCTTACTGAAATGAACAAATCTTTGAGAATAAAG	210
Arabidopsis	GCTGAACTTCAACAAAAGGAACAATCTCTTACTGAAATGAACAAATCTTTGAGAATAAAG	720

PEPPER	TTGGAAGAACTTGGTGTACCTTTCAAACATCATGGCATTGTGGTGAGCAAAGTGTACAA	270
Arabidopsis	TTGGAAGAACTTGGTGTACCTTTCAAACATCATGGCATTGTGGTGAGCAAAGTGTACAA	780

PEPPER	TATAGACATGAACAGCCTTCTCATCATGAGGGATTTTTCAACATGTAATTTGCAATAAT	330
Arabidopsis	TATAGACATGAACAGCCTTCTCATCATGAGGGATTTTTCAACATGTAATTTGCAATAAT	840

PEPPER	ACATTGCCTA-----	340
Arabidopsis	ACATTGCCTATAAGTATGGAACAAATCTTGAACGATATGAAAGATACTCATATGCAGAG	900

Figure 4. The RIN alignment between bell pepper and Arabidopsis sequences.

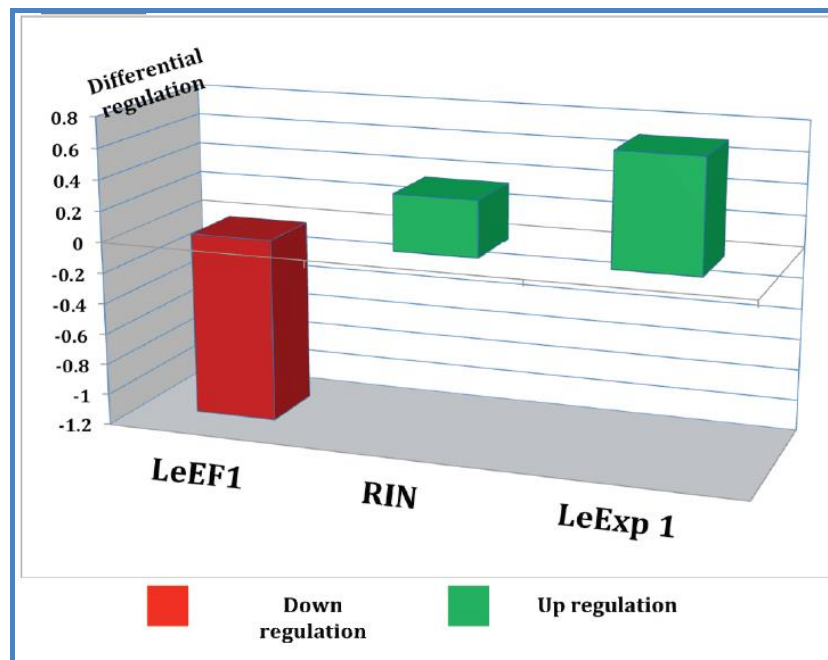
PEPPER	----ATGGGTATCATAATTTT-----CATCCTTGT-----CTTCT	32
Arabidopsis	CGGCACGAGCCTCTCCACACTGAGACACTTCCCATTTTGTCTCTCAAACAAATGGCTTCT	60
	* * . * . * : . * : * * : * * * : * *	
PEPPER	TT-----TTGTAGACTCATG-----TTTTCAACATT-----GTTGAAGGA	66
Arabidopsis	ATGGGTGTCATGGCCACTTACATTCTTGTCTCTCCTTTTCTCATTCTCTTGTGAAGCA	120
	: * * . * . * : * : * * . : * * * * * * * * * * * * * * * * *	
PEPPER	AGAATCCCTGGTGTITACTCTGGTGGTTCATGGGAAACTGCACATGCTACATTTTACGGC	126
Arabidopsis	AGAATCCCTGGCGTITACTCTGGTGGATCCTGGCAGACTGCTCATGCAACATTTTATGGT	180
	***** *	
PEPPER	GGAAGTGAIGCTTTCGGAACAATGGGCGGTGCGTGTGGTATGGAATTTATACAGCCAA	186
Arabidopsis	GCAGTGAIGCTTTCGGAACATGGGAGCAGCATGTGGTACGGCAATCTGTACAGCCAA	240
	* * . *	
PEPPER	GGATACGGAGTTAACACAGCAGCACTGAGTACTGCTTTTGTAAACAATGGATTAAGTGT	246
Arabidopsis	GGGTACGGAGTGAACACTGCAGCATTGAGCAGCAGCCTTGTTTAACAATGGGTTAAGCTGT	300
	* * . *	
PEPPER	GGAGCCTGTTTTGAACCTTAAATGTACAATACTCCTAATTTGGAATGGTGTCTTCCCTGGA	306
Arabidopsis	GGAGCATGCTTTGAGCTAAAATGTACAATACTCCTAATTTGGAATGGTGTCTTCCCTGGA	360
	***** *	
PEPPER	AACCCTTCCATTTTAAATCACAGCTACCAATTTCTGCCACCAAATACGCGTGTCCAAAT	366
Arabidopsis	AACCCTTCCATTTTAAATCACAGCCACTAATTTCTGCCCGCAAATACGCTAAGCCTAAT	420
	***** *	
PEPPER	GACAAATGGTGGCTGGTGTAAACCTTCTCGCCCTCACTTTGACCTCGCTATGCTATGTTT	426
Arabidopsis	GACAAATGGTGGCTGGTGTAAACCTTCTCGTTCATTTTGTATCTTGCCATGCTATGTTT	480
	***** *	
PEPPER	CTCAAACITGCTCAGTACCGGCTGGCATTGTTCTGTAACTTATCGCAGGATCCCAATGC	486
Arabidopsis	CTCAAGATCGCAGATACCGTGTGGCATTGTTCTGTAACTTATCGCCGGTACCTTGC	540
	***** . * * * : *	
PEPPER	CGAAAGCAAGGAGGAATCAGATTTACCATCAATGGATTCCGTTACTTCAACTTAGTGTG	546
Arabidopsis	CGCAAACAAGGAGGAATCAGTTTCAATGAACGGATTCCGTTACTTCAACTTAGTGTG	600
	* * . * * . *	
PEPPER	ATCACGAATGTAGCAGGTGACGGGGATATTATTAAGGTTTGGGTAAGGAAACAAAGACA	606
Arabidopsis	ATCCCTAACGTGGCGGGTGCAGGAGATATTATTAAGGTTTGGGTAAGGAAACAAAGACT	660
	* * . * * * * * . *	
PEPPER	AATTGGATTCCATTGAGCCGTAATTTGGGACAAAATGGCAATCAATGCGGTTTTAAT	666
Arabidopsis	AATGGATTCCGTTAAGCCGTAATTTGGGTCAAAATGGCAATCAATGCTTGGACT	720
	* * * * * * * * . * * . * * * * * * * * * * * * * * * * * * *	
PEPPER	GGTCAATCACTCTCTTTTCAAGTAAAGCTAGTGACCAATCGATCTTCTACCT-----	718
Arabidopsis	GGTCAATCACTTTCTTTTCAAGTAAAGCTAGTGACCAATCGATCTTCTACCT-----	780
	***** *	

Figure 5. The EXP1 alignment between bell pepper and Arabidopsis sequences.

Table 1. CT values and standard deviation of the fruit ripening genes in mature bell pepper fruits.

Genes	Pepper		Arabidopsis	
	C _T value	Std. deviation	C _T value	Std. deviation
EF-1	7.11	± 0.026	8.53	± 0.016
RIN	12.28	± 0.017	11.92	± 0.031
EXP1	9.67	± 0.021	8.95	± 0.008

Ct, Threshold cycle values.

**Figure 6.** Normalized differential expression of the *LeEF-1*, *rin* and *LeExp1* between the bell pepper and Arabidopsis.

to both climacteric and non-climacteric types (Paul et al., 2012). Partial sequencing of the genes under investigation revealed high similarity among the genes in Arabidopsis and bell pepper fruits. The differential display reverse transcriptase-PCR was used to extract EF-1a which is an ethylene-inducible gene (Kendrick and Chang, 2008). However, an 88.59% similarity has been detected between the partial sequence of EF-1a in bell pepper and Arabidopsis (1, 100 bp). Moreover, the expression analysis of EF-1a in the bell pepper was minor in comparison to its expression in the Arabidopsis. The expression studies indicated that the EF-1a gene is ripening-regulated, and illustrated changes in transcript accumulation at different fruit developmental stages. The analysis of transcript accumulation in different organs indicated a compelling bias towards expression in the fruit (Yokotani et al., 2009). However, the RIN fragment sequence had a complete similarity of a 100% to the RIN of the Arabidopsis.

The increase of the expression of RIN gene induces the ripening process of the fruits (Tieman et al., 2012). In the case of the EXP1 gene of the bell pepper, it had an 82.73% similarity to the Arabidopsis'. In addition, the gene expression of the bell pepper EXP1 was higher than the gene expression in the Arabidopsis fruit. Regarding the RT-PCR, there is a general consensus on using a single control gene for normalization purposes. Accurate normalization of gene-expression levels is an absolute prerequisite for reliable results, especially when the biological significance of subtle gene-expression differences is studied. Still, little attention has been paid to the systematic study of normalization procedures and the impact on the conclusions (Vandesompele et al., 2002). The Real-time PCR differential regulation results illustrated that LeEF-1a gene was significantly down-regulated in bell pepper in comparison to Arabidopsis. Low expression levels of the EF-1a decreased during fruit ripening process

(Pokalsky et al., 1989). Some studies showed high degree of conservation between other previously isolated EF-1a genes which suggested that a fungal EF-1a gene might serve as an appropriate probe for a plant EF-1a cDNA. The study also suggested that EF-1a is a multigene family due to the occurrence of six bands on hybridization with EF-1a probe in southern blot analysis.

The result of the RIN expression levels appeared adjacent in bell pepper and Arabidopsis. However, the level of the expression of RIN in the bell pepper was higher than the Arabidopsis. The expression of RIN increases dramatically during Arabidopsis ripening regardless of the temperature. However, it is influenced by both a developmental and ethylene factors (Bartley and Ishida, 2007). In addition to the expression level of the EXP1 in the bell pepper showed a higher score than Arabidopsis. Over-expression of the LeEXP1 gene resulted in enhanced fruit softening which resulted in an improved texture of the fruit, which may be useful for the tomato processing industry (Kaur et al., 2010). Another study by Fujisawa et al. (2011) found a link between the presence of LeEXP1 and CArG boxes in the promoters of several genes involved in fruit ripening.

Conflict of Interests

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

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Full Length Research Paper

Hydrogen 1 (1H) magnetic resonance spectroscopy (MRS) of 3.0T in assessment of steatosis to antiviral therapy for chronic hepatitis C

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This study investigates the utility of Hydrogen 1 (1H) magnetic resonance spectroscopy as a noninvasive test for steatosis of response to interferon and ribavirin treatment in patients developing different severity of hepatitis C virus (HCV). Ninety chronic hepatitis C patients undergoing antiviral therapy with interferon and ribavirin underwent 1H MR spectroscopy at 3.0T before treatment, 6 month after the start of treatment and one year after the start of treatment. Peak value of lipid, area under the peak of lipid, peak ratio of lipid, water and area ratio under the peak of lipid, water statistical difference between baseline of control group and antiviral group, and also between baseline and after the start of therapy 6 month of antiviral therapy group. 1H MRS is a noninvasive technique that can be used to provide liver steatosis information on hepatic metabolic processes. This study indicates that the 1H MRS can be used as an indicator of steatosis response to antiviral treatment in chronic hepatitis C patients.

Key words: Hydrogen 1 (1H), magnetic resonance spectroscopy (MRS), hepatitis C, antiviral therapy.

INTRODUCTION

For the reason of obesity and insulin resistance in non-alcoholic fatty liver disease (NAFLD), the prevalence of hepatic steatosis is increasing rapidly in the world (Angulo, 2002; Williams, 2006). Simple nonalcoholic steatosis can progress to more serious liver disease (nonalcoholic steatohepatitis and cirrhosis), representing a threat to public health.

