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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Somaclonal variation associated with oil palm (*Elaeis guineensis* Jacq.) clonal propagation: A review

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Somaclonal variation refers to any phenotypic or genotypic modifications that arise from *in vitro* culture. In the oil palm, it is characterized by fruit mantling and abnormal vegetative growth. Tissue culture remains the only means of micro propagation of oil palm as its biological characteristics do not allow for vegetative propagation by conventional means. The early success of plantlets production inspired many oil palm organizations to explore *in vitro* propagation technique. Though oil palm tissue culture is already well established, it is still faced with many challenges. Prominent among them is somaclonal variation which was first reported in 1986. They are only detectable when the palms start flowering; that is, after two to three years in the field. It has not been possible to fully eliminate or circumvent floral abnormality in the oil palm. However, the adoption of several measures such as reducing hormone level, avoiding fast growing callus and, reducing culture period, have reduced the problem to manageable levels of < 5%. Possible causes and factors influencing somaclonal variation in the oil palm are discussed.

Key words: Somaclonal, variation, propagation, *Elaeis guineesis,* ramets, embryogenesis.

INTRODUCTION

Somaclonal variation is a genotypic or phenotypic modification due to a variety of causes resulting in variation of the progeny of clonal propagation. This means a significant percentage of the regenerated plants may not be identical in genotype and phenotype to the plant from which the original explants were obtained (Evans et al., 2003). Simply put, somaclonal variation is mutation that occurs in tissue culture. It is not restricted to but is particularly common in plants regenerated from callus such as the oil palm. The oil palm, *Elaeis guineensis* Jacq, belongs to the Family Arecaceae. It is a perennial monocot and monoecious, that is, male and female flowers occur separately on the same plant, usually in distinct male and female inflorescences (Corley and Tinker, 2003). It is one of the most efficient oil bearing crops in the world with average yield of 4 to 5 tons of crude oil per hectare and up to 7 to 8 tons of crude oil per hectare (Te-Chato and Hilae, 2007). The oil palm produces seven times more vegetable oil per hectare than soybean which is the largest source of edible vegetable oil in the world (Ndon, 2006). The major centre of oil palm production is in South East Asia with Malaysia and Indonesia together accounting for around 83% of world palm oil production in 2001 (Wahid et al., 2004).

The oil palm is an important economic crop, producing food and raw materials for the food, confectionary, cosmetics and oleo-chemical industrial demands of oil palm products. In the past, planting material consisted solely of seed-derived and mainly of the *Tenera* hybrids (fruit with shell of intermediate thickness) originally from crosses between *Dura* (thick shell) and *Pisifera* (thin

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shell) types (Rival, 2000). Conventional plant breeding techniques have also been used to develop, improve and conserve elite genotypes. However, since each selection cycle lasts for around 10 years, genetic improvement is inherently very slow, and high heterogeneity is still observed among hybrids. When these characteristics are joined with the low planting density (generally 143 palms/ha) and the necessity of establishing seed orchards for the production of commercial material, it can be seen that oil palm improvement is labour intensive, time consuming and therefore expensive (Rival, 2000). The biological characteristics of the oil palm do not allow its vegetative propagation by conventional horticultural means such as cutting, bud grafting, suckers etc. It has a single growing point and does not produce offshoots like some other palm species as all the auxillary buds form inflorescences and there is no reported method for the establishment of cutting (Vovola and Lord, 2004).

These constraints which exist in oil palm breeding make desirable the development of a micropropagation technique such as tissue culture which is an efficient and effective method for rapid multiplication of uniform planting material with high genetic potential.

BACKGROUND OF OIL PALM TISSUE CULTURE

The tissue culture technique for oil palm was developed in the 1970s (Jones, 1974; Rabechault and Martin, 1976). The oil palm industry was quick to capitalize and commercialize this new technology as the clones hold a promise of an estimated 30% increase in yield compared to commercial seedlings (Hardon et al., 1987). One possible way to clonally propagate elite oil palm is by means of somatic embryogenesis on calli derived from various origins (Rival, 2000). The early success of plantlet production in the 1970s and increased yield potentials inspired many oil palm organizations to exploit the *in vitro* propagation techniques. Most of the laboratories involved in the oil palm industry now have well established tissue culture media and protocols. However, the large number of variables in the culture process has meant that different laboratories often obtain different results from superficially similar treatments. Even in the same laboratory, results are not always reproducible, so there remains some uncertainty as to the best procedure (Corley and Tinker, 2003). To date, whole plants have been successfully regenerated from various explants and callus derived from seedlings of the oil palm. They include mature and immature embryos, apical meristems, embryogenic cells, suspension cultures, friable, embryogenic tissues, roots, inflorescence and young leaves (Teixeira et al., 1993, 1994). However, in 1986, problem of clonal fidelity associated with somaclonal variation was reported (Corley et al., 1986).

It was noticed that some of the clones planted were not flowering normally, instead had a high incidence of flowers with 'mantled' character (conversion of stamen whorl into a carpel whorl). This led to the destruction of several hectares of clonal palms. Many laboratories reduced production, but maintained enough for field evaluation. Somaclonal variation has till date impeded commercial large-scale production of oil palm planting material via *in vitro* culture. The somaclonal variant can exhibit a marked heterogeneity in its occurrence and intensity between different clone lines, between palms of the same line, and between flowers of the same individual variant palm (Jaligot et al., 2000). With concerted research efforts in various tissue culture laboratories in the world, more information and understanding on the tissue culture process and the problems arising from it have accumulated. It is therefore hoped, that floral abnormality in oil palm may soon be overcome or circumvented. This review seeks to address the origins and mechanisms of somaclonal variation, its causes and factors influencing it; the phenomena in oil palm clones, symptoms and problems associated with it as well as control measures.

ORIGINS AND MECHANISMS OF SOMACLONAL VARIATION

According to Evans et al. (2003) and Morcillo et al. (2006), there can be two major classes of somaclonal variation: genetic and epigenetic variations:

Genetic (heritable) variability is caused by mutation or other changes in DNA such as changes in chromosome number and structure. Various molecular mechanisms are responsible for genetic variability associated with somaclonal variation.

Changes in ploidy level

This refers to changes in chromosome number originating from abnormalities that occur during mitosis for example, aneuploidy (having more or less than an integral multiple of the haploid number of chromosome), polyploidy [having three or more times the haploid (n) number of chromosomes] and mixoploidy (the presence of cell lines with a different genetic constitution in an individual).

Structural changes in nuclear DNA

This appears to be a major cause of somaclonal variation. The change can modify large regions of a chromosome and so may affect one or several genes at a time. Such modifications include: deletion (loss of gene), inversion (alteration of gene order), duplication (duplication of genes), translocation (segments of chromosomes

moving to new locations), activation of transposons (transposable elements) and point mutation (chance rearrangement of the four bases) in the DNA structure.

Epigenetic (non- heritable) variability

This is stably transmitted modifications that result in stable phenotype differences even when the parent cells are genetically identical. Epigenetic changes are often temporary and plants may revert to the normal phenotype relatively easily, but some can be long lasting and may even be transferred during sexual propagation (Smulders and De Klerk, 2011). Epigenetic variability maybe caused by DNA methylation, DNA amplification, histone modification and activation of transposable elements (transposons). These modifications may influence gene transcription.

One major characteristic difference bet-ween genetic and epigenetic changes is that, whereas genetic changes occur at random, same epigenetic changes can be 'reproduced' when same condition are imposed during the production of another population (Smulders et al., 1995; Smulders and De Klerk, 2011).

SOMACLONAL VARIATION IN OIL PALM CLONES

Oil palm clonal propagation though successful, has not been without some difficulties. Problems with clonal fidelity associated with somaclonal variation have been encountered. Somaclonal variation, specifically in the form of flower and fruit mantling, has impeded commercial large-scale production of oil palm planting materials through tissue culture. According to Kushairi et al. (2010), the first case of abnormality in clonal palm was brought to the attention of a conference organized by the International Society for Oil Palm Breeders (ISOPB) in 1985 and later reported by Corley et al. (1986). It was observed that some clones planted were not flowering normally, instead had a high incidence of flowers with 'mantled' character (rudimentary stamen primordial on female flowers developed into supplementary carpels). That is, feminisation of the male parts in flowers of both sexes. This abnormality is accompanied by parthenocarpic (seedless) fruit set and severe bunch failure. Approximately, 5% of somatic embryo-derived palms show abnormalities in their floral development (Corley et al., 2003; Jaligot et al., 2000; Rival, 2000). The somaclonal variant can exhibit a marked heterogeneity in occurrence and intensity between different clone lines, between palm of the same clone line, and between flowers of the same individual variant palm.

Interestingly, reversions to the normal phenotype over time have been found to occur, leading to a complete recovery of the normal phenotype for 100% of the slightly 'mantled' individuals, and for 50% of the severely ''mantled'' ones after nine years in the field (Jaligot et al., 2000).

Causes and factors influencing somaclonal variation in oil palm

The causes of clonal abnormality in oil palm remain unknown but experimental evidence suggests that the floral abnormality observed in clonal palm may often be epigenetic in nature although genetic causes cannot be ruled out (Alwee et al., 2001). However, the following have been implicated in the problem.

Genotype of explants

Genotypic differences exist in oil palm and this can confer susceptibility/tolerance to the abnormality (Soh et al., 2011; Eeuwens et al., 2002).

Hormone type and concentration

These have been associated with somaclonal variation especially high levels of the chlorinated auxin 2,4-dichlorophenoxy acetic acid (2,4-D), reported to cause mitotic spindle abnormality (Duval et al., 1988; Sogeke, 1998).

Length of culture period

The longer the cells that remains in culture, the greater their chromosomal instability. Prolonged culture period *in vitro*, especially the callus stage shows a higher frequency and degree of abnormality (Corley et al., 1986; Rohani et al., 2003).

Use/type of callus in culture

In vitro systems that uses callus have been found to produce aberrant clones the most. Nodular compact calli have been found to produce on the average, 5% variant palms with fast growing calli producing up to 100% (Jaligot et al., 2000).

In vitro culture regime

The composition of the growth medium can trigger changes in ploidy and cultures grown under nutrient limitations can develop abnormalities. Tissue culture media can change the level of DNA methylation and thus maybe one of the important causes of somaclonal variation (Evans et al., 2003; Morcillo et al., 2006).

Table 1. Estimated world production of oil palm tissue culture plantlets.

Country	Million ramets/year
Malaysia	2.5
Costa Rica	0.5
Indonesia	0.5
Total	3.5

Source: Kushairi et al. (2010).

Type of regenerative process (organogenesis or embryogenesis)

The oil palm currently uses the indirect somatic embryogenesis method and most somatic embryo-derived palms show abnormality in their floral development (Armstrong and Phillips, 1988). The frequency of somaclonal variation in regenerated plants depends on the genotype of the material, type of explants used, age of culture, type of regenerative process (organogenesis or embryogenesis), stress inherent in cellular deprogramming induced by plant growth regulators such as the synthetic auxin 2,4-dichlorophenoxy-acetic acid (2,4-D) (Morcillo et al., 2006). Other stress conditions could be water deficiency, osmotic stress, heat, extreme procedure in tissue culture, nutrient media conditions etc. The age of explants source also influence somaclonal variation as the frequency of changes in ploidy increases with age of plant cells and therefore in chromosomal instability (Evans et al., 2003; Smulders and De Klerk, 2011).

WORLD PRODUCTION STATUS OF OIL PALM PLANTLETS

About 20 oil palm laboratories with varying capacities are in operation throughout the world. According to Kushairi et al. (2010), it is estimated that there is a ready market for more than 100 million tissue culture plantlets annually based on current demand for oil palm seeds in Malaysia and other countries. Some five million oil palm plantlets are currently being produced annually worldwide (Table 1) with Malaysia alone contributing over 80% of the total production from 11 commercial tissue culture laboratories such as Advanced Agric-ecological Research (AAR) and Federal Land Development Agency (FELDA).

Oil palm clonal abnormality is epigenetic?

Experimental evidences abound that suggest or point to the epigenetic nature of oil palm clonal abnormality.

1) Reversion of abnormal palms in the field over a period of years (Jaligot et al., 2000; Morcillo et al., 2006).
2) Non-Mendelian transmission of the trait by convention genetic crossing that is, inherited in non-Mendelian fashion (Morcillo et al., 2006).
3) Absence of any detectable defect in DNA organization (Jaligot et al., 2000).

Plate 1. Normal oil palm fruits. Source: Soh et al. (2011).

Symptoms and problems associated with oil palm clonal variation

The oil palm clonal abnormalities are only detectable once palm start flowering; that is, after two to three years in the plantation. Therefore, the cost, time and labour spent on production constitute huge losses. The abnormality is characterized by:

1) Flowers with ‘‘mantled’’ character (Plates 1 and 2); that is, flowers develop a secondary carpel whorl instead of stamen (apparent feminization of male parts in flowers of both sexes) (Corley et al., 1986; Alwee et al., 2006; Jaligot et al., 2000).
2) Parthenocarpic (seedless) fruit set, small bunches and severe bunch failure, Plates 3 and 4 (Corley et al., 1986; Rival, 2000).
3) Partial or complete flower sterility, poor pollination, extended juvenility (Plates 5 and 6), fruit abortion and therefore no yield (Soh et al., 2011; Morcillo et al., 2006).
4) Abnormal vegetative growth (Plate 7 and Table 2) (Guzman and Peralta, 2010).

Control measures to floral abnormality

It has not been possible to fully eliminate or circumvent floral abnormalities in oil palm clones. However, the adoptions of the following measures have kept it to manageable levels of less than 5%.

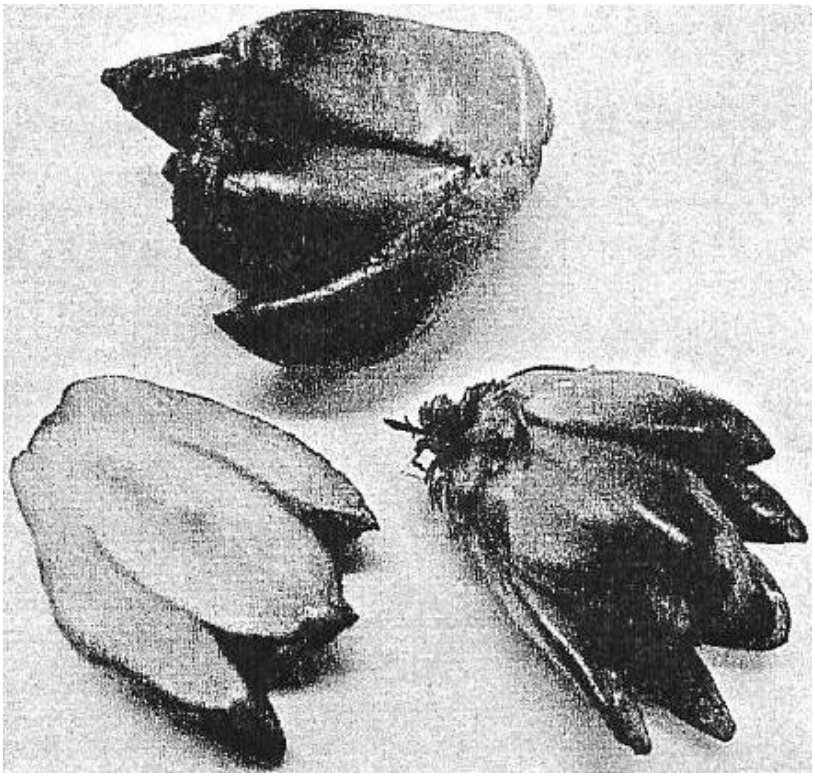

Plate 2. 'Mantled' oil palm fruits. Source: Soh et al. (2011).

Plate 3. (a and b) Normal and abnormal bunch.Source: Smulders and De klerk (2011).

1) Reducing hormone levels or total avoidance of them in certain culture stages (Duval et al., 1988).

2) Replacing chlorinated auxin, 2,4-D with non-chlorinated auxin, NAA (Sogeke, 1998).

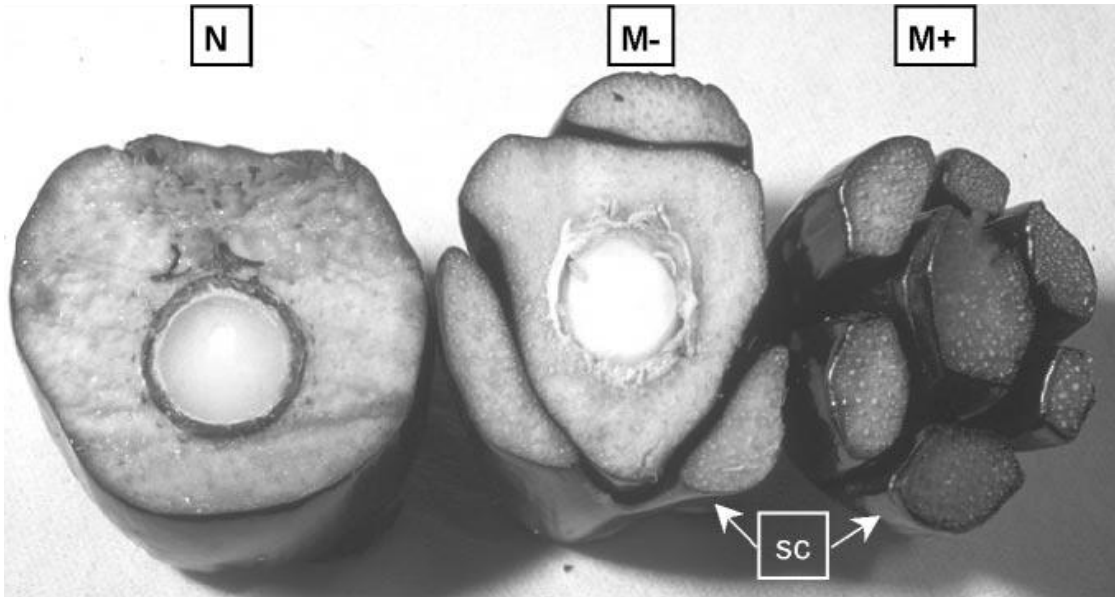

Plate 4. Cross-section of oil palm fruits originating from somaclones. N, fruit from normal; M, slightly mantled; M+, severely mantled; SC, supplementary carpel. Source: Jaligot et al. (2000).

Plate 5. Somaclonal oil palm with extended juvenility.Source: Soh et al. (2011).

Plate 6. Oil palm tree with normal fruits and leaves fruits/leaves. Source: Soh et al. (2011).

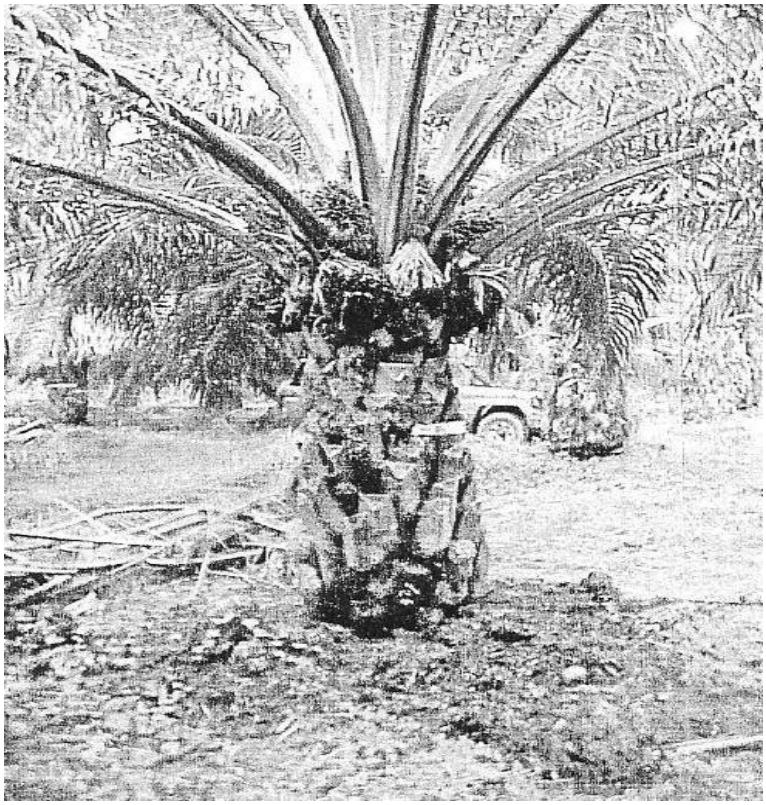

Plate 7. Oil palm with abnormal vegetative growth. Source: Soh et al. (2011).

Table 2. Comparison of normal and abnormal ramets.

Normal ramets	Abnormal ramets
Successive leaves in a shoot differ in shape.	Successive leaves are similar in shape
Lower (older) leaves are smaller than newly formed, expanded leaves	Older and new leaves attain similar size
Soft and fibrous texture of leaves	Hard and plastic-like texture of leaves.
Newly, expanded leaves with a prominent angle with respect to the vertical	Newly, expanded leaves with little or no angle with respect to the vertical
Pale green new leaves	Dark green new leaves

Source: Guzman and Peralta (2010).

3) Reducing culture period by reducing the number of subculture cycles and number of plantlets produced. Discard after about two years (Rohani et al., 2003).
4) Avoid fast-growing callus as high multiplication rates magnify any problem (Jaligot et al., 2000; Smith et al., 2010).
5) Stringent selection/strict culling at the earliest stages as a problem may not appear until after plants have been grown in permanent location (Kushairi et al., 2010; Guzman and Peralta, 2010).
6) Practicing strict process quality control (low contamination, optimum growing condition, stringent culture selection at each transfer (Soh et al., 2011).
7) Developing diagnostic tools for predicting genetic predisposition to abnormality. These include global gene expression analysis via DNA microarray, genetic mapping and candidate gene approach (Wahid et al., 2004; Morcillo et al., 2006).
8) Cloning a large pool of selected palms with different genetic background (genotypes) as there are genetic/clonal differences in susceptibility/tolerance to the mantling abnormality (Soh et al., 2011).
9) Direct organogenesis to by-pass the callus stage but this is not yet feasible in the oil palm though direct or 'true' somatic embryogenesis has been reported (Smith et al., 2010).