Diagnosis and quantification of hepatic steatosis is

important. In NAFLD, steatosis is the hepatic manifestation of the metabolic syndrome and the earliest biomarker for the development of liver fibrosis in the more severe condition of non-alcoholic steatohepatitis (NASH). Early diagnosis and treatment of NASH can prevent the potential development of cirrhosis and hepatocellular carcinoma (HCC) (Adams et al., 2005; Farrell and Larter, 2006; Rector et al., 2008). In hepatitis C, steatosis is

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associated with more severe fibrosis and rapid disease progression (Adinolfi et al., 2001; Rubbia-Brandt et al., 2004). Liver biopsy remains the gold standard for providing the evaluation of hepatic steatosis, despite well-established drawbacks regarding its invasiveness and sampling error due to small sample size and inter-observer variability (Bravo et al., 2001). But this invasive procedure is not without risk. There is a small mortality rate but a high error rate, predominantly owing to under sampling, whereby typically less than 1/50,000 of the liver volume is obtained for histologic evaluation. Histological assessment of a needle biopsy specimen is potentially inaccurate since heterogenic manifestation of hepatic steatosis can lead to underscoring of the degree of steatosis or to false-positive results (Ratziu et al., 2005). These factors highlight the need for a noninvasive test to characterize diffuse liver disease. For ethical reasons and because most patients are unwilling to undergo repeated procedures, treatment algorithms rarely mandate or require serial liver biopsies.

Noninvasive modalities such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) have been employed for the assessment of hepatic steatosis (Saadeh et al., 2002; Mehta et al., 2008; Cho et al., 2008). However, these modalities do not specifically measure hepatic fat content, are semi-quantitative, and lack high sensitivity and specificity (Cho et al., 2008). Many studies have focused on the role of imaging techniques as a non-invasive alternative to liver biopsy for detecting and quantifying hepatic steatosis (Charatcharoenwithaya and Lindor, 2007; Hamer et al., 2006; Joseph and Saverymuttu, 1991; Szczepaniak et al., 2005). The reported sensitivities and specificities between different imaging techniques and between different studies investigating the same technique vary substantially.

¹H MRS is widely used to measure intramyocellular and intrahepatocellular lipid content *in vivo* (Szczepaniak et al., 1999). ¹H-MRS measures the resonance signals derived from protons in triglycerides, which can be quantified and used as a noninvasive measure for the degree of steatosis. The lipids observed in ¹H MRS arise mainly from triglycerides (TGs) in lipid droplets (Opstad et al., 2008) as these are NMR visible, whereas lipids bound to membranes and proteins are too rigid to generate a ¹H MRS-observable signal. This property of ¹H MRS to detect mobile lipids in lipid droplets has made it the standard method for the quantification of liver fat content (LFAT) (Szczepaniak et al., 2005). The purpose of this essay was to assess the value of ¹H MRS in diagnosis of hepatic steatosis in non-alcoholic fatty liver disease.

MATERIALS AND METHODS

Patients

From January 2010 to June 2010, after institutional and ethics board review and approval and after providing informed consent,

120 patients with chronic hepatitis C were enrolled. The diagnosis of decompensated HCV-induced cirrhosis was based on the American Association for the Study of Liver Diseases Clinical Guideline for Hepatitis C (2004).

All enrolled patients were also naive to any anti-viral treatments. Other inclusion criteria were (1) HCV RNA >500 copies/mL; (2) absence of complications such as gastrointestinal bleeding, hepatic encephalopathy and primary liver cancer; and (3) liver function defined as Child-Pugh grade A or B based on serum bilirubin, serum albumin, presence of ascites, presence of hepatic encephalopathy and prothrombin time. Patients with hypersplenism were also enrolled. Exclusion criteria included: (1) infection with hepatitis A, B, D or F virus, Epstein-Barr virus, cytomegalovirus or human immunodeficiency virus; and (2) presence of alcoholic or drug-induced liver diseases, Child Cor severe heart, brain or kidney disease.

A total of 120 patients that meet the inclusion criteria were enrolled. Patients were considered as part of the treatment group (n = 90) or control group (n = 30) based upon whether they opted to receive anti-viral therapy. The study was approved by the Institutional Review Board of the hospital, and informed consent was obtained from all study participants.

Clinical evaluation

Determination of therapeutic efficacy

The primary endpoints were (1) SVR, defined as HCV RNA undetectable or < 500 copies/mL for at least 24 weeks after treatment discontinuation (Pearlman, 2012); (2) relapse, defined as HCV RNA undetectable or < 500 copies/mL during antiviral therapy, but becomes detectable at 24 weeks after treatment discontinuation. The secondary endpoint was assessment of disease progression, (defined as an increase of 2 or more in the Child-Pugh score), presence of primary hepatocellular carcinoma, renal dysfunction, spontaneous bacterial peritonitis, variceal bleeding or death due to liver disease (Liaw et al., 2004)

Measures

Patients in the treatment group were evaluated for serum HCV antibodies, liver function, HCV RNA, coagulation function, thyroid function, and alpha-fetoprotein as well as liver computed tomography. Routine blood and urine tests were performed before the start of the study. Routine blood and liver function tests were performed weekly in the first month, then once every 4 weeks during the study period and once every 8 weeks for 24 weeks after discontinuation of treatment. Quantitative detection of HCV RNA was done immediately prior to treatment (baseline), at 24 and 48 weeks after treatment, and 6 months after discontinuation of treatment. HCV RNA levels were quantitated by real-time polymerase chain reaction (PCR) using a kit from the Roche company.

Patients in the control group were evaluated for liver function and HCV RNA levels. Routine blood tests and color ultrasonography of the liver were done once every 12 weeks. All patients were assessed for disease progression.

Treatment regimen and follow-up

All participants received symptomatic and supportive treatment, including treatment for reducing levels of transaminase and bilirubin and supplemental albumin. For patients in the treatment group, those who had a neutrophil count of $\geq 1.0 \times 10^9/L$, platelet count of $\geq 50 \times 10^9/L$ and hemoglobin of $> 10 \text{ g/L}$ were treated additionally with both Peg-IFN α -2a and RBV. The initial dose of Peg-IFN α -2a

was 180 µg/kg subcutaneously. Peg-IFN α -2a dosage was reduced to 90 µg/kg once weekly when neutrophil or platelet counts decreased to $\leq 0.75 \times 10^9/L$ or $< 50 \times 10^9/L$, respectively. The dose was returned to 180 µg/kg if neutrophil and platelet counts increased to $> 0.75 \times 10^9/L$ and $\geq 50 \times 10^9/L$, respectively, after two weeks. Treatment was discontinued if neutrophil count was $\leq 0.5 \times 10^9/L$ or platelet count was $< 30 \times 10^9/L$. Patients tolerating the standard Peg-IFN α -2a dose of 180 µg/kg weekly were treated for 48 weeks. Patients who could not tolerate the standard dose were treated with the reduced dose of 90 µg/kg once weekly for up to 72 weeks.

Patients with hemoglobin of > 100 g/L were initially treated with standard dose of RBV (genotype 1: 1200 mg/d for patients with body weight >75 kg and 1000 mg/d for patients with body weight ≤ 75 kg; non-genotype 1: 1000 mg/d for patients with body weight > 75 kg and 800 mg/d for patients with body weight ≤ 75 kg). RBV dosage was reduced when hemoglobin levels decreased to ≤ 100 g/L after the dosage increase. RBV treatment was discontinued when hemoglobin levels were ≤ 80 g/L. Patients tolerating the standard dose of RBV were treated for 48 weeks. Patients developing cytopenia during the treatment period were treated with cell growth-stimulating factor and/or erythropoietin. All patients were followed up for 3 years.

MR imaging and 1H-MR spectroscopy

MR measurement was performed on a clinical Philips Achieva 3.0 T TX scanner (Philips Healthcare, Best, The Netherlands). The Sense Torso coil was positioned on the abdomen and scout image were acquired to localize the liver and surrounding structures. T1-, T2-weighted, were performed in all patients and controls. TR/TE 2000/40 ms, FOV =35mm*35mm*35mm, 96 averages, 3.4mm, PA w/s exc angle 250.

1H-MR spectrum was acquired with and without water suppression. Localized single voxel point resolved spectroscopy (PRESS) BH with TR/TE=3,000 ms/35 ms and number of averages =64 were taken. A voxel of $2 \times 2 \times 2$ cm³ was located mainly in right parietal region of the liver in all the subjects. First a layer selection gradient plus 90. Radio frequency pulse (RF), while followed by two layer selection gradient plus 180. RF reunion pulse. Three orthogonal vertical, reach the three-dimensional spatial orientation, the spin echo signal. Data acquisition during the breath hold to ensure that the constant of the scanning area of interest and reduce the impact of cardiac pulsatility. Liver tissue contains more water and fat, and the strongest signal of 1H-MRS detected are water and fat.

Analysis of 1H-MR spectroscopy

All the data was calibrated and calculated with spectroview of extended MR workspace 2.6.3.2. The peak value of lipid, peak value of water, area under the peak of lipid and lipid/water ratios of all patients were analyzed and the results of different groups were compared.

Statistical analysis

Age and baseline HCV RNA level were normally distributed and presented as mean and standard deviation. Differences in age and baseline HCV RNA levels between the two groups were tested with the independent two samples t-test. Child-Pugh scores were non-normally distributed and presented as median and inter-quartile range (IQR). Differences in Child-Pugh scores between the two groups were tested with the non-parametric Mann-Whitney test. Other categorical variables were presented as number and

percentage, and the associations with categorical variables versus treatment group were tested with the Fisher's exact test. Statistically significant variables from the univariate analyses were used in the multivariate analysis. All statistical tests were two sided, and a p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL).

RESULTS

Patient demographics and baseline characteristics

As shown in Table 1, 120 patients, who met the inclusion criteria, were enrolled. Among them, 90 patients had sufficient blood cell counts for anti-viral therapy. The remaining 30 patients, who refused anti-viral therapy, were placed in the control group. Patients in the treatment group were significantly younger than those in control group (mean age 52.7 vs. 58.3 years, $p < 0.001$). There were also no significant differences between the two groups in baseline HCV RNA levels. In addition, baseline MELD scores were not significantly different between treatment and control groups (Table 1). Although baseline Child-Pugh scores, total bilirubin, and hepatic encephalopathy were not different between the two groups, significant differences in serum albumin, international normalized ratio (INR) for prothrombin time, and ascites were observed between the treatment and control groups ($p = 0.002$, $p = 0.018$, and $p < 0.001$, respectively).

Comparison of the 1H MRS between before and after antiviral therapy

Peak value of lipid, area under the peak of lipid, peak ratio of lipid/water and area ratio under the peak of lipid/water had statistical difference between baseline of control group and antiviral group: peak value of lipid: (0.39 \pm 0.18) vs (0.05 \pm 0.05); area under the peak of lipid: (26.01 \pm 17.08) vs (2.22 \pm 1.76); peak ratio of lipid/water: (0.42 \pm 0.17) vs (0.43 \pm 0.21); area ratio under the peak of lipid/water (0.58 \pm 0.25) vs (0.60 \pm 0.21), $P < 0.05$. Peak value of lipid, area under the peak of lipid, peak ratio of lipid/water and area ratio under the peak of lipid/water also had statistical difference between baseline and after the start of therapy 6 months of antiviral therapy group: peak value of lipid: (0.28 \pm 0.11) VS(0.39 \pm 0.18); area under the peak of lipid: (21.53 \pm 13.28) vs (26.01 \pm 17.08); peak ratio of lipid/water: (0.14 \pm 0.13) vs (0.42 \pm 0.17); area ratio under the peak of lipid/water (0.24 \pm 0.12)VS(0.58 \pm 0.25), $P < 0.05$. (Table 2)

Change of peak ratio of lipid/water and area ratio under the peak of lipid/water between baseline and after treatment of response group and non-response group

69 patients responded to antiviral treatment with a

Table 1. Patient demographics and baseline characteristics.