CONCLUSION

The oil palm is an important economic crop and the most efficient oil-bearing plant in the world. Due to increasing demand for oil palm planting materials and products coupled with the slow conventional breeding methods for its improvement, there is need for alternative means for improvement of elite hybrids. As oil palm has no known method of conventional vegetative propagation like other crops, clonal micropropagation becomes a viable means of rapid multiplication of elite genotype with desirable characteristics. The world production of oil palm tissue culture plantlets is about five million with Malaysia contributing above 80%; although, oil palm tissue culture has been successful but not without some difficulties. Major among these difficulties is somaclonal variation (epigenetic) expressed as vegetative and floral abnormalities which was first reported in 1986. This caused a major setback in the industry leading to the destruction of hundreds of hectares of oil palm plantation worldwide. Though these abnormalities still exist but at minimal and manageable levels of less than 5%. It is hoped that with current myriads of research and developmental efforts going on in various laboratories in the world to further resolve or circumvent the amenability and fidelity deficiencies in tissue culture, clonal palms are expected to eventually replace seed-derived planting materials on a commercial scale.

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Virulence, serotype and phylogenetic groups of diarrhoeagenic *Escherichia coli* isolated during digestive infections in Abidjan, Côte d'Ivoire

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The virulence, serotype and phylogenetic traits of diarrhoeagenic *Escherichia coli* were detected in 502 strains isolated during digestive infections. Molecular detection of the target virulence genes, *rfb* gene of operon O and phylogenetic grouping genes *Chua, yjaA* and TSPE4.C2 was performed. Prevalence of strains harbouring virulent genes was 7.8%. The virulent genes *eaeA, bfp, stx2, st1, lt, aggA, east1, ipaH, ial, cnf1* and *afa* were detected. EAEC (36%) and both EPEC and ATEC (25.6%) are the most detected pathovars (p<0.05). STEC (5.1%), NFEC (7.7) and DAEC (7.7) are less represented. Serogroups are overall diversified (89%), however, serogroups O157, O103 and O86, previously known to be associated with virulence were revealed. Most of the *E. coli* pathovars (53%) belonged to phylogenetic group A and in decreasing importance order, to D (23.5%), B1 (11.7%) and B2 (11.7%) groups. The study shows a diversified population of intestinal strains (84.6%), with a low phenotypic and phylogenetic link lower (p<0.05). Due to the great diversity of pathotypes, continuous monitoring should be implemented to identify risk factors and major pathways of contamination that help defining strategies to reduce infections associated with *E. coli*.

Key words: *Escherichia coli*, virulence gene, serogroup, phylogenetic group, diversity.

INTRODUCTION

Escherichia coli are known as a component of the intestinal microflora of humans and most homeothermic or warm-blooded animals (Bettelheim, 1997). However, strains that have acquired virulence factors are involved in digestive and extra-intestinal infections (Donnenberg, 2002). The epidemiological significance of virulent *E. coli* is well known and of public health concern (EFSA, 2011, 2012). The most recent outbreak occurred in Germany, and has spread in many countries of the European Union (Bielaszewska et al., 2011; Frank et al., 2011).

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Abbreviations: EPEC, enteropathogenic *Escherichia coli*; **ATEC,** atypical *E. coli*; **STEC,** shiga-toxin producing *E. coli*; **EAEC,** enteroaggregative *E. coli*; **ETEC,** enterotoxigenic *E. coli*; **NFEC,** necrotizing factor producing *E. coli*; **DAEC,** diffusely adherent *E. coli*; **EIEC,** enteroinvasive *E. coli*.

This outbreak was linked to the emergence of unusual serotype O104:H4 of Shiga toxin-producing *E. coli* (STEC), although with genetic proprieties related to enteroaggregative *E. coli* (EAEC) (Rasko et al., 2011). The outbreak has made an impact of over 3332 STEC infections in patients, 818 haemolytic uremic syndrome (HUS) cases with nervous system complications and 36 deaths (Wieler et al., 2011). The alternative for the control of such infections is usually based on epidemiological surveillance, itself based on spatio-temporal documentation of phenotypic and molecular determinants of pathotypes (OMS/FAO, 2008). The production of this data reveals the diversity of strains, risk factors or the presence of infectious chains which constitute elements for the implementation of outbreaks prevention models. Phylogenetic characteristics play an important role for traceability and knowledge of the evolutionary history of pathovars (Chaudhuri and Henderson, 2012). Multilocus sequence typing (MLST) is considered as the gold standard for phylogenetic characterization (Goldberg et al., 2006). However, Clermont et al. (2000) have developed a method easier and faster, based on triplex PCR detection of *Chua* gene, involved in the transport of heme in *E. coli* O157:H7 (Bonacorsi et al., 2000), yjaA gene characterized in the genome of *E. coli* K-12 (Blattner et al., 1997) and the anonymous fragment TSPE4.C2 (Bonacorsi et al., 2000). Using this method, it was possible to determine the relationship between virulent clones of *E. coli* and their phylogenetic group (Clermont et al., 2000).

Data on the molecular characteristics of pathogenic *E. coli* are rare in Côte d'Ivoire, while their involvement in frequent infectious diarrhea is established (Guessennd et al., 2008; Dadie et al., 2000). Recent studies on the subject are limited to a few virulence determinants and did not take into account many of the ten listed pathovars (Dadie et al., 2010; 2000). In addition, it has not been mentioned in previous studies of phylogenetic characteristics of strains.

The objective of this study was to determine the diversity of *E. coli* strains isolated during diarrheas, based on virulence determinants, serogroups and phylogenetic groups.

MATERIALS AND METHODS

Strains and sampling

Study material consisted of *E. coli* strains isolated from stool during infectious diarrheas among children and adults patients. They were collected from 2009 to 2011 in the laboratories of bacteriology at the Pasteur Institute of Côte d'Ivoire, Centre Hospitalier Universitaire (CHU) of Yopougon, CHU of Treichville, and the National Institute of Public Health of Adjamé (INSP). Reference and control strains of *E. coli* were used to validate the tests. These are *E. coli* ATCC 35401, which harbored virulence genes *lt, st*; *E. coli* M19 (*stx1, stx2*) obtained from the University of Brno, Czechoslovakia; *E. coli* Ec-368 (*stx1, stx2*), B2F1 (saa), received from Pasteur Institute, Paris, France; *E. coli* ATCC 43887 (*eaeA, bfpA*); *E. coli* O157:H7 EDL 933 (Schmidt et al., 1995). The sampling included strains isolated during a waterborne poisoning, which occurred in March 2010 in the municipality of Attécoubé in Abidjan. A total of 502 strains of *E. coli* were collected. The distribution of strains collected was as follows: 104 strains from children younger than 5 years, 197 from young children aged from 5 to 14 years and 201 patients aged more than 14 years. The control of collected strains identity was carried out, using API 20E (BioMérieux, Marcy l'Etoile France). Among the strains analysed, 230 were kept for at least 6 months at -70°C before the detection of virulence factors.

Virulence genes detection

A 200 µl suspension of bacterial culture of 24 h was boiled for 10 min at 100°C and the 10^5 g centrifuged solution resulting of this thermal shock was used as template DNA for virulence genes detection. The primers used are shown in Table 1. The amplification reaction was performed by PCR in a 25 µl reaction mixture consisting of 10X buffer (Bio-Rad, Marnes-La-Coquette, France), 1.5 mM $MgCl_2$ (Bio-Rad, Marnes-La-Coquette, France), 200 µM of each deoxynucleotide triphosphate (dNTP) (Fischer-Canada), 20 pmol of each primer (Sigma-Aldrich, Canada Ltd), 5 U/µl of Taq DNA polymerase (Bio-Rad, Marnes-La-Coquette, France) and 5 µl of the template DNA. The amplification was performed in a thermal cycler, Perkin Elmer 9700 (Applied Biosystems, USA). The amplification program includes an initial denaturation at 94°C for 3 min; 30 cycles of 1 min denaturation at 94°C, 45 s annealing at 56°C and 1 min elongation at 72°C; followed by a final extension for 5 min at 72°C. The revelation of the amplification products was performed on agarose gel 1.5% with ethidium bromide, 0.5 mg / ml.

Molecular serotyping

DNA extraction and purification

The molecular serotyping was performed using a purified DNA extract, according to the protocol of the Promega®Kit, "Wizard Genomic DNA Purification Kit". The *E. coli* strain to be studied was cultured in a tryptic-casein-soy broth (BioMérieux, Marcy l'Etoile France) for 18 h at 37°C. From the obtained suspension culture, 1 ml was collected in a 1.5 ml Eppendorf tube and centrifuged at 13000 *g* for 2 min. The pellet obtained was taken up in 600 µl of "nuclei lysis" solution (Promega, USA) and cell lysis was performed for 5 min at 80°C. To this lysate was added 3 µl of RNAse solution (Promega, USA). After homogenization, the mixture was incubated for 1 h at 37°C and vortexed after the addition of 200 µl of "Protein Precipitation" solution (Promega, France). The mixture was incubated for 5 min in an ice bath and centrifuged at 13000 *g* for 3 min. The supernatant was mixed with 600 µl of isopropanol, homogenized and centrifuged at 13000 *g* for 2 min. The pellet was resuspended in 600 µl of 70% ethanol and centrifuged at 13000 *g* for 2 min. After aspiration of ethanol, the tube was air dried for 15 min and the pellet was dissolved in rehydration solution and stored at 4°C.

PCR-RLFP for detection of operon O rfb gene

Typing procedure was performed according to Coimbra et al. (2000) method, slightly modified. The reaction was carried out, using kit reagents "Expand Long Template PCR System" (Boehringer, Mannheim, Germany). The primers used, 412.5'-CAC TGC CAT ACC GAC GCC GAT CTG TTG CTT GG-3' and 412.5'-ATT GGT AGC TGT AAG CCA AGG GCG GTA GCG T-3', are respectively complementary to JUMPstart and *gnd* (Wang and Reeves, 1998). A

Table 1. Primers used for PCR and amplicon size expected.

Gene	Primer sequence	Molecular size (pb)	References Gen bank/EMBL, Number
stx1	stx1f-5'-GAAGAGTCCGTGGGATTACG-3' stx1r-5'-AGCGATGCAGCTATTAATAA-3'	130	AF461172
stx2	stx2-5'-ACCGTTTTTCAGATTTTACACATA-3' stx2-5'TACACAGGAGCAGTTTCAGACAGT-3'	298	AY143337
st1	fp: 5′-TTTCCCCTCTTTTAGTCAGTCAACTG- bp: 5′-GGCAGGATTACAACAAAGTTCACAG-3′	160	M25607
east1	East1F-5'- CCATCAACACAGTATATCCGA-3' East1R- 5'- GGTCGCGAGTGACGGCTTTGT-3'	111	Monteiro-Nato et al. (1997)
cnf1	CNF1F-5'- GCTCAACGAGACTATGCTCTG-3' CNF1R-5- ACGCTGCTAAGTACCTCCTGG-3'	278	Falbo et al. (1992)
eaeA	eae5'-CACACGAATAAACTGACTAAAATG-3' eae5'-AAAAACGCTGACCCGCACCTAAAT-3	376	AE005595
bfpA	bfp f- 5'-TTCTTGGTGCTTGCGTGTCTTTT-3' bfp r'-5'-TTTTGTTTGTTGTATCTTTGTAA-3'	367	Yatsuyanagi et al. (2002)
ipaH	ipaHf-5'- TGGAAAAACTCAGTGCCTCT-3' ipaHr-5'- CCAGTCCGTAAATTCATTCT-3'	423	Lüscher et Altwegg (1994)
ial	ial F -5'-CTGGATGGTATGGTGAGG-3' ial R'-5'-GGAGGCCAACAATTATTTCC-3'	320	Frankel et al. (1989)
aggA	aggA -5'- AGACTCTGGCGAAAGACTGTATC -3' aggA5' ATGGCTGTCTGTAATAGATGAGAAC-3'	194	Schmidt et al. (1995)
lt	LT.f-5'- TCTCTATGTGCATACGGAGC-3' LT.r-5'- CCATACTGATTGCCGCAAT-3'	322	Frankel et al. (1989)
afa	afa f- 5'- CAGCAAACTGATAACTCTC-3' afa r-5'-CAAGCTGTTTGTTCGTCCGCCG-3'	750	Le Bouguenec et al. (1992)

50 µl reaction mixture, was constituted with 30.25 µl of PCR water (Fischer-Canada), 2.5 µl of dNTP, 10 mM, 1.5 µl of each primer, 15 pM, 5 µl of 10 X buffer, 1 µl of Promega®kit extract of template DNA and 3.5 U/µl of Taq polymerase (Bio-Rad, Marnes-La-Coquette, France). The amplification program consisted of two phases. Phase 1 composed of initial denaturation at 94°C for 2 min; 10 cycles composed of denaturation at 94°C for 10 s, annealing at 63°C for 30 s and elongation 68°C for 15 min. Phase 2 consist in 20 cycles of denaturation at 94°C for 10 s, annealing at 63°C for 30 s, elongation at 68°C for 15 min, 20 s and final elongation at 72°C for 7 min. Amplification products were subjected to a restriction by *Mbo*II (Amersham-Pharmacia-Biotech), in a reaction mixture of 25 µl, composed of 21 µl of amplification products, 2.5 µl of 10 X buffer and 1.5 µl of *Mbo*II, 12 U/µl (endonuclease). The restriction was performed at 37°C for 3 h and an irreversible denaturation of *Mbo*II was carried out by thermic shock at 72°C for 10 min. The digestion products were run on 2% gel consisting of 1% standard agarose and 1% Metaphor agarose in TAE buffer 0.5 X. The molecular weight marker used was composed of five volumes Amplisize (Bio-Rad, Marnes-La-Coquette, France) and a volume of lambda *Hind*III (Promega, USA). The data was processed with the software RestrictoTyper®(Institut Pasteur, Paris, France) and molecular profiles were translated into O antigen.

Determination of phylogenetic groups

The method of Clermont et al. (2000) was adapted to the experimental conditions of this study, by changing in certain components concentration of the reaction mixture and the amplification parameters. This method is based on the realization of a triplex PCR, using characteristic primers of genes *Chua* (Whittam, 1996; Bonacorsi et al., 2000), *yjaA* (Blattner et al., 1997) and the anonymous fragment TSPE4.C2 (Bonacorsi et al., 2000). The sequences of the three primers pairs used are reported in Table 2. The 25 µl reaction mixture consists of 1.5 mM of each dNTP,

Table 2. Primers sequences used for determination of phylogenetic groups.

Gene	Primer sequence	Molecular Size (pb)	Reference
ChuA	ChuA.1 -5'-GACGAACCA ACGGTCAGGAT-3' ChuA.2 5'-TGCCGCCAGTACCAAAGACA-3'	279	Whittam (1996)
YjaA	YjaA.1- 5'-TGAAGTGTCAGGAGACGCTG-3' YjaA.2 5'-ATGGAGAATGCGTTCCTCAAC-3'	211	Bonacorsi et al. (2000)
TspE4C2	TspE4C2.1.5'-GAGTAATGTCGGGGCATTCA-3' TspE4C2.2 5'-CGCGCCAACAAAGTATTACG-39	152	Clermont et al. (2000)

Table 3. Isolation frequency of virulent *E. coli.*

Patients class of age (year)	*E. coli* strains isolated	Virulent *E. coli* detected	Frequence SV (%)
< 5 years	104	19	18.2
5 - 14 years	197	11	5.6
> 14 years	201	9	4.5
Total	502	39	7.8

SV: Strains harboring virulence gene.

$MgCl_2$, 25 mM, 20 pmol of each of the three primers pairs, 10 X buffer, Taq DNA polymerase and 5 µl of DNA extract. Gene amplification was performed using an initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 15 s and elongation at 72°C for 30 s; followed by a final elongation for 6 min at 72°C. Interpretation of the results for classify species respectively in phylogenetic groups B2, D, A and B1 was performed according to the diagram of Clermont et al. (2000).

Statistical analysis

Categorical variables were compared using the Chi-squared test and Ficher's exact test (Armitage and Berry, 1987). The clonal relationship was assessed using Ward's method (Ward, 1963), which allows us to estimate the aggregation distance, through the creation of a dendrogram.

RESULTS

Prevalence of virulents strains of *E. coli*

The search for virulence factors, performed on a total of 502 *E. coli* strains revealed 39 (7.8%) harboring virulence genes, of which 19 (18.2%) isolated from younger than 5 years children; 11 (5.6%) from young children of 5 to 14 years and 9 (4.5%) of persons aged more than 14 years (Table 3).

Pathovars and their virulence factors

Virulence genes corresponding to pathovars are presented in Table 4. Virulence factors *east1* and *aggA*, which are characteristic of EAEC pathovars, were the most frequently detected (38.5%). Virulence genes specific to EPEC or ATEC (*eaeA, bfp*) were also detected in 10 (29%) strains, among which 5 (12.8%) were typical EPEC and 5 (12.8%) atypical EPEC (ATEC). The results also showed the presence of *stx2* genes, *ial, ipaH, afa* and *cnf1* that are respectively some characteristic pathovars of STEC, EIEC, DAEC and NFEC.

In this study, eight of nine pathovars known as agents associated with diarrheas caused by pathogenic *E. coli* were detected. The EAEC pathovars were more frequently ($p<0.05$) isolated (36%), followed by typical or atypical EPEC (25.6%). STEC were rarely detected (5.1%). The majority of EPEC and almost all DAEC were isolated from the population of children under 5 years. However, EAEC were detected in the same proportions among both children and adults. The frequencies of isolates are respectively 2.7% for EAEC, 2.4% for typical and atypical EPEC and 0.6% for each pathovar EIEC, DAEC and NFEC.

Molecular profile of operon O *rfB* gene and serogroups

The electrophoresis of restriction products of the amplified gene rfb of the operon O is shown in Figure 1, for nine virulent strains. It shows a variety of profiles compared to the molecular weight marker at 6 strains. However, the profile of pathovar HE5 is apparently identical to that of pathovar Ha102. Pathovars HE10 and He80 also have a profile different from the previous, but apparently similar for both strains. Some profiles obtained (LC26, Lc71, Lf19) did not correspond to serogroup

Table 4. Frequency of pathovars and their virulence factors.

Pathovar	Frequency Nb (%)	Virulence gene	Frequency Nb (%)
EPEC and ATEC	10 (25.6)	*eae* *bfp*	9 (17.3) 6 (11.5)
STEC	2 (5.1)	*stx1* *stx2*	0 2 (4)
EAEC	14 (36)	*east1* *aggA*	12 (23.1) 8 (15.4)
ETEC	5 (13)	*st1* *lt*	2 (4) 3 (5.7)
NFEC	3 (7.7)	*cnf1*	3 (5.7)
DAEC	3 (7.7)	*afa*	3 (5.7)
EIEC	2 (5.1)	*ipaH* *ial*	2 (4) 2 (4)

Nb: Number; EPEC: enteropathogenic *E. coli*; ATEC: atypical *E. coli*; STEC: shiga-toxin producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*; NFEC: necrotizing factor producing *E. coli*; DAEC: diffusely adherent *E. coli*; EIEC: enteroinvasive *E. coli*:

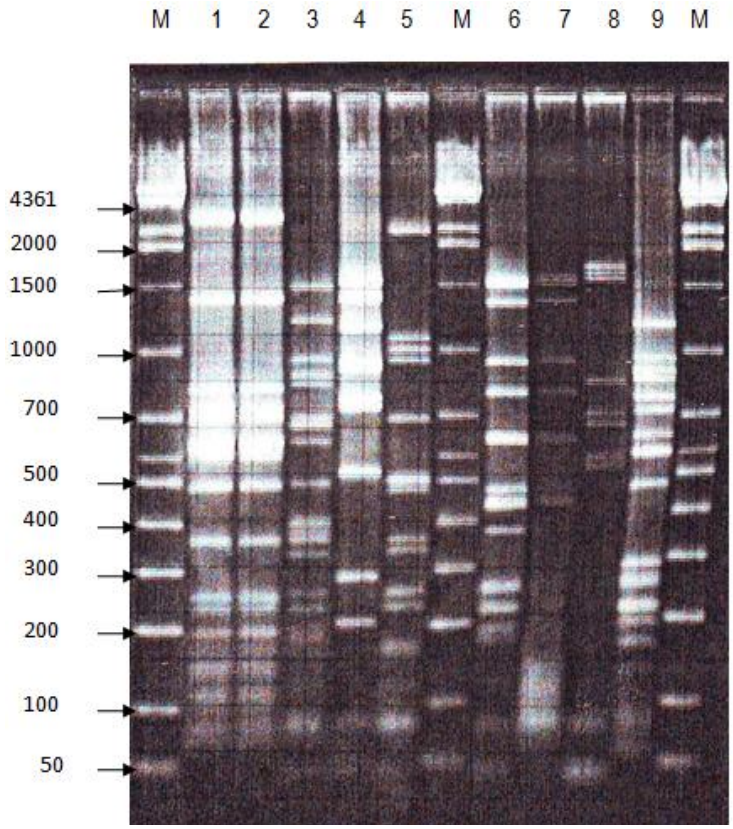

Figure 1. Electrophoresis of restriction products of the operon O *rfb* gene of *E. coli* strains. M: molecular weight marker, Pb: size in base pair; 1: He5 (O103); 2: Ha102 (O103); 3: He89 (O150); 4: Ha17 (O22); 5: He94 (O96); 6: He10 (O85); 7: He80 (O85); 8: He98 (O105); 9: Ha 87(O107).

Table 5. Pathovars distribution acording to their phylogenetic groups.