Parameter		Treatment (n = 90)	Control (n = 30)	P-value
Age (y)		52.7 ± 10.1	58.3 ± 12.5	< 0.001*
Gender	Male	36 (40.0%)	14 (46.7%)	0.451
	Female	54 (60.0%)	16 (53.3%)	
Baseline HCV RNA level (log ₁₀ copies/ml)		5.30 ± 1.18	5.23 ± 1.15	0.641
Baseline MELD score		12.6 (9.8, 15.2)	12.5 (9.4, 15.8)	0.637
Baseline Child-Pugh score		5.0 (4.0, 7.0)	5.0 (3.0,7.0)	0.791
Total bilirubin (mg/dL)	<2	9 (12.3%)	5 (15.0%)	0.660
	2-3	40 (43.8%)	12 (46.5%)	
	>3	41 (43.8%)	13 (38.6%)	
Serum albumin (g/dL)	>3.5	9 (10.8%)	3 (11.0%)	0.002*
	2.8-3.5	40 (42.3%)	19 (62.2%)	
	<2.8	41 (46.9%)	8 (26.8%)	
Prothrombin time INR	<1.7	26 (29.2%)	8 (29.1%)	0.018*
	1.7-2.3	50 (55.4%)	13 (41.7%)	
	>2.3	14 (15.4%)	9 (29.1%)	
Hepatic encephalopathy	None	90 (100.0%)	30 (100.0%)	NA
Ascites	Absent	90 (100.0%)	26 (87.4%)	<0.001*
	Easily controlled	0 (0.0%)	4 (12.6%)	

HCV, hepatitis C virus; MELD, model for end-stage liver disease; INR, international normalized ratio. *indicates a significant difference between two groups. Age and baseline HCV RNA level were normal distributed and presented by mean and standard deviation. Baseline Child-Pugh score was non-normal distributed and presented by median and inter-quartile range (IQR). Other category variables were presented by number and percentage.

Table 2. 1H MRS parameters between the control and NAFLD group.

Parameter	Peak value of Lip	Area under the peak of lip	Peak value of water	Area under the peak of water	Peak ratio of lipid/water	Area ratio under the peak of lipid/water
Control baseline	0.05±0.05	2.22±1.76	1.05±0.53	51.36±39.28	0.43±0.21	0.60±0.21
control After the start of therapy 6month	0.06±0.07	2.37±1.89	1.23±0.67	53.27±41.72	0.45±0.23	0.62±0.25
Antiviral therapy group baseline	0.39±0.18*	26.01±17.08*	0.96±0.54	69.84±30.50	0.42±0.17*	0.58±0.25*
Antiviral therapy group After the start of therapy 6month	0.28±0.11#	21.53±13.28#	0.89±0.47	62.47±27.08	0.14±0.13#	0.24±0.12#

*Compared with control group, P<0.05, *compared with antitherapy group baseline, P<0.05.

sustained viral response. In 54 of these patients, peak ratio of lipid/water and area ratio under the peak of lipid/water had decreased on 6 months follow-up MR spectroscopy; P<0.05. Figure 1 is the graph of a responder whose spectra changed after treatment,

showing a decrease in peak (Figure 2) ratio of lipid/water and area ratio under the peak of lipid/water. 15 of the 21 virologic non-responders had peak ratio of lipid/water and area ratio under the peak of lipid/water on follow-up imaging similar to the baseline values.

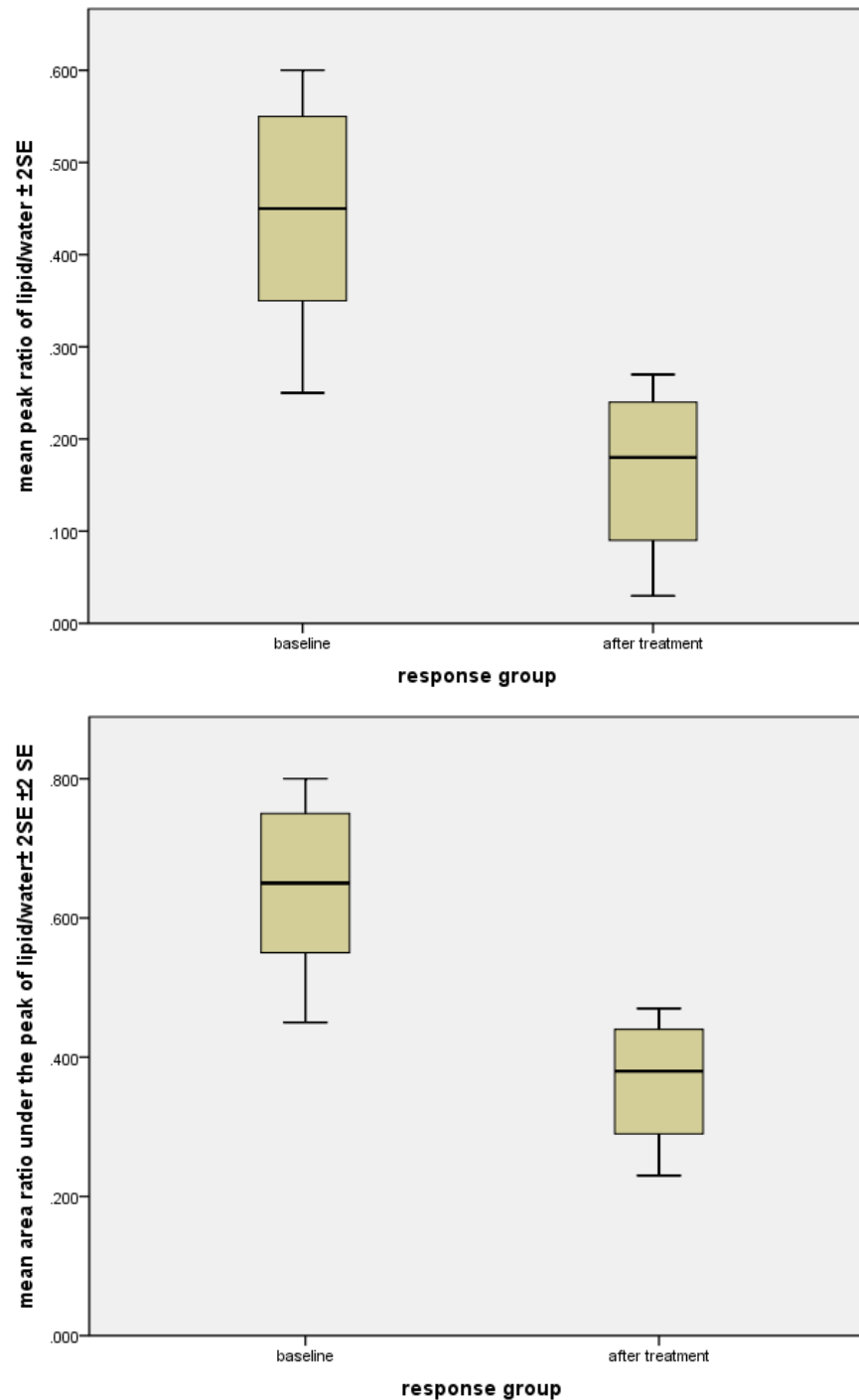


Figure 1. Change of peak ratio of lipid/water and area ratio under the peak of lipid/water between baseline and after treatment of response group.

DISCUSSION

It is estimated that approximately 3% of the global population has chronic infection with the hepatitis C virus (HCV) and that approximately 4 million persons are newly infected each year (Hoofnagle, 2002). In 55 to 85% of

patients with infection develops into chronic liver disease, which in many cases remains asymptomatic. In approximately 20% of cases, fibrosis develops into cirrhosis, which leads to hepatocellular cancer in 5% of cases each year (Brook et al., 2010). Liver biopsy is the reference standard for staging and grading chronic liver disease,

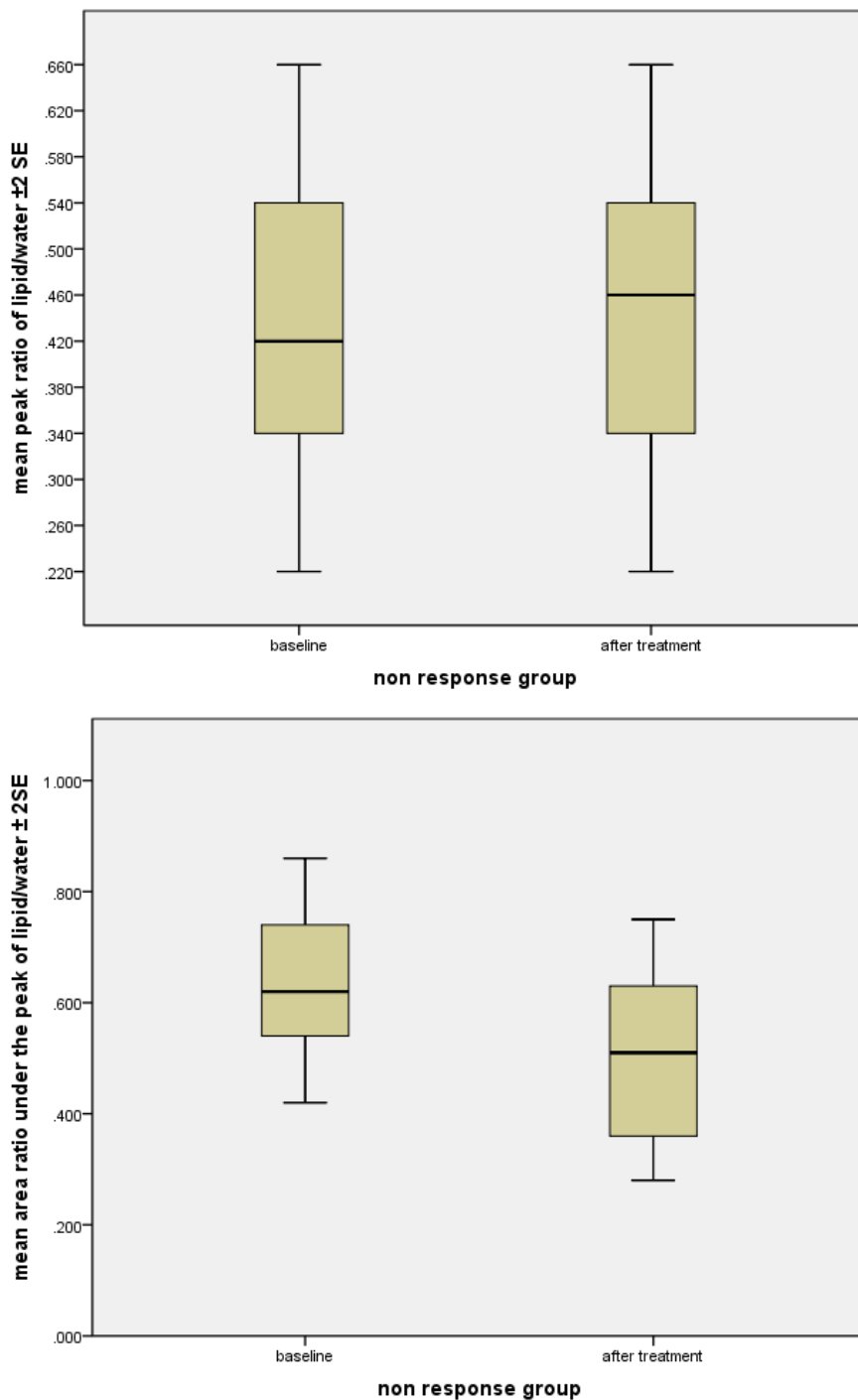


Figure 2. Change of peak ratio of lipid/water and area ratio under the peak of lipid/water between baseline and after treatment of nonresponse group.

but this invasive procedure is not without risk. There is a small mortality rate but a high error rate, predominantly owing to under sampling, whereby typically less than 1/50,000 of the liver volume is obtained for histologic evaluation (Ishak et al., 1995; Regev et al., 2002). As a result of the problems associated with biopsy, a steady

drive to find an effective noninvasive method of evaluating liver damage has led to developments both in testing with serologic biomarkers of disease and in imaging. For ethical reasons and because most patients are unwilling to undergo repeated procedures, treatment algorithms in the United Kingdom rarely allow serial liver

biopsy. The impetus to find a reliable and repeatable biomarker of disease activity and response to treatment thus has renewed focus (Cobbold et al., 2006).