Phylogenetic group	EPEC	ATEC	ETEC	EIEC	STEC	EAEC	NFEC	DAEC	Total Nb (%)
A	2	1	4	1	0	7	1	2	18 (53)
B1.	0	2	0	0	0	1	0	1	4 (11.7)
D	2	0	0	0	2	4	0	0	8 (23.5)
B2	1	0	0	0	0	1	2	0	4 (11.7)
Total	5	3	4	1	2	13	3	3	34(100)

Nb: Number; EPEC: enteropathogenic *E. coli*; ATEC: atypical *E. coli*; STEC: shiga-toxin producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*; NFEC: necrotizing factor producing *E. coli*; DAEC: diffusely adherent *E. coli*; EIEC: enteroinvasive *E. coli*:

Table 6. Virulence, serotype and phylogenetic groups of *E. coli* strains**.**

Reference	Virulence	Sero group	Phylo group	Reference	Virulence	Sero type	Phylogroup
He2	*aggA*	O137	A	He83	*eae*		
He5	*cnf1*	O103	B2	He88	*aggA, east1*		A
He8	*afa*	O9	A	He89	*afa*	O150	A
He10	*eae, bfp*	O85	D	He94	*east1*	O96	A
He17	*lt*	O147	A	He96	*ipaH*		
He29	*east1, afa*		B1	He98	*east1*	O105	A
He33	*eae*	O109	A	He99	*aggA*		D
He45	*eae , bfp*	O43	A	He101	*eae, bfp*	O12	D
He78	*aggA, east1*	O130	D	He104	*ial*		A
He80	*eae, bfp*	O85	B2				
Ha11	*lt*	O110	A	Ha167	*stx2*		D
Ha17	*east1*	O22	D	Hd25	*lt*	O53	A
Ha31	*cnf1*	O39	A	Hd39	*aggA,*		B1
Ha41	*St1*	O141	A	Hd48	*aggA, east1*	O76	D
Ha46	*aggA, east1*		A	Hd79	*eae*	O80	B1
Ha55	*eae*	O86	B1	Hd91	*ipaH, ial*		
Ha68	*east1*		A	Hd108	*eae, stx2*	O157	D
Ha87	*bfp*	O107		Hd136	*east1*	O91	A
Ha102	*cnf1*	O103	B2	Hd140	*St1*	O17	A
Ha137	*eae, bfp*	O32	A	Hd177	*aggA, east1*	O40	B2

He: Strain from children <5 years, Ha: strains isolated from young children aged 5-14 years Hd: strains isolated from adults (> 14 years).

models in the database available after Taxotron treatment. The method used has revealed somatic antigen for 28 of 39 strains (72%). Strains HE5 and Ha102 belonged to the same O103 serogroup (Table 6). This was also the case of strains HE10 and He80 which belonged to serogroup O85. Serogroups where generally diverse and did not appear to be linked with age.

Electrophoretic profiles and phylogenetic groups of strains

The electrophoretic pattern of amplification products of genes obtained in our experimental conditions is shown in Figure 2. It shows bands of the four phylogenetic groups A, B1, B2 and D expected. It is a unique band of 279 base pairs (bp) of the *Chua* gene, indicating membership of strains HE10, HD48 and Ha17, to group D, the three bands of 279, 211 and 152 bp of the strain HE5, corresponding respectively to *Chua*, *YjaA* and TspE4C2X, which reflect their membership to group B2 strain; the 211 bp single band of strain Cg27 (group B1) and 152 bp single band (VH50) for belonging to the group A.

The phylogroup was revealed in 34 (87.2%) strains on a set of 39 isolated (Table 5). In descending importance order, the pathovars belonged to phylogenetic group A (53%), D (23.5%), B1 (11.7%) and B2 (11.7%). There was no pathotype belonging to a specific phylogenetic group. In addition, phylogenetic group membership was not also related to specific serotypes and strains ecosystem.

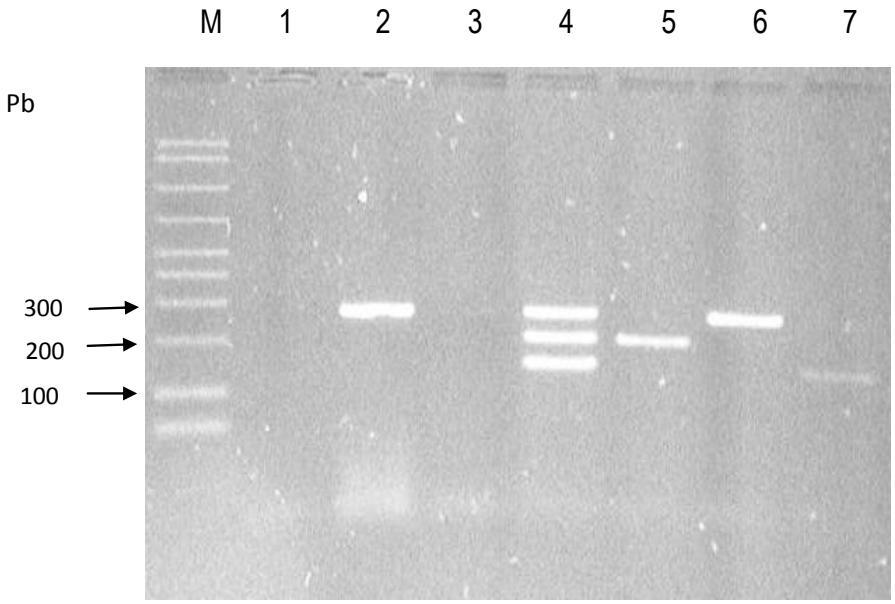

Figure 2. Electrophoretic pattern of amplification products of genes characteristic of phylogenetic groups. M: molecular weight marker, Pb: size in base pair; 1: He9; 2: He10; 3: Ha17; 4: He5; 5: Cg27; 6: Hd48; 7: Vh50

Table 6 shows the diversity on the distribution of pathovars in conventional phylogenetic groups. However, strains Ha102 and HE5, which shared the same virulence factor, as well as serotype, belonged to the same phylogenetic group (B2). As for pathovars HE10 and He80 which were bound by the virulence and serotype, they differed on their phylogenetic group. HE10 belongs to D and He80 to B2 group.

Clonal relation

The dendrogram of Figure 3 shows a low phylogenetic distance and thus a clonal link between pairs of species HE8 - He89 (A), HE10 - HE101 - He80 (D), He55-Hd79 (B1) and HE5 - Ha102 (B2). Only the last pair belongs to the same serotype and phylogroup.

DISCUSSION

E. coli pathovars and human diarrheas

The use of PCR revealed the diversity of strains on the basis of traits related to virulence. A total of 502 strains of *E. coli* were isolated, of which 39 (7.8%) harbored virulence factors associated with agents of infectious diarrhea. The prevalence of virulent strains is similar to that (7.3%) obtained by Rappelli et al. (2005), in a study of agents of childhood diarrhea in Mozambique. However, similar studies, conducted in children less than five years in South Africa (Galane and Leroux, 2001) and Tunisia (Al-Gallas et al., 2007) gave respectively 32.6 and 65%. The low prevalence of virulent *E. coli* in humans obtained in this study may be due to the fact that sampling took into account the strains isolated during a waterborne poisoning, occurred in March 2010 in the municipality of Attécoubé in Abidjan. A set of 170 strains of *E. coli* were isolated, of which only 5 (3%) had virulence factors. In addition, the fact that several strains were stored at -70°C for at least six months before analyses could lead to a loss of virulence factors (Markoulatos et al., 2002) and therefore also have an impact on reducing overall prevalence of virulent strains.

The results of our study show that almost half of the virulent strains (49%) were isolated from children less than 5 years. This result confirms those reported in previous work (Okeke et al., 2003; Valentiner-Branth, 2003; Rappelli et al., 2005), namely that children of this age group, represent the population most exposed and vulnerable to diarrhea of virulent *E. coli*. Among the 39 virulent strains of *E. coli*, 14 (36%) were entero-aggregative *E. coli* (EAEC). The EAEC pathovars would therefore be most frequently involved in human diarrhea in our environment. This observation not only confirms our previous results (Dadie et al., 2000), but also shows the emerging nature of this pathovar, as reported by other authors (Veilleux and Dubreuil, 2005; Franck et al., 2011). It is also known that EAEC play a special role in chronic diarrhea in people with HIV/AIDS infection (Kelly et al., 2003; Gassama-Sow et al., 2004). The high proportion of these pathovars in human strains studied may be due to the fact that several strains collected during sampling came from CHU of Treichville (Abidjan),

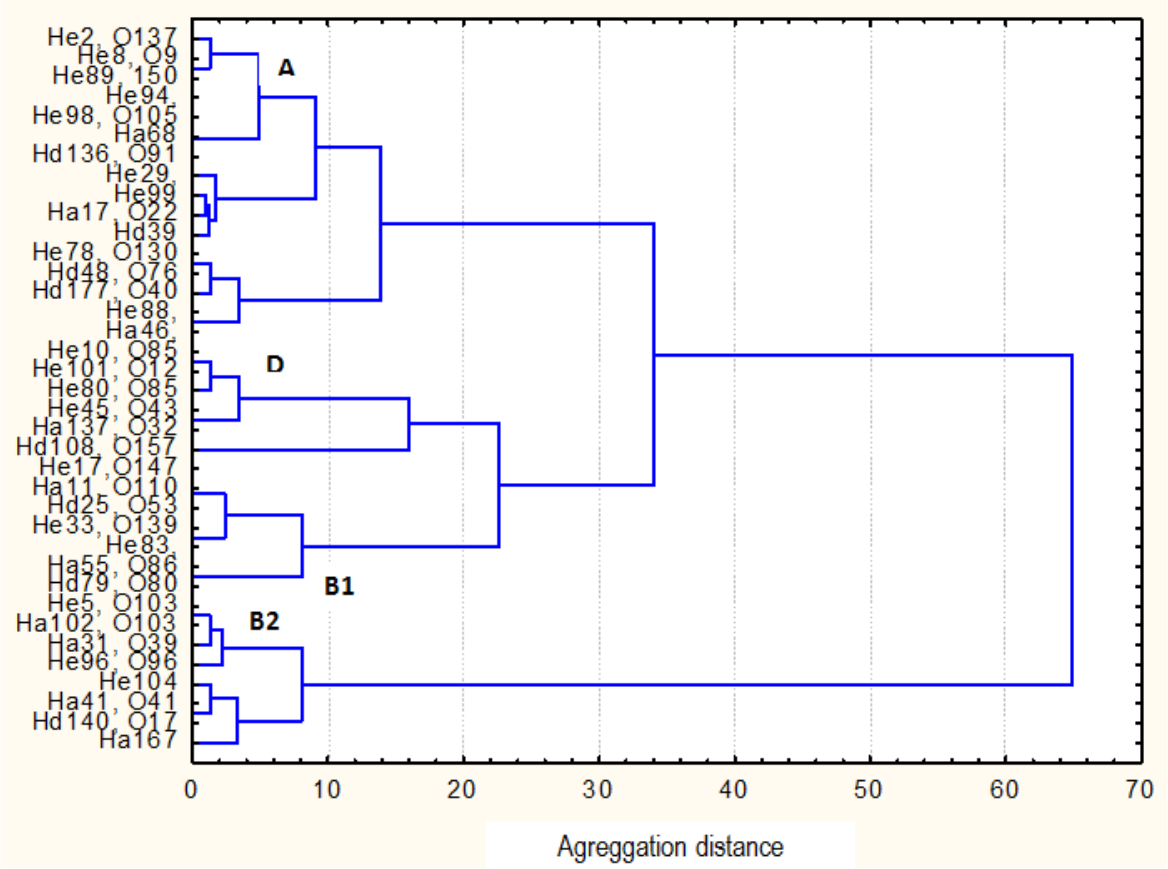

Figure 3. Dendrogram showing the phenotypic and phylogenetic relationship between pathovars of *E. coli* of human origin.

in a service receiving preferentially seropositive patients for HIV/AIDS. In this study, the rate of EPEC was 25.6 and in 60% of cases strains came from children under 5 years old. Many studies of EPEC on this part of the population showed that these pathovars were the leading cause of diarrhea in infants and children less than five years in Nigeria (Okeke et al., 2000), India (Bhan et al., 1989) and Bangladesh (Albert et al., 1991). Classification based on virulence determinants distinguishes the typical EPEC (*eae, bfp*) from atypical EPEC or ATEC (*eae*) which lacks EAF plasmid, encoding the *bfp* gene (Nataro et Kaper, 1998; Tobe et al., 1999).