Magnetic resonance spectroscopy is a valuable tool for the non-invasive assessment of metabolic processes *in vivo*. Because of the presence of certain compounds form in the organization of the nucleus proton, these compounds or metabolites would produce a certain chemical shift in certain chemical environment. Small changes in the magnetic resonance peak caused by these changes could be collected by magnetic resonance scanner and converted to numerical spectrum. Neuronal markers, membrane constituents, osmolytes and the energy status can be measured for the diagnosis of various diseases and therapeutic monitoring in humans (Ross and Bluml, 2001). ¹H magnetic resonance spectroscopy generates a spectrum of the various resonances of protons that are embedded in different chemical bonds. Because the protons are surrounded by various nuclei and electrons with their own magnetic properties, small magnetic field perturbations occur in a systematic manner, leading to slight differences in the received frequencies of protons in different chemical bonds. Thus, the chemical shift values occur essentially as a consequence of the variable electronegativity of adjacent chemical moieties in the molecule. The chemical shift scale describes the position of resonances in the spectrum in parts per million (p.p.m.), irrespective of the field strength, relative to a reference set at 0 p.p.m. The underlying frequency shift, however, measured in Hertz (Hz), is directly proportional to the strength of the magnetic field, for example 1 p.p.m. of the proton spectrum at 1.5 T refers to 64 Hz and at 3.0 T to 128 Hz. Therefore, with higher magnetic fields the resonances are better separated. The frequency separation of the resonances or peaks describes the resolution of the spectrum.

The clinical use of localized ¹H magnetic resonance spectroscopy (¹H MRS) *in vivo* first in the brain and then in the prostate has been well established and refined over the last two decades (Ross and Bluml, 2001; Michaelis et al., 1993; Cunningham et al., 2005; Hom et al., 2006). Single volume spectroscopy with stimulated echo acquisition mode or the point-resolved spectroscopy sequence (PRESS) technique is recommended, because of longer acquisition times and reduced SNR for multi-voxel liver MRS with chemical shift imaging (Fischbach and Bruhn, 2008).

The ratio of the fat peak (1.3 ppm) to the water peak (4.7 ppm) is a common definition of hepatic fat percentage by ¹H-MRS (Thomas et al., 2005). Using this definition, Thomas et al. (2005) reported on the relationship between body adiposity and steatosis in 11 NASH patients and measured hepatic fat percentages of up to 75%. But clinical study by Longo et al. (1995), a proposal for the ([AUC total fat peaks/ AUC total peaks]) method for calculating hepatic fat content from ¹H-MR spectra was advocated. The same method was applied in a large study by Szczepaniak et al. (1999), evaluating the

prevalence of hepatic steatosis in over 2300 participants of the Dallas Heart Study population.

In this study, Philips Achieva 3.0 T TX scanner and ¹H torso coil were used to get the signal. Localized single voxel point resolved spectroscopy (PRESS) BH was used. In this study, data was analysed by Philips Achieva 3.0T spectroview of extended MR workspace 2.6.3.2, quantitative spectral analysis chemical shifts, the calculation Product of metabolite peak and the area below peak and so on. Peak value of lipid, area under the peak of lipid, peak ratio of lipid/water and area ratio under the peak of lipid/water had statistical difference between baseline of control group and antiviral group, and also between baseline and after the start of therapy 6 month of antiviral therapy group. It suggested that liver steatosis of chronic HCV steatosis was modified significantly by antiviral therapy, which is the same result of study of K. R. Reddy [Reddy, 2008 #3469].

In short, ^{3.0}T ¹H-MRS may be an effective technology in assessing of lipid metabolism in chronic HCV. Use of MRS technology is based on triglycerides (TG) methylene chemical shift difference between TG accumulations in fine Cell sources for fatty liver quantitative determination of a new idea. However, the study samples are relatively small, pending further in-depth exploration.

Conflict of Interests

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

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Full Length Research Paper

Determination of aflatoxin B1 in food products in Thailand

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Aflatoxin B1 is a secondary metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*. It can be formed in commodities before and after harvest. This mycotoxin possesses a variety of toxic effects, potent carcinogen to both animal and human health. Aflatoxin B1 is generally found in feed and food stuff, such as cereal and all products derived from cereals, including processed cereals since it has been proven to be at least partly resistant to food processing methods. Hence, the aim of this study was to determine the possibility of contamination of aflatoxin B1 in food products in Thailand. The 100 food samples were purchased from markets around Bangkok. They were divided into five categories: seven samples of local fermented alcoholic beverages, five samples of imported blue cheese, 18 samples of fermented soybean products, 70 samples of raw peanuts (30) and peanut derived products (40). They were determined for aflatoxin B1 by ELISA method. The revealed rates of aflatoxin B1 contamination were 71.42, 100, 83.33, 86.67 and 90% for the alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived samples, respectively. The individual values with each category samples, ranged from 0.3 to 2.15 µg/kg (average 0.48 µg/kg), 0.5 to 1.25 µg/kg (average 0.95 µg/kg), 0.2 to 3.2 µg/kg (average 1.54 µg/kg), 0.2 to 8.05 µg/kg (average 6.83 µg/kg) and 0.1 to 73.85 µg/kg (average 5.6 µg/kg) for alcoholic beverages, blue cheese, soybean, raw peanuts and peanut derived samples, respectively.

Key words: Mycotoxins, aflatoxin B1, carcinogen.

INTRODUCTION

Mycotoxins are a group of toxic secondary metabolites with no apparent function in the normal metabolism of fungi. They are produced by certain fungi infesting agricultural products. They induce a variety of toxic response in humans and animals when foods or feeds

containing these compounds are ingested. Therefore, human ingestion of mycotoxins mainly occurs from the consumption of mycotoxins in residues or metabolites in animal-derived foods or contaminated food products (Smith et al., 1995). Aflatoxins (B1, G1, B2, G2) are the

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best known and most widely studied mycotoxins. Since, Aflatoxins are found in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are optimum for growth of the molds and for production of the toxin. More than 300 to 400 classes of mycotoxins are identified, but the most significant and toxic group is aflatoxins. Aflatoxins have been considered as the most prevalent mycotoxins contaminating human food and animal feed (Bhat and Vashanti, 1999). They are highly toxic, mutagenic and carcinogenic compounds that have involved as a potential agent in human hepatic carcinogen (Wogan, 1999; 2004). Among aflatoxins, aflatoxin B1 (AFB1) is a natural toxin produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. AFB1 is a potent carcinogen, teratogenic and mutagenic and WHO-International Agency for Research on Cancer (IARC) have classified AFB1 as carcinogenic agent to humans as it is responsible for human primary hepatocellular carcinoma (IARC, 2002).

Aflatoxin M1 (AFM1) is the metabolite of AFB1 and has been reported that AFM1 was detected in milk within 12 to 24 h after the first ingestion of AFB1 (Asi et al., 2012). AFM1 is also classified as toxic like AFB1 and is resistant against heat, pasteurization and sterilization (Abdulrazzaq et al., 2003; Sadeghi et al., 2009). Aflatoxins are generally found in feed and foodstuffs, such as beans, cereals fruits and seeds. It has proven to be at least partly resistant to food processing methods meaning it is also present in derived products and thus, finds its way into humans. Contamination of foods and feeds by the aflatoxin-producing species *A. flavus* and *A. parasiticus* cannot be completely avoided and may lead to significant economic losses and health risks (Shane, 1994). As Thailand is in the tropical area, it is hard to avoid mold-contaminated food and feed. Therefore, mycotoxins production is unavoidable and depends on different environmental factors in the field or during storage. As a result, the high incidence rate of contamination of cereal grains and animal feed has been reported worldwide. It can be assumed that about 25% of food products, mainly from cereals, are substantially contaminated with aflatoxins (Magan et al., 2004). Mycotoxins are responsible for generating huge economic losses (Bhat and Vasanthi, 2003) and 25 to 40% of cereals consumed in the world are contaminated by these toxic compounds (Pitter, 1998). Due to this reason and their toxicity, the EU fixed the stringent maximum residue levels for total aflatoxins and AFB1 levels in human commodities to 4 and 2 µg/kg, respectively (Moss, 2002).

The Codex Alimentarius commission (Joint FAO/WHO) also adopted the total aflatoxins limit at 15 µg/kg in peanut (Codex, 2001), but the WHO prescribed the maximum limit for AFB1 in various food stuffs at 5 µg/kg (Papp et al., 2002). Owing to these adverse health effects of AFB1, a survey was conducted in various food products

in Thailand. Thus, the main purpose of this study was to investigate the possible incident of AFB1 in food products by using a 96-well micro-titer plates ELISA test kit for AFB1 determination, and to compare the obtained levels to those set by the European commission.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, methanol, phosphate buffer saline (PBS) and phosphoric acid used, were of analytical grade and were purchased from Sigma (MO, USA) and Merck (Darmstadt, Germany). One milligram per milliliter of AFB1 standard was purchased from Supelco, USA, prepared as an aqueous stock and working solution in concentrations of 10,000 and 10 ng/ml, respectively, and stored at 4°C until analyses.

Sample collection

The 100 food samples were randomly purchased from different markets around Bangkok, Thailand during 2012 (November to December) and 2013 (February to April). They were divided into five groups: the first group was local alcoholic beverages, the second was imported blue cheese, the third was fermented soybean (pickled bean, curd soybean paste and salted soybean), the fourth was peanuts (raw peanuts with and without shells) and the last was peanut derived products (fried peanuts, roasted salted peanut, roasted ground peanuts, peanut sesame, crispy peanut cake, peanut cookies and etc.).

Aflatoxin B1 extraction

The extraction of AFB1 was carried out according to the AOAC method (AOAC, 1990) with a slight modification. Briefly, 20 g of each sample was mixed with 100 ml of 70% methanol and shaken for 300 rpm/10 min. After that, each sample was filtered through filter paper number 4 (Whatman International Ltd., Maidstone, UK) and centrifuged. Then, the supernatant was taken to determine the aflatoxin B1 by using DOA-AFLATOXIN B1 ELISA test kit from Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives, Thailand. This test kit is the direct competitive ELISA with polyclonal antibody specific to AFB1 with the sensitivity of detection of 0.4 µg/kg.

ELISA determination

After the extraction and filtration as mentioned above, the sample was ready for the determination of AFB1 by following the test kit directions. The results were measured by micro ELISA Reader (Stat Fax 303 Plus, USA) at 450 nm. Briefly, AFB1 standard solutions, used for making the calibration curve, contained AFB1: 0, 4, 10, 20, 40, and 80 µg/l.

The samples were diluted with 200 µl of 0.01 M PBS-T and 50 µl of each diluted sample was dropped into a well of Micro ELISA plate which was coated with antibody and 50 µl of enzyme conjugate (AFB1-HRP) was added to the well afterward. The plate was allowed to stand in the dark for 30 min at room temperature. Thereafter, the wells were emptied by inverting the micro-plate upside down, tapped vigorously against absorbent paper and washed with 200 µl of 0.01 M PBS-T for 3 times. 100 µl of substrate (tetramethylbenzidine, TMB) was added to the wells and left in the

dark for 10 min at room temperature to produce yellow color. To stop the reaction, 100 µl of 0.5 M phosphoric acid was added. The intensity of the color is inversely proportional to the concentration of AFB1 in the sample or standard, which was measured at 450 nm. Each sample was analyzed in triplicates. The optical density of the standards from the standard curve, and the samples optical densities are plotted against the curve to calculate the exact concentration of AFB1.

Recovery detection

To test the sensitivity of the method, the AFB1 standard solution at different concentrations (2, 4, 10, 20 and 40 µg/kg) were added to the food samples suspected to contain less than 0.4 µg/kg of AFB1. Due to the fact that no certified sample was available for the recovery tests, the confirmation of recovery in various food samples was also done by spiking AFB1 at different concentrations (2, 4, 10, 20 and 40 µg/kg) into the food samples. The extraction of the spiked samples was done as the same as non-spiked samples which described above.

$$\% \text{ Recovery} = (A_s - B_n) \times 100 / C_{\text{std}}$$

A_s and B_n are the representative of the concentration of AFB1 in the food sample for spiked and non-spiked AFB1 standards, whereas C_{std} is the concentration of AFB1 standard.

The recovery tests were performed twice, and each instance included 5 replicates. Then, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated (Miller and Miller, 2000).

Statistical analysis

The data was analyzed using SPSS software IBM SPSS (PASW Statistics 19) and presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Food contamination with mycotoxins is a serious issue in developing countries that poses a significant health risks for humans as well as for animals. Mycotoxins are natural occurring toxin that cannot be 100% controlled. The presence of moulds in food stuffs could lead to the possible formation of mycotoxins, which is well known to be carcinogenic or genotoxic to both animals and humans' health and present a severe health hazard because these toxins are very stable even if passing through quite severe processes, especially AFB1, the most ubiquitous form and the most toxic (Moss, 1996; Creppy, 2002; Kamika and Takoy, 2011; Wild and Gong, 2010). Consequently, they can be a problem in processed foods and lead to health risk. Hence, the purpose of this study was to determine the concentration of AFB1 in various food product samples that are popular for Thai people. In this study, we assessed the natural occurrence of AFB1 in various food samples by using ELISA method because it has been shown to be reliable, simple, and it could be standardized for routine analysis of the mycotoxins present in food and feed materials. The LOD and LOQ estimated for the method were 0.36 and 1.2 µg/kg for local alcoholic beverages, 0.51 and 1.7 µg/kg for blue

cheese, 0.33 and 1.1 µg/kg for fermented soybean, 0.36 and 1.2 µg/kg for raw peanuts and 0.45 and 1.5 µg/kg for peanut derived products, respectively (Table 1a). In our study, the average of % recovery rate in food samples (2 to 40 µg/kg) was 65.55 to 87.7% as shown in Table 1a and b. A total of 100 samples, consisting of 5 samples of imported blue cheese, 7 samples of local fermented alcoholic beverages, 18 samples of fermented soybean products, 30 samples of raw peanuts and 40 samples of peanut derived products, was analyzed to determine the amount of AFB1.

The results of the occurrence and level of AFB1 in the different food samples revealed that the rates of AFB1 contamination were 71.42, 100, 83.33, 86.67 and 90% for the alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived products, respectively (Table 2). The individual values, for each category sample, ranged from 0.3 to 2.15 µg/kg (average 0.48 µg/kg), 0.5 to 1.25 µg/kg (average 0.95 µg/kg), 0.2 to 3.2 µg/kg (average 1.54 µg/kg), 0.2 to 8.05 µg/kg (average 6.83 µg/kg) and 0.1 to 73.85 µg/kg (average 5.6 µg/kg) for alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived products, respectively (Table 2). Our results showed that the concentration of AFB1 in the samples did not exceed the world accepted level (FAO, 2004; CAC, 2001) except raw peanuts and peanut derived products. The WHO prescribed the maximum limit for AFB1 in various food stuffs at 5 µg/kg (Papp et al., 2002). The limits of Aflatoxins (B1, G1, B2, G2) for human consumption were 15 µg/kg in raw peanuts and 10 µg/kg in processed peanuts (CAC, 2001). From the results, it can be concluded that because our country is in tropical area, the climate is suitable for fungal growth and peanuts are considered to be one of the most susceptible food materials for fungal growth and Aflatoxins productions. Several researchers have investigated peanuts for the presence of Aflatoxins, particularly AFB1 (Bankole et al., 2005; Barro et al., 2002; Mutegi et al., 2009). Iqbal et al. (2013) reported the level of AFB1 ranging from 2.4 to 12.3 µg/kg but Wagacha et al. (2013) reported AFB1 level in peanut products ranging from 0 to 1629 µg/kg.

Therefore, our study is in agreement with other previous studies in other countries on the natural occurrence of AFB1 in peanut samples. The contamination of aflatoxins in peanuts is related to climatic conditions (Nakai et al., 2008). It is known that the majority of food and feed products can allow the growth and development of toxic fungi during their processing, transport and storage (Frisvad and Samson, 1991). The ingestion of AFB1 by human occurs mainly through eating contaminated plant products and animal products (Smith et al., 1995). Keeping that in mind AFB1 can be found in a daily diet (nut, spices, dried fruits, wine, coffee, cereal, milk, cheese, meat, etc.) because they are able to contaminate a wide range of food commodities that are commonly consumed by all age groups including raw

Table 1a. Mean and %recovery test of AFB1 in spiked food samples using ELISA.

Type of food samples	LOD ($\mu\text{g}/\text{kg}$)	LOQ ($\mu\text{g}/\text{kg}$)	Spiked level ($\mu\text{g}/\text{kg}$)	Mean \pm SD	Recovery (%)
Local alcoholic beverages	0.36	1.2	2	1.61 \pm 0.12	80.5
			4	3.04 \pm 0.22	75.9
			10	7.42 \pm 0.67	74.2
			20	16.65 \pm 0.84	82.8
			40	28.2 \pm 0.18	70.49
Blue cheese	0.51	1.7	2	1.58 \pm 0.17	79.1
			4	3.3 \pm 0.18	82.5
			10	7.57 \pm 0.24	75.7
			20	13.52 \pm 0.72	67.6
			40	28.23 \pm 0.67	70.58
Fermented soybean	0.33	1.1	2	1.51 \pm 0.11	75.4
			4	3.22 \pm 0.71	80.5
			10	8.08 \pm 0.45	80.8
			20	13.11 \pm 0.16	65.55
			40	28.05 \pm 0.58	70.12
Raw peanuts	0.36	1.2	2	1.75 \pm 0.12	87.7
			4	3.26 \pm 0.45	81.5
			10	7.47 \pm 0.37	74.7
			20	17.02 \pm 0.91	86.1
			40	32.59 \pm 0.27	81.48
Peanut derived products	0.45	1.5	2	1.65 \pm 0.15	82.6
			4	3.19 \pm 0.17	79.7
			10	8.59 \pm 0.18	85.9
			20	16.17 \pm 0.27	80.27
			40	31.8 \pm 0.98	79.5

7 replicates were done for 2 $\mu\text{g}/\text{kg}$ samples, 3 replicates were done for 4, 10, 20 and 40 $\mu\text{g}/\text{kg}$ samples (n = 3).

Table 1b. %recovery test of AFB1 in spiked food samples using ELISA.

Food sample	As ($\mu\text{g}/\text{kg}$)	Bn ($\mu\text{g}/\text{kg}$) (Mean \pm SD)	Cstd ($\mu\text{g}/\text{kg}$)	%Recovery (Mean \pm SD)
Local alcoholic beverages	3.65 \pm 0.51	1.9 \pm 0.12	2	85.5 \pm 8.41
Blue cheese	4.95 \pm 0.23	1.9 \pm 0.41	4	75.25 \pm 10.25
Fermented soybean	12.7 \pm 0.35	3.2 \pm 0.23	10	94.5 \pm 5.86
Raw peanuts	34.7 \pm 0.28	16.45 \pm 0.95	20	90.5 \pm 12.48
Peanut derived products	35 \pm 0.14	1.0 \pm 0.17	40	83.14 \pm 10.15

Values are mean \pm SD Limit of detection was 0.36. Five replicates were done. n=3, % Recovery= (As-Bn) x 100/Cstd, As and Bn are the representative of the concentration of AFB1 in the food samples that were spiked and not spiked with AFB1 standard, whereas Cstd is the concentration of AFB1 standard.

materials or cereal- based products, processed cereals and ready to eat foods such as snacks. So, total dietary AFB1 intake could likely be underestimated.

Conclusions

This study revealed the occurrence of AFB1 in various

food products collected in Thailand. Approximately 87% of the food samples analyzed were positive but in most of the samples it was found that AFB1 levels did not exceed the maximum limit of 5 $\mu\text{g}/\text{kg}$ prescribed by WHO except peanuts and peanut derived products and since they are used as an ingredient or snack, they must be considered for food safety. However, further research study should

Table 2. Occurrence and concentration of Aflatoxin B1 in food samples.

Food sample	Sample (n)	Positive sample	%	Min-Max ($\mu\text{g}/\text{kg}$)	Average, $\mu\text{g}/\text{kg}$ (Mean \pm SD)
Local alcoholic beverages	7	5	71.42	0.3-2.15	0.48 \pm 0.71
Blue cheese	5	5	100	0.5-1.25	0.95 \pm 0.18
Fermented soybean	18	15	83.33	0.2-3.2	1.54 \pm 0.25
Raw peanuts	30	26	86.67	0.2-8.05	6.83 \pm 0.56
Peanut derived products	40	36	90	0.1-73.85	5.6 \pm 0.12

Limits of detection was 0.36 ppb. Three replicates were tested. 1. The MRL of total Aflatoxins and AFB1 in human foods are 4 and 2 $\mu\text{g}/\text{kg}$, respectively, (EU -regulation). 2. The total Aflatoxins of unprocessed peanut and ready to eat peanut are 15 and 10 $\mu\text{g}/\text{kg}$, respectively, (Codex, 2001). 3. WHO prescribed the maximum limit for AFB1 is 5 $\mu\text{g}/\text{kg}$ in various foodstuffs.

be conducted by using more samples and using other methods such as HPLC together with immuno-affinity column to confirm results and reduce uncertain results so as to assess the risk of AFB1.

Conflict of Interests

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

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Full Length Research Paper

Callus induction and RAPD analysis of *Simarouba glauca* DC

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Callus induction for somatic embryogenesis from *Simarouba glauca* DC leaf explants of three genotypes (*S. glauca* 5, *S. glauca* 19 and *S. glauca* 21) was studied. Leaf explants (leaf segments from basal, middle and tip of the leaves) were cultured on two types of nutrient media; SGC1 and SGC2. Both media contained Murashige and Skoog (MS) medium with vitamins: 100 mg/L ascorbic acid, 0.5 mg/L 6-benzylaminopurine (BAP), 0.5 to 5.0 mg/L NAA (1-naphthaleneacetic acid), and 3.0 g/L sucrose. The SGC2 media additionally contained 0.5 to 5.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). SGC2 media generated better callusing response compared to SGC1, thus displaying the importance of using 2,4-D in combination with NAA for callus induction. MS medium containing 2.5 mg/L NAA (SGC1.5) was noted to be the most effective in the initiation of friable embryogenic callus. On the other hand, MS medium containing a combination of 2.0 mg/L NAA and 2.0 mg/L 2,4-D was effective in the early initiation of friable embryogenic callus. In addition, a higher frequency of callus formation was observed from basal leaf segment as compared to that from middle and apical leaf segments. A random amplified polymorphic DNA (RAPD) analysis was also performed to see the genetic differences between the three *S. glauca* genotypes used in this study. The performance of *S. glauca* 5 and *S. glauca* 19 for higher callus frequency over the *S. glauca* 21 could be attributed to the genotypic differences between these genotypes. Overall, our protocol using SGC 2.4 media yielded optimal results and is suitable for large scale micropropagation of *S. glauca*.

Key words: *Simarouba glauca*, somatic embryogenesis, callus, plant growth regulators, RAPD and biofuel.