In this study, on all 10 EPEC suspected, 5 (50%) presented characteristics of typical EPEC and 5 others, those of ATEC. The results show that ATEC are common in our environment and are involved in diarrheas like ECEP. Data confirms those of some authors (Nguyen et al., 2005; Orlandi et al. (2006), showing that the ATEC are widespread and sometimes dominant in many developing countries, but others found rather a predominance of typical EPEC on ATEC (Sooka et al., 2004: Moyo et al., 2007). Moreover, Nataro and Kaper (1998) indicated the difficulty of detecting specific *bfp* gene. The consequence is that the rate of EPEC detected in humans, could be better than the reality.

The ETEC pathovars represented 13% of the strains isolated during the infectious diarrhea associated with virulent *E. coli* in this study. The rates obtained by Okeke et al. (2003) in Nigeria and Sooka et al. (2004) in South Africa, respectively, were 8.8 and 8.42%. A greater importance of ETEC (32.4%) was found in Tunisia during the study of Al-Gallas et al. (2007). Each pathovar EIEC, DAEC and NFEC was detected, respectively, at a rate of 5.1 and 7.7%. The revelation of EIEC was rare or impossible in the studies respectively carried out in Guinea-Bissau (Valentiner-Branth et al., 2003), Mozambique (Rappelli et al., 2005), South Africa (Sooka et al., 2004) and Nigeria (Okeke et al., 2000; 2003). However, during a study on human diarrheas in Tunisia, EIEC pathovars were isolated by Al-Gallas et al. (2007) with a prevalence of 11.3%. The DAEC were all isolated from children under 5 years. Giron et al. (1991) reported that these pathovars were involved in diarrhea occurring most frequently in children less than five years, according to a mechanism of cell invasion similar to EPEC. However, some authors (Gunzberg et al., 1993; Nataro and Kaper, 1998) showed that DAEC did not always induce diarrhea in children. According to them, children

and adults are carriers of this type of pathovars, without evidence of specific symptoms. In addition, *E. coli* is known as the first agent of urinary tract infections (Stamm, 2002) in which the DAEC and NFEC pathovars are mostly involved (Santos et al., 2006), so the significance of the isolation of this type of pathovars in infectious diarrhea remains questionable.

Two strains of STEC harboring the *stx2* gene were isolated. The study confirms in this regard that STEC are not important etiological agents of diarrhea associated with *E. coli* in our environment, as we previously reported (Dadié et al., 2000). However, due to the evolving of epidemiological role of STEC in several African countries (Isaacson et al., 1993; Koyange et al., 2004; Hiko et al., 2008, Al-Gallas et al., 2007; Okeke et al., 2003), the establishment of a monitoring mechanism is necessary.

Serogroups and phylogenetic groups

The results of molecular serogrouping by detecting the *rfb* gene of operonO, do not generally show significant relationships between serotypes and pathotypes, but show a diversity of serogroups. Identified serogroups, including O157, O103 and O86 are known to be related respectively to pathovars STEC (O157 and O103) (Coimbra et al., 2000) and EPEC (O86) (Donnenberg, 2005; Kaper, 2004). If in the course of this study, the virulence genes classically attributed to these pathotypes were detected for some (O157 and O86); it is not for serogroup O103 which was rather carrying the gene *cnf1* instead of Shiga-toxin. The serogroup O86 was also not systematic to EPEC class, but may be characteristic of ETEC according to Germani (1995).

The multilocus sequence typing (MLST) is considered as the gold standard for phylogenetic grouping of species (Goldberg et al., 2006). However, the application of the method of Clermont et al. (2000) on our strains gave a sensitivity of 87% and a diverse phylogenetic distribution, with the majority (53%) of pathovars belonging to group A. The pathovars belonging to B2 group accounted for 11.7% and group D, 23.5%. During a study on 64 clinical strains, most of which composed of extra intestinal species, Clermont et al. (2000) found a distribution of 58% of B2 group agents and 17.1% of group D. It is established that most extra intestinal pathogenic strains belong to group B2 and to a lesser extent to group D (Bonacorsi et al., 2000). The low pathovars belonging to group B2 obtained in our study may be justified by the fact that the strains studied were of intestinal origin.

Both STEC (stx2) isolated in this study belonged to phylogenetic group D. The phylogenetic grouping of 10 strains of *E. coli* O157: H7, carried by Clermont et al. (2000) also showed that all isolates belonged to group D. Overall, the intestinal strains of our environment belonged to diverse phylogenetic groups, in contrast to what is known of extraintestinal strains that have a clonal population more than homogeneous (Clermont et al., 2000).

Clonal relationship between pathovars

The pathovars of the human ecosystem resulting from our study, were characterized by a high (89%) diversified population. The clonal relation is not significant between pathovars and there is no chain of contamination, may be due to one or some specific pathotypes. However, the markers of diversity used, show similar characteristics for two NFEC pathovars belonging to phylogenetic group B2. We know that the majority of extra intestinal pathovars belong to group B2 (Bonacorsi et al., 2000) and that NFEC also are frequently involved in urinary tract infections (Bielaszewska, 2007). The NFEC O103 clone detected could be a strain originally extra intestinal, probably an agent of urinary tract infection that transiently contaminated the digestive tract to be isolated during infectious diarrhea. In our study, two EPEC with similar phenotypic and genotypic factors were also isolated. It could be a single clone associated to infantile diarrhea and circulating in our environment. But to support this assertion, this clone should be isolated more frequently and an additional differentiation of strains of pulsed field gel electrophoresis (PFGE) type should be performed.

Conclusion

The study of strains diversity based on their virulence traits revealed the eight pathovars sought with greater frequency of EAEC and EPEC. The rare STEC highlighting should be seen as a warning, given to the epidemiological significance of these pathovars in several countries. Molecular serotyping performed by detecting the *rfb* gene of the operon O shows a variety of serogroups. Some serogroups conventionally known to have a link with specific pathovars were however identified. The majority of pathovars belongs to phylogenetic group A, although the phylogenetic group B2 agents have been highlighted. Belonging to group B2 is not specific for particular pathotypes. The study shows overall a relatively weak link between pathotypes. The great diversity of pathovars requires surveillance of virulent *E. coli*, for successfully identifying risk factors and the major routes of contamination, which determines the control of infections associated with pathovars.

Conflict of interests

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

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Full Length Research Paper

Evaluation of two okra species [*Abelmoschusesculentus* (L.) Moench and *Abelmoschuscaillei* (A. Chev.) Stevels] exposed to crude oil contaminated soil in Auchi, Edo State, Nigeria

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Six accessions of cultivated okra [*Abelmoschus caillei* (A. Chev.) Stevels and *Abelmoschus esculentus* (L.) Moench] were evaluated for growth parameters in crude oil contaminated soil. Morpho-agronomic characters such as numbers of days from sowing to germination, dry and fresh weight of the accessions in both soil samples was determined. Others were copper (Cu), zinc (Zn), manganese (Mn), cadmium (Cd) and Lead (Pb) concentration in plant parts (leaves and fruits). The growth responses of the different accession varied considerably. Soil chemical analysis revealed decreased levels of pH, phosphorus and potassium in the contaminated soil. The chemical analysis of plants grown in these soils showed that heavy metals like Cu, Zn, Mn, Cd and Pb were present in all the organs of the accession.

Key words: Crude oil, soil, growth parameters, *Abelmoschus esculentus*, *Abelmoschus caillei*, heavy metals.

INTRODUCTION

Okra is a widely cultivated vegetable in tropical and subtropical regions where it is grown for its leaves, fruits, seeds, flora parts and stems. These are edible when young and succulent and dried in powdery form. In these regions, the fresh unripe and tender pods (fruits) are sliced, grated, boiled or steamed or fried and beaten into a gelatinous soup. This soup facilitates the swallowing of relatively rough or coarse textured starchy foods (Schippers, 2000). It is also used raw in salads with egg plants. The mucilaginous exudes is an excellent thickener for stews and soups. The leaves are eaten as spinach; sun dried whole or sliced. Fruits are conserved for year-round consumption. Seeds are removed from the matured pod and sun dried. These are made into powder and used for flavoring. The genus *Abelmoschus* is said to have originated from South East Asia (Hamon and Hamon, 1991). The basis for this claim was because wild relatives abound there. Siemonsma (1991) recognized nine species in *Abelmoschus* based on cytogenetic evidence. The morphological characters such as number, dimensions and persistency of the epicalyx segment, form and dimensions of the capsule (including pedicel) and characteristics of the indementums are unique. In West Africa, the wide varieties of okra cultivars are of two distinct species, the common okra, *Abelmoschus esculentus* (L) Moench and West African okra *Abelmoschus caillei* (A. chev.) Stevels (Charrier, 1984).

A. esculentus is adapted to the Sudano-sahelian zone and the *A. caillei* is referred to as Guinean type in relation to its zone of cultivation (Charrier, 1984; Chedda and Fatokun, 1991; Schippers, 2000). This study focuses on these two cultivated species. Osawaru and Dania-Ogbe

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Table 1. *Abelmoschus* accessions used in the study, their origin and location.

Accession	Origin	Latitude and longitude
47-4	NIHORT	07° 22' N; 03° 52' E
LD-88	NIHORT	07° 22' N; 03° 52' E
OS/AC/001	Benin	06° 20' N; 05° 37' N
OS/AC/002	Benin	06° 20' N; 05° 37' N
OS/AE/006	Benin	06° 20' N; 05° 37' N
OS/AE/OO7	Benin	06° 20' N; 05° 37' N

(2010) reported that both species are wide spread between 12°N and 12°S and most commonly found between 5 and 12°N. The common okra is mainly grown for market gardening in areas with limited rainfall, sometimes under irrigation. West African okra, found throughout the high rainfall zone is cultigens of the region where it is liked for its ability to draw-well. Both are grown mainly for subsistence economy. Cytologically, Singh and Bhatnagar (1975), Siemonsma (1982), Ariyo (1993) and kehinde (1999) reported that West African okra contain 194 diploid chromosomes as against 130 of the common okra thereby indicating that West African okra is a separate species. However, there are overlapping characters in these features that are not clearly or exclusively defined (Josh and Hardas, 1976). *A. caillei* is highly polymorphic and unique in fruit and indumetum characters. The nutritional, industrial and cultural roles of these okra species is documented by Siemonsam and Hamon (2002), Schipper (2000), Grubben (1977), Obire (2002), Chevalier (1940) and Charrier (1984).

In this present study, we investigated the growth performance of *Aesculentus* and *A. caillei* in crude oil contaminated soil to access the metal sink. and Onofeghara (1984) and Udo and Fayemi (1975) reported that "at high concentrations of oil in soils, most plant species suffers serious depression in growth which has been attributed to poor soil conditions, dehydration and impaired nutrient uptake by the roots even when they are present, they are not usually in the absorbable form (ions) rather they are present as compounds. This is created by the presence of crude oil". Wong et al. (2001) reported that acidic condition (pH) favors nutrients absorption and availability of some heavy metals. The bioavailability of components of the contaminants in the plant parts (fruit and leaves) was also determined. Crude oil in soil makes the soil condition unsatisfactory for plant growth.

MATERIALS AND METHODS

Study area

The study area lies within the humid tropical rainforest vegetation at the Experimental Plot of the University of Benin, Department of Plant Biology and Biotechnology (6.20°N and 5.37°E) in a completely randomized block experimental design. Soil sample for the study were obtained from Auchi (7.04°N and 6.16°E), located at the Northern axis of Edo State, Nigeria. It lies within the derived savannah vegetation.

Plant collections

The accessions obtained from home gardens in Benin City were made and identified based on the identification given by the gardeners due to their high yield and superior quality (Table 1). Further identification was done using IBPGR (1984) and Stevels (1988, 1990).

Soil collection and analysis

Two samples of about 100 kg each were collected at the premises of Nigeria National Petroleum Corporation (NNPC) sub-station measuring 2 × 2 km^2 in Auchi. One of the soil samples was collected 30 m away from a site that was reported to have been contaminated with crude oil in February, 2008 during a test run on newly fixed pipelines. The crude oil was reported to have overflow from a pit of about (60 × 60 × 30) m^3 into adjoining farms from a radius distance of 100 m^2 away from the terminal point of the spill. After seven days, 2.75 kg of each soil was prepared and transferred into thirty polythene bags for field trials. The two soil samples collected for experimentation were subjected to soil analysis at the Soil Science Laboratory, Nigerian Institute for Oil Palm Research (NIFOR). The method used for the analysis is as outlined by Ogunwale and Udoh (1990). Total elemental content, organic nutrient and presence of heavy metals were determined.

Planting and plant husbandry

The 30 bags from the two soil samples were transferred to the experimental plot. Two plots of (6 × 3) m^3 one each for the contaminated and control were demarcated on the site with a distance of 3 m apart, spacing bags was done 7 m in each plot. These bags were left on the field for seven days before planting was done. Seeds of the six accessions collected were subjected to floatation test and viable seeds were selected for field trials. Planting was done simultaneously on the two plots. Bags for each soil sample were arranged in a randomized block design and each accession with five replications. Crops were rain fed throughout the period of experimentation. There was no fertilizer application during trials. After 2 weeks of sowing, each stand on both plots (where germination occurred) were thinned to a plant per stand. Weeding was done weekly. Pest control was done using methods outlined by Osawaru and Dania-Ogbe (2010).

Determination of germination and germination percentage

Daily visit was made to the plots for 14 days. Emerged seedlings

Table 2. The chemical properties of the two soil samples.

Soil sample	% (ppm)				Meq/100 soil						
	pH	C	N	P	Na	K	Ca	Mg	H+	Al+	ECEC
Control	7.7	0.93	0.10	11.25	0.34	0.24	9.60	3.84	0.10	0.00	14.12
Contaminated	7.6	1.38	0.14	6.41	0.35	0.13	9.84	3.90	0.10	0.00	14.32

Soil sample	mg/kg soil							
	Cu	Mn	Pb	Fe	Zn	Cr	Ni	Cd
Control	2.79	1.46	3.56	7.58	2.69	0.13	-	0.78
Contaminated	7.26	5.54	8.44	50.60	5.58	0.49	-	1.46

Table 3. The particle size analysis in percentage of the two soil samples.

Soil sample	Clay (%)	Silt (%)	Sand (%)
Control	4.10	1.70	94.20
contaminated	3.10	4.70	92.20

were counted in each plot and from each bag for the accessions and percentage determined using method outlined by Osawaru and Dania-Ogbe (2010).

Analysis of data and plant organs composition

Plant parts (leaves and fruits) from the six accessions grown in the control and contaminated soil samples were harvested. Leaves from the third to fifth nodes and the first fruits of the accessions were harvested and weighed fresh. The parts were oven dried for three days. After which the dry weight of the samples were taken. The oven dried materials were ground using an electric grinding machine. The ground samples were then subjected to heavy metal analysis using dry ash method as outlined by Ogunwale and Udoh (1999) at the Nigerian Institute for Oil Palm Research near Benin City, Nigeria. All the data obtained in the study was subjected to analysis using Microsoft Excel 2007 windows.

RESULTS

Chemical analysis of soil

The result of the soil samples used for the study is presented in Tables 2 and 3. The values obtained for C, N, I, Na, Ca, Ma and ECEC were higher in the crude oil contaminated soil. The values obtained for P and K were higher in the control. $A1^{+}$ was undetected in both soil samples H^{+}. The pH value of the contaminated soil was slightly closer to mental value than the control uncontaminated soil. The particle size analysis (Table 2) shows that the contaminated soil had less clay and more silt in comparison with the control soil.

Germination and germination percentage

The results of germination are presented in Figures 1 and 2. The result from the control soil shows that OS/AC/001, OS/AE/006 and OS/AE/007 had high percentages when compared with other accessions while that obtained from contaminated soil showed low germination percentages. Figures 3 and 4 show the concentration of Cu in the leaves and fruits of okra accessions. In the control and contaminated soil, OS/AC/001 and OS/AC/002 had no Cu in their fruits. Accession OS/AC/002 had the highest amount of Cu concentration in the control soil with accession OS/AE/006 having the highest value in the contaminated soil. Accession LD-88 and OS/AE/006 had the highest concentration of copper in their fruits in the contaminated and control soils, respectively. Figures 5 and 6 show the concentration of Zn in the leaves and fruits of okra accessions. In the control, soil LD-88 had the highest concentration of Zn in leaves and fruits. In the contaminated soil, OS/AE/007 had highest amount Zn in the leaves while accession LD-88 had the highest concentration of Zn in the fruits. Figures 7 and 8 shows the concentration of Pb in the leaves and fruits of okra accessions. In the control, soil OS/AE/006 had the highest concentration of Zn in leaves while 47-4 had the highest concentration in the fruits. In the contaminated soil, LD-88 had highest amount of Pb in the leaves while accession 47-4 had the highest concentration of Pb in the fruits.

Figures 9 and 10 shows the concentration of Cd in the leaves and fruits of okra accessions. In the control, soil OS/AE/006 had the highest concentration of Cd in leaves while LD-88 had amount in the fruits. In the contaminated soil, OS/AC/001 had the highest amount of Cd in the leaves while accession 47-4 had the highest concentration of Cd in the fruits. Figures 11 and 12 show the concentration of Mn in the leaves and fruits of okra accessions. In the control, soil OS/AC/001 had the highest concentration of Mn in leaves while 47-4 had

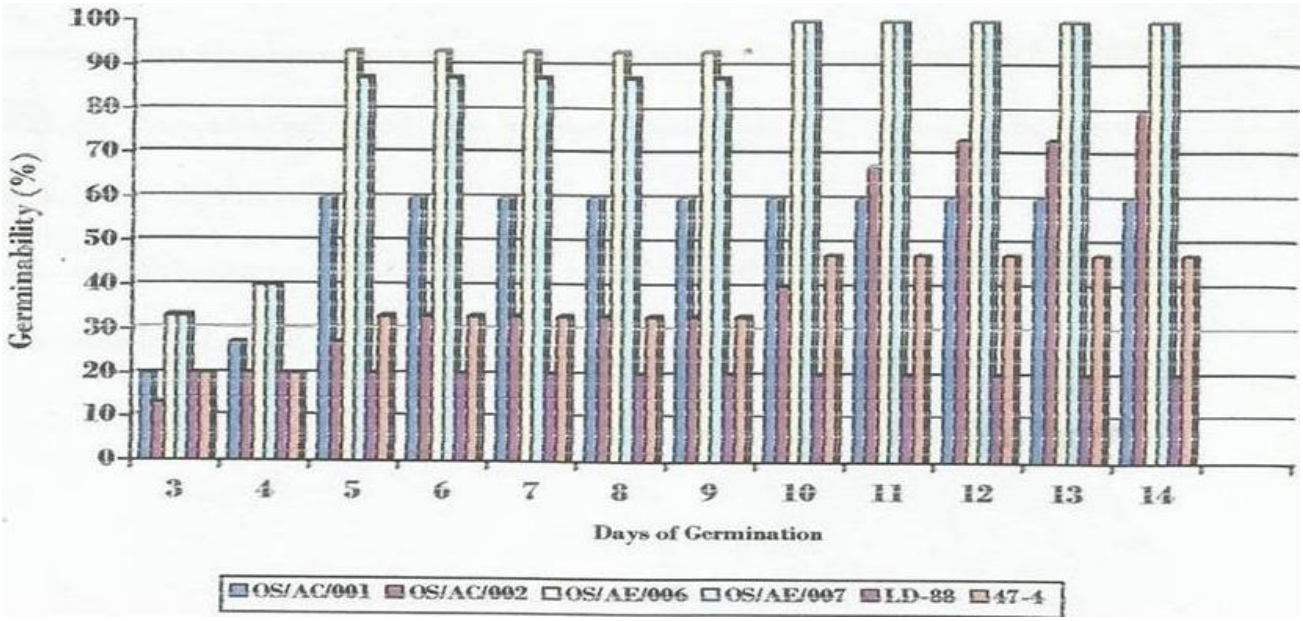

Figure 1. Germination percentage in control soil. Germination was initiated on the third day. Accession OS/AC/001, OS/AE/006 and OS/AE/007 had higher germination percentages than the other accessions.