INTRODUCTION

Simarouba glauca DC, commonly known as “paradise tree”, is an emerging and promising plant for biofuel production. At approximately 65% oil seed content, when compared to current sources of biofuels, such as *Camelina*'s 43% oil seed content, the *S. glauca* tree

produces a much higher oil yield (Zubr, 1997). Each fully grown tree yields 15 to 30 kg nutlets, which is equivalent to 2.5 to 5.0 kg of oil. Although, *S. glauca* has the potential to be an effective second generation biofuel crop, it requires a long pre-bearing period (5 to 6 years) to

mature and bear seeds (Mansai and Gaikwad, 2011). The tissue culture technique, micropropagation, can rapidly multiply elite *S. glauca* germplasm. Somatic embryogenesis via callus route is preferred to the *in vitro* regeneration method because it is a useful tool for recovery of large number of plants with little somaclonal variation and also to recover genetically modified plants (Manasi and Gaikwad, 2011). Despite its promise as an effective biofuel source, little scientific research has been performed on *in vitro* propagation of *S. glauca*. Rout and Das (1994) reported somatic embryogenesis from immature cotyledon of *S. glauca* using 6-benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA). Of these, 20 to 25% of somatic embryos were regenerated into plants. It is well known in tissue culture that different genotypes of a species respond differently to a combination of hormones due to their underlying genomic variation (Rutkowska-Krause et al., 2003; Bregitzer et al., 1998). However, most *Simarouba* genotypes respond quite well to different combinations of auxins and cytokinins for induction of callus (Das, 2011).

Using a similar technique, Das (2011) showed that 2,4-D was effective for callus induction from cotyledons. *S. glauca* is an outcrossing species, thus, the genotype of cotyledon explants are ambiguous. Hence, to clone an elite *S. glauca* tree, regeneration from a vegetative part of the tree (shoot, leaf explants) is essential. A callus based regeneration system is necessary to assist genetic transformation of *S. glauca* to achieve quick genetic improvement. Therefore, we studied regeneration of *S. glauca* from leaf explants through a callus based, somatic embryogenesis system. There was a significant difference in the *in vitro* responses (callus induction and subsequent regeneration) of the three *S. glauca* genotypes we studied. Seeds were obtained from an open pollinated *Simarouba* nursery in Florida, USA. Each seed was grown individually as a plant in a greenhouse under controlled conditions and assigned a number. Three plants with promising physiological characteristics were chosen for the current study (SIM 5, SIM 9 and SIM 21). For this reason, we used a Random Amplified Polymorphic DNA (RAPD) analysis, which uses random primers to detect DNA polymorphisms in order to determine if there was identifiable genetic variability between the genotypes studied. Endogenously present PGRs in leaves are known to play an important role in tissue culture, by interacting with PGRs added to the nutrient media. For example, different segments of leaves have different levels of auxin (Davies, 2004). For a clearer understanding, each leaf blades were cut into four

segments: the tip, two middle segments and the base (near the petiole).

MATERIALS AND METHODS

Plant materials and culture method

The three different genotypes (two year old seedlings) of *S. glauca* used in this study were maintained in the climate controlled greenhouse at Penn State University Harrisburg. Young, fully developed leaves (1st to 3rd leaf) from the tip were examined. The leaves were washed with 5% Tween 20[®] for 10 min and moved to the laminar flow cabinet for surface sterilization and inoculation. Washed leaves were surface sterilized first with 10% Clorox[®] bleach for 5 min, then followed by 100 mg/l mercuric chloride for 4 min, which were then rinsed several times with sterile distilled water.

After surface sterilization, the leaves were cut into 4 equal size pieces; the apex, two middle parts (mid₁, mid₂), and the basal segment. All explants were inoculated on different media's (SGC1.1- SGC1.10 and SGC21.1- SGC21-10). The explants were placed with their adaxial or abaxial side touching the media in equal numbers, all of which were incubated in a TC60 growth chamber (Conviron[®]) at 25 ± 2°C in the dark. Sixteen explants were cultured into each media type and of the 16 explants, four were tip, four were mid₁, four were mid₂, and four basal explants. The explants were sub-cultured into fresh media every four weeks. The experiment was repeated with a similar number of replicates.

Media preparation

To assess the effect of NAA alone and in combination with 2,4-D on callus induction from *S. glauca* leaf explants, ten levels of NAA and 2,4-D (0.5 to 5 mg/L) were used in combination with a fixed amount of BAP as described in Table 1. The composition of SGC1 was MS medium (Murashige and Skoog, 1962) + 30 g/L of sucrose, 0.5 mg BAP, 100 mg of ascorbic acid, varying concentrations of NAA (0.5 to 5.0 mg/L), 0.7% agar, and pH adjusted to 5.8 before autoclaving at 121°C and 1.2 Kg/cm² pressure for 20 min to sterilize the medium. The composition of SGC2 was the same as SGC1 except that, in addition to NAA, another cytokinin, 2,4-D (0.5 to 5.0 mg/L) was added to the medium. SGC Control media only contained MS nutrients, 30 g/L sucrose, 7.0 g/L Agar and pH was adjusted to 5.8. Shoot induction media composed of MS nutrients, 30 g/L sucrose, 0.1 mg IBA, 0.5 mg/L adenine sulfate (AdSO₄) and 100 mg/L ascorbic acid, with varying levels of BAP (0.5 to 5 mg/L) and pH was adjusted to pH 5.8 (Table 1).

Observations

Observations were taken every three to four weeks. For each type of media, the number of calli growing from each of the 16 explants was noted and the percent of callus was obtained. Letters "L" for Loose and "F" for Friable were assigned to the type of morphology the callus exhibited. The callusing frequency was calculated as:

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Abbreviations: BAP, 6-Benzylaminopurine; NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog; PCR, polymerase chain reaction.

Table 1. Composition of growth media for callus and shoot induction.

Callus induction media				Shoot regeneration media		
SGC1*		SGC2*		SGS**		
Media #	NAA (mg/L)	Media #	NAA (mg/L)	2,4-D (mg/L)	Media #	BAP (mg/L)
Control	--	Control	--	--	Control	--
SGC1.1	0.5	SGC2.1	0.5	0.5	SGS1.1	0.5
SGC1.2	1.0	SGC2.2	1.0	1.0	SGS1.2	1.0
SGC1.3	1.5	SGC2.3	1.5	1.5	SGS1.3	1.5
SGC1.4	2.0	SGC2.4	2.0	2.0	SGS1.4	2.0
SGC1.5	2.5	SGC2.5	2.5	2.5	SGS1.5	2.5
SGC1.6	3.0	SGC2.6	3.0	3.0	SGS1.6	3.0
SGC1.7	3.5	SGC2.7	3.5	3.5	SGS1.7	3.5
SGC1.8	4.0	SGC2.8	4.0	4.0	SGS1.8	4.0
SGC1.9	4.5	SGC2.9	4.5	4.5	SGS1.9	4.5
SGC1.10	5.0	SGC2.10	5.0	5.0	SGS1.10	5.0

*The composition of SGC1 and SGC2 was 4.43 g/L MS-Vit. + 30 g/L Sucrose + 0.5 mg/L BAP + 100 mg/L ascorbate with the indicated levels of NAA and 2,4-D. **The composition of SGS was 4.43 g/L MS-Vit. + 30 g/L Sucrose + 0.1 mg/L IBA + 0.5 mg/L AdSO₄ + 100 mg/L ascorbate with the indicated levels of BAP.

Table 2. Primer sequences used for random amplified polymorphic DNA analysis.

Primer #	Primer sequence
OPG-1	5' AAGACGACGG 3'
OPG-2	5' AATCCGCTGG 3'
OPG-3	5' AGTCGGCCCA 3'
OPG-4	5' AACAGGGCAG 3'
OPG-5	5' TGGAAGCACC 3'
OPG-6	5' AGGCAGCCTG 3'
OPG-7	5' GATGCGACGG 3'
OPG-8	5' CCAGATGGGG 3'
OPG-9	5' GGGGGCTTGA 3'
OPG-10	5' TCCCGGTCTC 3'
OPG-11	5' GAAGGCTCCC 3'
OPG-12	5' GGGAGCGCTT 3'
OPG-13	5' GTTCTCGGAC 3'
OPG-14	5' AACTGGCCCC 3'
OPG-15	5' TGTCATCCC 3'

RAPD analysis

Total genomic DNA was extracted from young leaves using DNAzol (Life Technologies). The DNA quality and quantity was determined by Nanovue spectrophotometer and the comparison was done with standard DNA electrophoresis on 0.8% agarose gel. Initially, a total of 15 primers were screened to achieve reproducible PCR product (Table 2). All primers were analyzed for understanding polymorphic relationships between SIM 5, 19 and 21 and to build the UPGWA tree in Figure 4. The primer OPG-3 was selected for PCR amplification and the subsequent RAPD analysis. PCR amplifications were performed in triplicate using a PCR master mix from Promega in a 25 µL reactions containing DNA template. The amplification program was as follows: initial denaturation at 94°C for 1 min followed by 40 cycles of 94°C for 1 min; 36°C for 45 s; 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated on 1.2% agarose gel and photographed. For RAPD analysis reproducible, unambiguous DNA bands were scored as 1 for the presence or 0 for the absence of bands in each lane. The RAPD data obtained was analyzed using the online tool <http://genomes.urv.cat/UPGMA/index.php?entrada>.

RESULTS

The genotypes in the present study showed a wide range of response for callus induction on SGC1 and SGC2 media with different levels of NAA and 2,4-D. The frequencies of callus induction of leaf tissues from genotypes *S. glauca* 5, *S. glauca* 19, and *S. glauca* 21 ranged from 27.08 to 77.08% after 30 days, which lead to the production of friable callus. The friable callus frequency of 41.67 to 100% was observed within 120 days of incubation on SGC1 and SGC2 media (Figure 1, Table 3). Further sub-culturing calli lead to the formation of a friable callus in all genotypes on SGC1 and SGC2 media with varying frequencies (Figure 1).

$$\text{Callusing Frequency} = \frac{\text{Number of explants callusing}}{\text{Total number of explants cultured}} \times 100\%$$

The callus induction frequency of a particular leaf segment (tip, mid₁, mid₂, or base) was calculated by taking the average callus induction frequencies from genotype *S. glauca* 5, *S. glauca* 19 and *S. glauca* 21 on a particular medium using the above formula. The callusing frequency and callus type were also noted individually of the three different genotypes. Friable callus frequency was calculated as:

$$\text{Friable Callus Frequency} = \frac{\text{Number of friable callus}}{\text{Total number of explants callusing}} \times 100\%$$

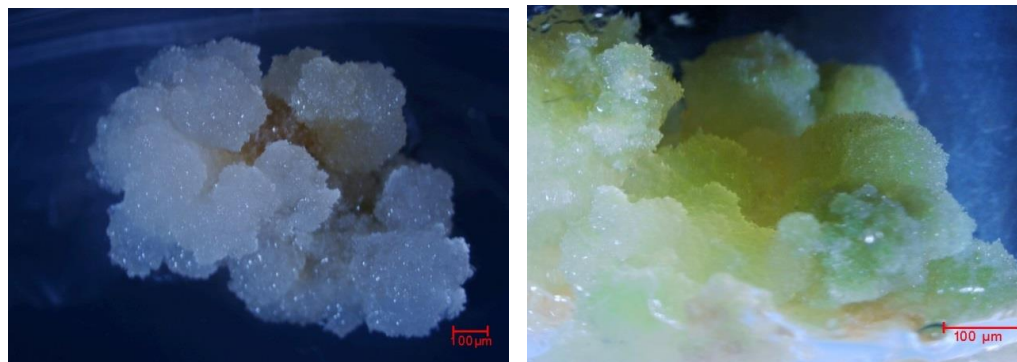


Figure 1. *Simarouba glauca* callus (a) after 30 days of culture and (b) after 120 days of culture.

Table 3. Effect of auxins on callus initiation of *Simarouba glauca*.

Media	SIM 5*			SIM 19*			SIM 21*		
	30 Day	90 Day	120 Day	30 Day	90 Day	120 Day	30 Day	90 Day	120 Day
Control	0.00 ± 0.00	0.00±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SGC1.1	47.92±3.65	72.92±3.42	50.00±7.22	31.25±2.71	60.42±1.88	0.00 ± 0.00	50.00±.77	68.75±4.69	41.67±7.51
SGC1.2	50.00±0.90	89.58±2.60	66.67±4.17	43.75±4.77	70.83±3.93	66.67±8.33	52.08±4.97	70.83±3.42	50.00±6.25
SGC1.3	54.17±2.76	89.58±1.38	91.67±2.08	29.17±3.76	58.33±4.77	66.67±8.33	43.75±4.13	75.00±3.93	83.33±4.17
SGC1.4	56.25±3.25	85.42±2.27	100.00±0.00	54.17±3.76	91.67±2.76	75.00±6.25	52.08±4.97	77.08±4.97	100.00±0.00
SGC1.5	41.67±5.21	81.25±3.25	83.33±2.08	70.83±5.13	100.00±2.90	100.00±0.00	54.17±4.45	83.33±3.42	91.67 ± 2.08
SGC1.6	64.58±2.27	95.83±1.04	100.00±0.00	41.67±5.51	68.75 ± 5.80	100.00±0.00	43.75±3.93	62.50±6.25	91.67 ± 2.08
SGC1.7	68.75±1.56	95.83±1.04	100.00±0.00	52.08±4.26	83.33 ± 4.07	100.00±0.00	41.67±4.13	77.08±3.17	100.00±0.00
SGC1.8	58.33±0.52	93.75±0.90	75.00 ± 6.25	52.08±6.89	64.58 ± 7.57	100.00±0.00	50.00±4.26	79.17±3.76	91.67 ± 2.08
SGC1.9	52.08±3.65	87.50±3.13	66.67 ± 5.51	29.17±4.54	64.58 ± 5.63	100.00±0.00	47.92±6.01	60.42±7.67	50.00 ± 6.25
SGC1.10	25.00±4.13	56.25±5.63	58.33 ± 7.51	39.58±2.76	81.25 ± 4.26	91.67±2.08	27.08±5.29	54.17±5.73	41.67 ± 7.51
SGC2.1	31.25±4.51	87.50±2.27	41.67 ± 7.51	43.75±2.39	68.75 ± 0.00	41.67±2.08	37.50±3.61	72.92±6.77	50.00 ± 7.22
SGC2.2	58.33±2.76	83.33±1.38	75.00 ± 6.25	47.92±1.38	68.75 ± 0.90	75.00 ± 0.00	50.00±4.69	64.58±6.65	75.00 ± 6.25
SGC2.3	62.50±3.25	93.75±2.60	75.00 ± 6.25	60.42±1.38	91.67 ± 1.04	91.67 ± 2.08	45.83±5.73	60.42±7.67	91.67 ± 2.08
SGC2.4	66.67±1.38	100.00±2.39	100.00±0.00	66.67±1.04	89.58 ± 2.27	91.67 ± 2.08	56.25±5.63	70.83±5.13	100.00±0.00
SGC2.5	66.67±0.52	93.75±2.27	100.00±0.00	70.83±1.38	75.00 ± 1.88	100.00±0.00	50.00±3.61	75.00±3.61	100.00±0.00
SGC2.6	64.58±1.38	87.50±3.17	100.00±0.00	72.92±2.90	79.17 ± 1.04	100.00±0.00	54.17±4.63	56.25±5.49	100.00±0.00
SGC2.7	72.92±2.27	89.58±0.90	100.00±0.00	77.08±3.42	83.33 ± 2.27	100.00±0.00	70.83±5.80	79.17±2.76	100.00±0.00
SGC2.8	72.92±2.27	91.67±1.38	100.00±0.00	70.83±4.97	75.00 ± 3.65	100.00±0.00	66.67±6.01	66.67±6.14	100.00±0.00
SGC2.9	62.50±0.90	81.25±1.88	100.00±0.00	70.83±1.88	87.50 ± 3.13	100.00±0.00	68.75±5.63	83.33±3.42	100.00±0.00
SGC2.10	56.25±0.90	95.83±1.04	91.67 ± 2.08	64.58±1.38	83.33 ± 4.17	91.67 ± 2.08	54.17±5.29	60.42±4.26	100.00±0.00

*Percent of explants with calli (Mean of three replications ± SE).

Table 4. Friable callus frequency after 120 days of culturing.

Media	SIM 5*	SIM 19*	SIM 21*
SGC0 (Control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SGC1.1	50.00 ± 7.22	0.00 ± 0.00	41.67 ± 7.51
SGC1.2	66.67 ± 4.17	66.67 ± 8.33	50.00 ± 6.25
SGC1.3	91.67 ± 2.08	66.67 ± 8.33	83.33 ± 4.17
SGC1.4	100.00 ± 0.00	75.00 ± 6.25	100.00 ± 0.00
SGC1.5	83.33 ± 2.08	100.00 ± 0.00	91.67 ± 2.08
SGC1.6	100.00 ± 0.00	100.00 ± 0.00	91.67 ± 2.08
SGC1.7	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC1.8	75.00 ± 6.25	100.00 ± 0.00	91.67 ± 2.08
SGC1.9	66.67 ± 5.51	100.00 ± 0.00	50.00 ± 6.25
SGC1.10	58.33 ± 7.51	91.67 ± 2.08	41.67 ± 7.51
SGC2.1	41.67 ± 7.51	41.67 ± 2.08	50.00 ± 7.22
SGC2.2	75.00 ± 6.25	75.00 ± 0.00	75.00 ± 6.25
SGC2.3	75.00 ± 6.25	91.67 ± 2.08	91.67 ± 2.08
SGC2.4	100.00 ± 0.00	91.67 ± 2.08	100.00 ± 0.00
SGC2.5	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.6	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.7	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.8	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.9	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.10	91.67 ± 2.08	91.67 ± 2.08	100.00 ± 0.00

*Percent of explants with calli (mean of three replications ± SE)

Callus induction on SGC1 media

Callus initiation of genotype *S. glauca* 19 was exception with the callus initiation frequency of 70.83 on SGC1.5 medium. After 90 days of incubation on callus induction media, the highest callusing response of 100% was seen in genotype *S. glauca* 19 on SGC1.5. In genotype *S. glauca* 5, the highest callus induction frequency was found to be 95.83% on day 90 of culturing on SGC1.7. Genotype *S. glauca* 21 yielded the highest callus induction frequency of 83.33% on day 90 of incubation on SGC1.5. After 120 days of sub-culturing, genotype *S. glauca* 19 performed well on SGC1 in comparison to genotypes *S. glauca* 5 and *S. glauca* 21 for friable callus production. It yielded 100% friable callus on SGC1.5, SGC1.6, SGC1.7, SGC1.8, and SGC1.9. 100% friable callus formation was observed in genotype *S. glauca* 5 on SGC1.4, SG 1.6 and SG 1.7 whereas genotype *S. glauca* 21 could produce 100% friable callus from SGC2.4 and SGC1.7 (Table 4).

Callus induction on SGC2 media

The highest callusing response of 100% was seen in genotype *S. glauca* 5 on SGC2.4 after 90 days. In genotype *S. glauca* 19, the highest callus induction frequency was found to be 91.67% on day 90 of culturing

on SGC2.3. Genotype *S. glauca* 21 yielded the highest callus induction frequency of 83.33% on day 90 of incubation on SGC2.9. After 120 days of sub-culturing, the three genotypes produced friable callus with different frequencies. An additional number of friable calli was obtained from SGC2 media than from SGC1. 100% friable callus formation was observed in genotype *S. glauca* 5 on SGC2.4, SGC2.5, SGC2.6, SGC2.7, SGC2.8, and SGC2.9. Genotype *S. glauca* 19 yielded 100% friable callus on SGC2.5, SGC2.6, SGC2.7, SGC2.8, and SGC2.9. 100% friable callus was obtained in genotype *S. glauca* 21 on SGC2.4, and SGC2.5, SGC2.6, SGC2.7, SGC2.8, SGC2.9 and SGC2.10 (Table 4). When the selected friable calli were transferred to the shoot regeneration media with BAP, tiny shoots began to appear (Figure 2). Observations were made after 60 days of culturing on shoot regeneration media. Friable calli on SGS1.6 with 3.0 mg/L BAP produced highest number of shoots in genotype *S. glauca* 5 and genotype *S. glauca* 19 (7.5 and 5.5, respectively). SGS2.5 produced the highest number of shoots/callus (5.5 shoots) in genotype *S. glauca* 21 (Table 5).

Callus induction from different leaf segments

Callusing frequency by leaf segments was noted to be highest on base followed by Mid₁, Mid₂, and the tip of the

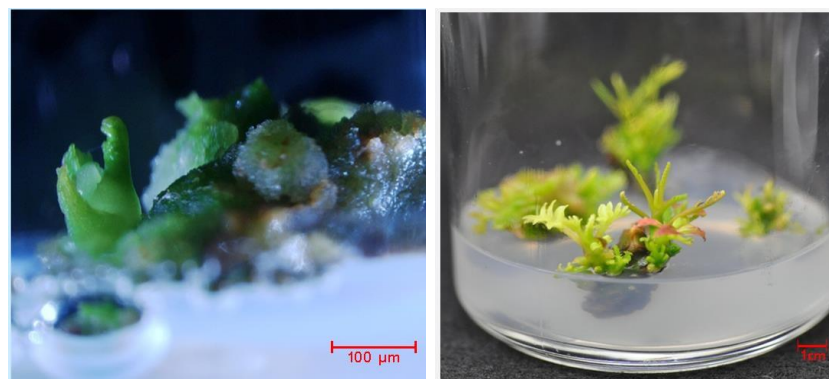


Figure 2. *Simarouba glauca* shoot formation (a) after 150 days of culture and (b) after 180 days of culture.

Table 5. Callus Induction frequency by leaf segments.

Media	Tip*		Mid 1*		Mid 2*		Base*	
	30 day	120 day	30 day	120 day	30 day	120 day	30 day	120 day
Control	0.00% ± 0.00	0.00% ± 0.00	0.00% ± 0.00	0.00%±0.00	0.00% ± 0.00	0.00%±0.00	0.00% ± 0.00	0.00%± 0.00
SGC 1.1	12.50% ± 6.94	62.50%±5.51	20.83% ± 5.81	54.17%±5.01	37.50% ± 5.81	87.50%±4.86	54.17% ± 3.67	95.83% ± 0.00
SGC 1.2	25.00% ± 5.81	70.83%± 5.81	29.17% ± 5.42	91.67%±2.20	50.00% ± 4.39	100.00%±3.03	54.17% ± 4.66	83.33% ± 0.00
SGC 1.3	16.67% ± 6.91	58.33% ± 5.68	33.33% ± 6.05	75.00%±5.51	41.67% ± 5.15	87.50% ± 4.22	45.83% ± 6.29	83.33% ± 0.00
SGC 1.4	37.50% ± 4.36	87.50% ± 2.08	37.50% ± 4.66	83.33%±3.67	45.83% ± 5.01	100.00%±1.39	54.17% ± 3.61	100.00% ± 0.00
SGC 1.5	33.33% ± 5.68	83.33% ± 1.84	37.50% ± 5.89	95.83%±1.39	45.83% ± 6.51	100.00%±1.39	58.33% ± 5.15	83.33% ± 0.00
SGC 1.6	25.00% ± 5.89	66.67% ± 4.66	37.50% ± 5.29	83.33%±2.95	54.17% ± 4.86	91.67% ± 2.20	62.50% ± 5.89	79.17% ± 0.00
SGC 1.7	37.50% ± 5.10	87.50%±2.95	50.00% ± 4.55	87.50%±3.03	62.50% ± 4.39	95.83% ± 2.08	70.83% ± 6.59	95.83% ± 0.00
SGC 1.8	29.17% ± 5.68	79.17% ± 3.67	37.50% ± 5.68	91.67%±2.78	50.00% ± 5.01	95.83% ± 1.84	62.50% ± 5.10	100.00% ± 0.00
SGC 1.9	33.33% ± 4.66	79.17% ± 5.51	41.67% ± 4.86	79.17%±5.42	50.00% ± 6.59	79.17% ± 5.51	54.17% ± 6.91	91.67% ± 0.00
SGC 1.10	29.17% ± 3.61	70.83% ± 6.71	33.33% ± 4.05	75.00%±6.29	41.67% ± 5.68	79.17% ± 6.62	45.83% ± 6.17	100.00% ± 0.00
SGC 2.1	25.00% ± 6.17	79.17% ± 2.78	33.33% ± 5.51	79.17%±4.39	50.00% ± 3.87	87.50% ± 5.89	50.00% ± 3.87	91.67% ± 3.61
SGC 2.2	29.17% ± 6.40	83.33% ± 3.03	41.67% ± 4.17	83.33%±2.95	41.67% ± 4.55	87.50% ± 5.68	66.67% ± 2.20	95.83% ± 5.68
SGC 2.3	33.33% ± 5.68	87.50%±2.94	45.83% ± 4.55	91.67%±3.03	58.33% ± 4.66	87.50%±4.66	62.50% ± 4.66	95.83% ± 2.08
SGC 2.4	41.67% ± 4.55	95.83% ± 1.84	54.17% ± 4.71	95.83%±1.84	62.50% ± 4.22	95.83% ± 3.61	83.33% ± 4.17	100.00% ± 4.66
SGC 2.5	50.00% ± 5.15	91.67% ± 1.39	62.50% ± 2.95	100.00%±4.17	66.67% ± 4.22	100.00%±5.01	75.00% ± 5.10	95.83% ± 4.55
SGC 2.6	50.00% ± 4.71	100.00%±2.78	54.17% ± 4.71	95.83%± 5.29	62.50% ± 3.67	100.00%±5.42	83.33% ± 5.01	100.00% ± 7.05
SGC 2.7	54.17% ± 4.55	95.83% ± 1.39	62.50% ± 4.05	95.83% ± 2.20	62.50% ± 5.01	95.83% ± 4.17	79.17% ± 4.22	100.00% ± 5.01
SGC 2.8	50.00% ± 5.89	87.50% ± 4.22	45.83% ± 5.93	95.83% ± 3.03	66.67% ± 4.66	100.00%±4.22	75.00% ± 4.17	100.00% ± 3.67
SGC 2.9	45.83% ± 5.15	100.00%±1.84	54.17% ± 4.17	100.00%±2.08	62.50% ± 4.17	100.00%±4.71	79.17% ± 3.61	100.00% ± 4.71
SGC 2.10	37.50% ± 5.29	83.33% ± 2.94	45.83% ± 3.87	87.50% ± 5.68	62.50% ± 3.03	100.00%±4.71	70.83% ± 3.67	95.83% ± 5.42

*Percent of explants with calli (mean of three replications ± SE).

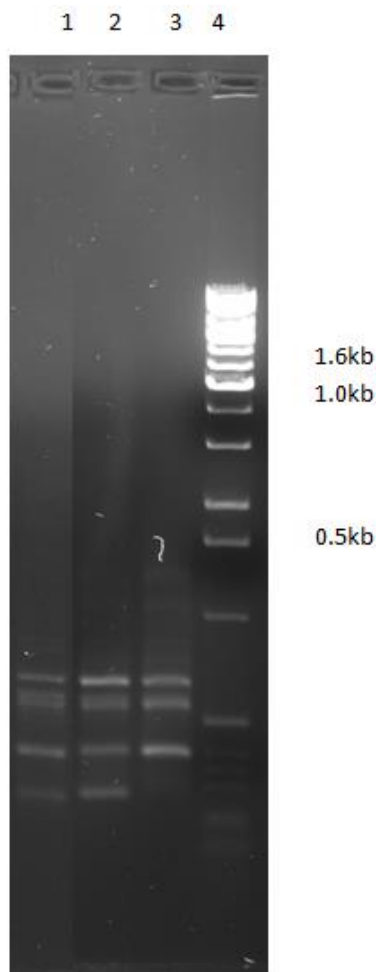


Figure 3. Gel electrophoresis of PCR with Primer 3 of genotype *Simarouba glauca* 5, *S. glauca* 19, and *S. glauca* 21. Lanes 1-4, *S. glauca* genotypes *S. glauca* 5, *S. glauca* 19 and *S. glauca* 21, respectively; lane 4: 1 Kb DNA extension ladder (Invitrogen).

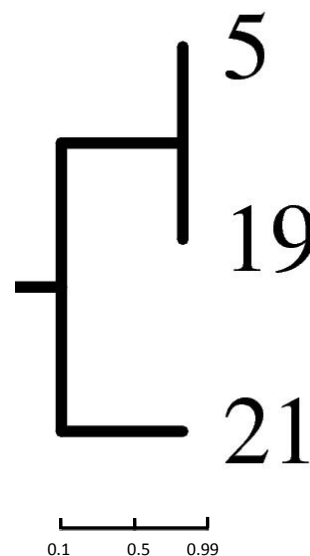


Figure 4. UPGWA tree inferred from the simple match coefficients of *Simarouba glauca* genotypes.

leaf. The more distal the segment was the lower callus induction frequency was observed (Table 5). A trend of increase in callus induction frequency was observed in all the segments with longer sub-culturing on SGC1, as well as on SGC2 media. However, SGC2 produced higher number of calli compared to SGC1 in a shorter time. After 30 days the highest callusing frequency on SGC1 of up to 70.83% was observed on the base segments. Mid₁ and Mid₂ segment of leaves could produce up to 50 and 62.50% callus, respectively. The callus induction frequency reduced in the leaf tip with the highest frequency of 37.50%. Similarly, on SGC2 medium, the base segment had the highest callusing frequency (83.33%). The callus induction frequency from Mid₂ and Mid₁ segments was up to 66.67 and 62.50%, respectively. The leaf tip could produce 54.17% callus.

After 120 days of culturing on SGC1 media, the callusing frequency of up to 100% was noted on the base and Mid₂ segments. A reduction in the callus induction frequency was observed in Mid₁ and tip segments; 91.67 and 87.50%, respectively. Callus induction frequency of 100% was seen on SGC2 media from all the segments used in the study after 120 days.

Genetic variation among *S. glauca* genotypes was studied using random amplified polymorphic DNA method. The genomic polymorphisms of the three genotypes of *S. glauca* produced with primers OPG-4 are presented in Figure 4. All genotypes yielded 1-10 PCR - amplified DNA fragments that ranged between 100 and 2000 bp (Figure 3). Only fragments with a high intensity were taken into account. The DNA polymorphism of SIM 19 and 21 were similar to each other, but different from that of genotype *S. glauca* 5 (Figure 4). Thus, the genotypes with high callus induction frequencies were found to be similar than the genotype with low callus induction rate.

DISCUSSION

Paradise tree, *S. glauca*, is a promising biofuel plant for both its high oil content and its adaptability to grow in a wide range of climate. In the present study, an optimum system of callus generation in *S. glauca* was investigated. Different combinations and concentrations of auxins and cytokinins have been used to induce callus in *S. glauca* (Rout and Das, 1994; 1999). In the present study, we tested two sets of media, SGC1 and SGC2.

Table 6. Effect of BAP on shoot regeneration from callus after 60 days of culturing on shoot regeneration media.

BAP mg/L	SIM 5	SIM 19	SIM 21
Control	0.00	0.00	0.00
0.5	4.50	3.00	2.00
1.0	6.00	3.50	4.50
1.5	4.50	4.50	4.50
2.0	5.00	4.00	4.50
2.5	6.50	5.00	5.50
3.0	7.50	6.00	5.00
3.5	6.00	6.00	5.00
4.0	4.50	3.50	5.00
4.5	3.50	1.50	3.00
5.0	2.50	1.50	1.50

Callus induction media SGC1 had increasing concentrations from 0.5 to 5.0 mg/L, while SGC2 had increasing concentrations of both NAA and 2,4-D from 0.5 to 5.0 mg/L. Previous studies have shown that 2,4-D when combined with NAA has a synergistic effect on the callus formation (Das, 2011). Our study shows that NAA, along with 2,4-D, is more efficient in initiating callus from leaf explants than NAA alone. The leaf explants showed faster response on SGC1 compared to SGC2 at 30, 60, 90 and 120 day time point. After 120 days, SGC2 had comparatively higher callusing frequencies in each media compared to SGC1. The combination of NAA with 2,4-D was also effective in obtaining friable callus from an already induced callus. The observation taken on 120 day time point indicated that 100% friable callus was obtained from media SGC1.5 - 2.9. Moreover, previous reports have indicated that the combination of 2,4-D with other auxins improved callus initiation (Debeaujon and Branchard, 1993; Khan et al., 2011; Qin Mao et al., 2006). Once the callus initiation is started, one auxin is sufficient to produce friable callus as in case of cucumber (Elmeir and Hennerty, 2008). Previous studies showed that high levels of auxins are required for the induction of embryogenic callus; however, cytokinins are critical for somatic embryo formation (Palmer and Keller, 2011; Webster et al., 2006). Shooting was also initiated from friable callus. Our experiments recorded an average number of 7.5 shoots per callus mass on media supplemented with 3.0 mg/L BAP (Table 6).

Callus induction and plant regeneration of many plant species is genotype-specific. Genotype *S. glauca* 19 performed well on the SGC1.5 medium in obtaining both the initial callus and friable callus. Medium type SGC1.5, which contained only one auxin (NAA, 3.5mg/L), initiated 70.83% callus production and lead to the production of 100% friable callus in genotype *S. glauca* 19. Our result supports the previous findings with other plants. Our results indicate that, genotype *S. glauca* 19 could be used in future studies that require the production of friable callus with NAA as a sole source of callus initiator.

However, a combination of NAA and 2,4-D could be used on a wider selection of *S. glauca* genotypes for callus initiation leading to the production of friable callus; in this case, all the genotypes performed well in obtaining friable callus on SGC2 media and could be used in future studies of plant regeneration and transformation. Separating the leaf segments revealed that the base of the leaf gave the highest callus responses than the tip of the leaf. This indicated the role of important endogenously present PGRs at the basal level of the *Simarouba* leaf that interact with exogenous substances in the media supplemented. Levels of endogenous growth regulators vary, not only among different plant organs, but also among different tissues of the same organ. Our study indicates that the highest callus response comes from the basal part of the leaf rather than the tip of the leaf. The Ye et al. (2012) study on callus induction in *Zizyphus jujuba* found that the basal section had higher frequency of callus formation compared to the apical and middle sections of the same leaf, underlining the fact that harmonized levels of endogenous and exogenous growth regulators are required to yield better results (Davies, 2004).

Different genotypes can respond to tissue culturing differently. This differential response perhaps is because of the genetic differences among these genotypes (Hadrys et al., 1992). In the present study, genotype *S. glauca* 5 and *S. glauca* 19 showed higher callusing frequencies compared to genotype *S. glauca* 21. RAPD analysis with OPG-3 primer revealed difference in banding patterns of PCR-amplified product. Genotype *S. glauca* 5 and genotype *S. glauca* 19 shared more DNA bands as compared to those with genotype *S. glauca* 21. Our results indicate that genotypic differences noticed through RAPD analysis directly reflect the differences noticed in the callusing frequencies. Previous studies using RAPD analysis have shown a strong association between the genetic diversity and geographical distribution of crops (Li et al., 2012). The genotypic differences that appeared in RAPD also suggest that the genotypes used in the present study came from different geographical locations.

Since, *S. glauca* is highly cross-pollinated, the genetic variation observed in the present study could be due to heterozygosity at some marker loci. However, further studies are required to ascertain if these loci could be associated to the difference in callus induction in these genotypes. These loci could be used as molecular markers to screen the *S. glauca* genotypes for high callus initiation response. Our results indicate that SGC 2.4 media produced optimum results and is economical in the long run for large scale micropropagation of *S. glauca* or for establishing a regeneration system for genetic transformation.

Conflict of Interests

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

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