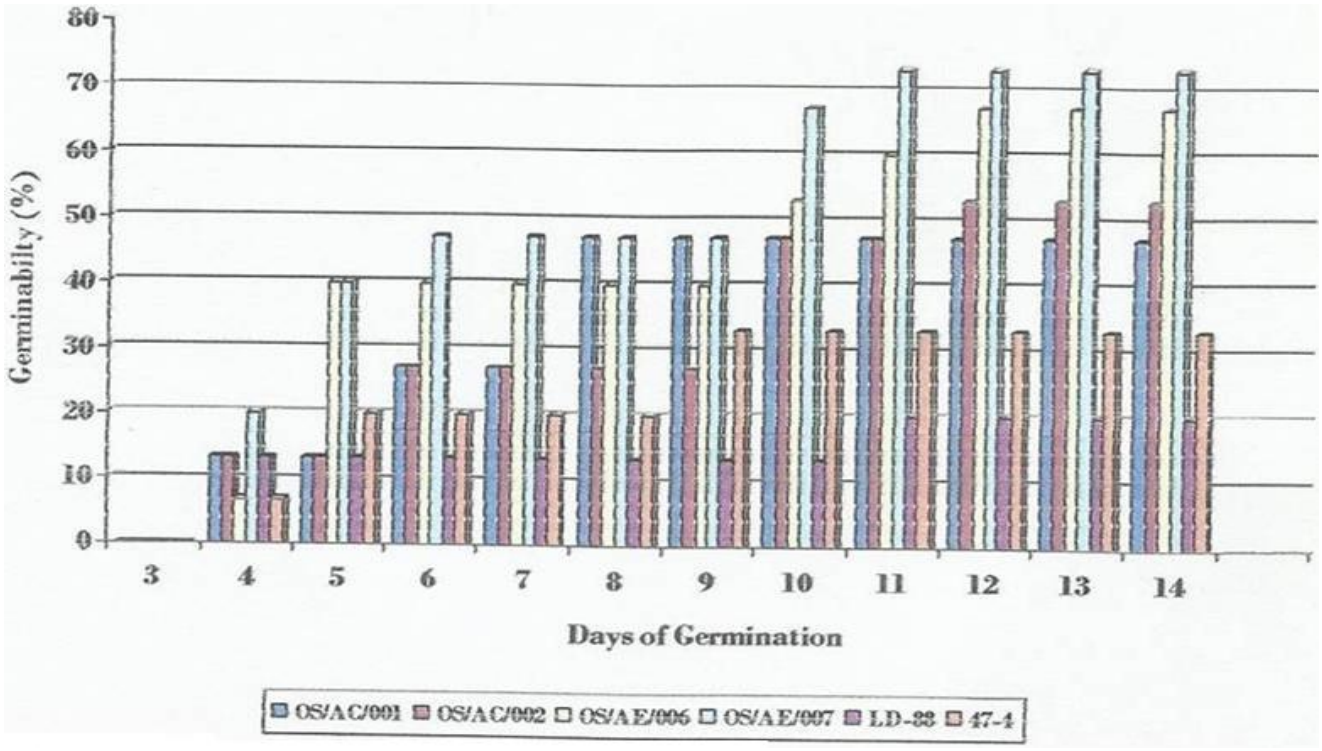

Figure 2. Germination percentage in the crude oil contaminated soil. Germination was initiated on the fourth day. Accession LD-88 and 47-4 never attained 50% germination.

amount in the fruits. In the contaminated soil, OS/AC/001 had the highest amount Mn in the leaves while accession LD-88 had the highest concentration of Mn in the fruits. Table 4 shows the fresh and dry weight of harvested

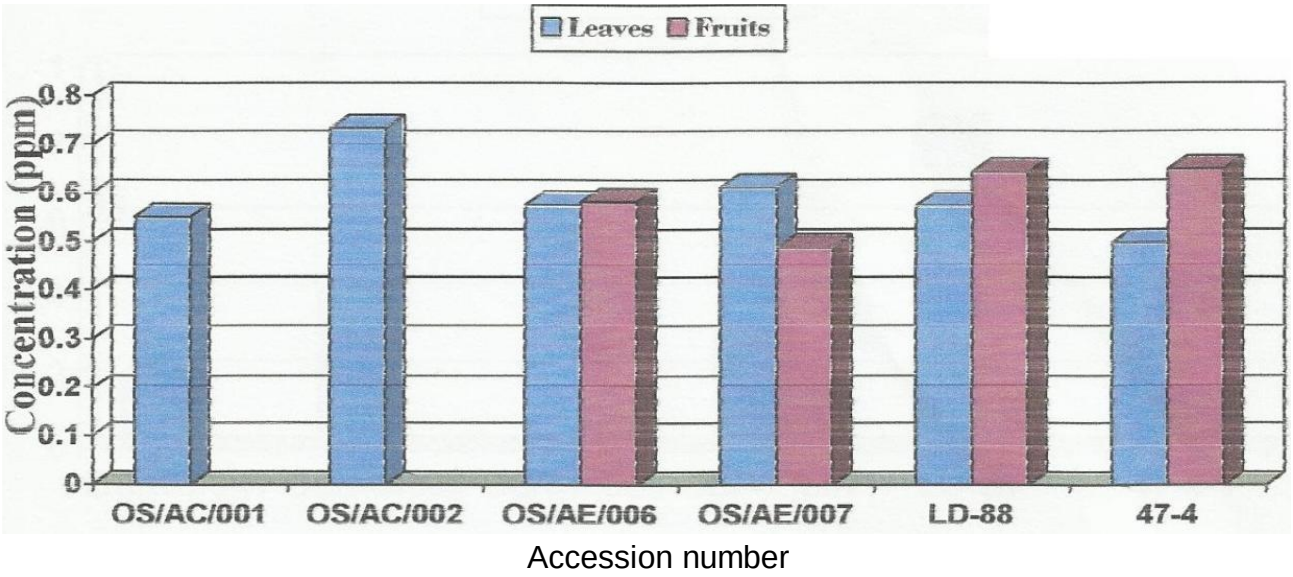

Figure 3. Concentration of copper in the leaves and fruits of the six accessions in control soil sample.

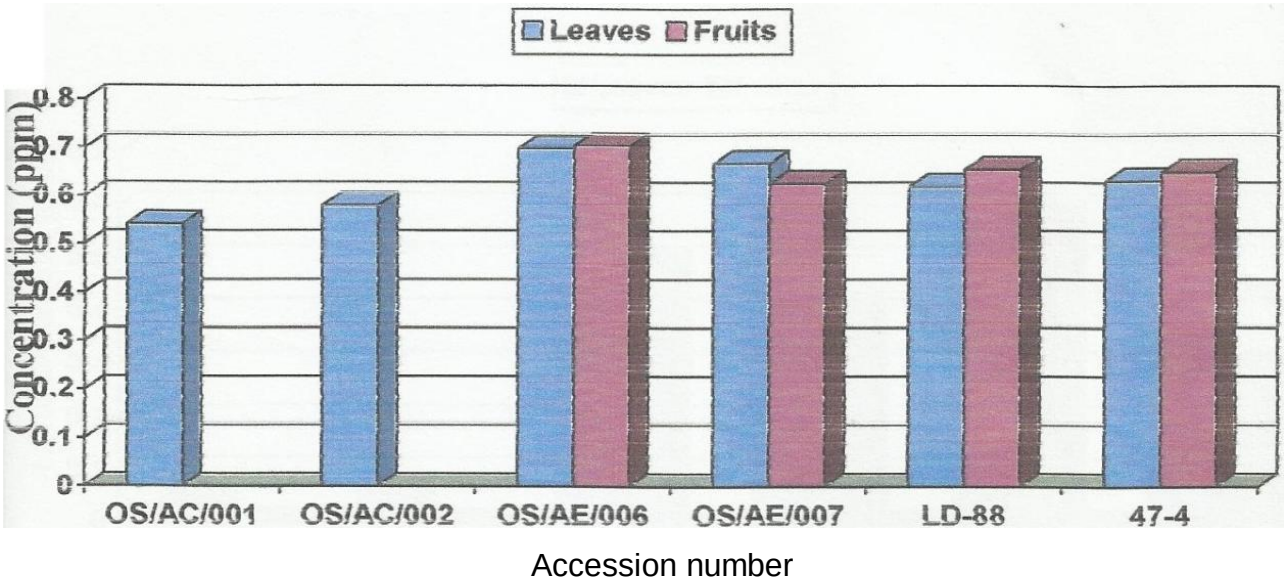

Figure 4. Concentration of copper in the leaves and fruits of the six accessions grown in contaminated soil sample.

organs of the okra accessions in the control and contaminated soil. The highest fresh weight of fresh fruit and leaves was obtained from OS/AE/006.

DISCUSSION

Growth in plants is generally adversely affected when exposed to crude oil (Baker, 1970; De-jong, 1980). The degree to which the plant growth is affected depends greatly on the level of contamination (Anoliefo, 1991). Similarly, the growth of six accessions especially those of the land races (OS/AC/001, OS/AC/002, OS/AE/006 and OS/AE/007) were generally mostly affected in the crude oil contaminated soil. The substantial damage on the soil by the crude oil was observable in the cultivated accessions of common okra and West African Okra. In the control soil: OS/AC/001, OS/AC/002, OS/AE/006 and OS/AE/007 showed variation in growth and development from the growth in the contaminated soil. It can be suggested that the depression in growth is as a result of the poor soil condition occasioned by the crude oil.

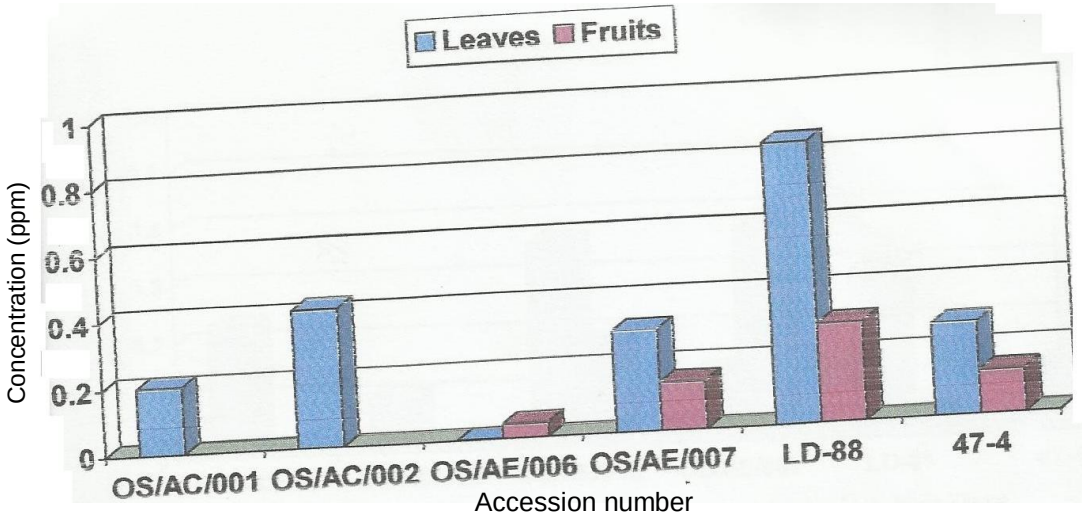

Figure 5. Concentration of zinc in the leaves and fruits of the six accessions grown in the control soil sample.

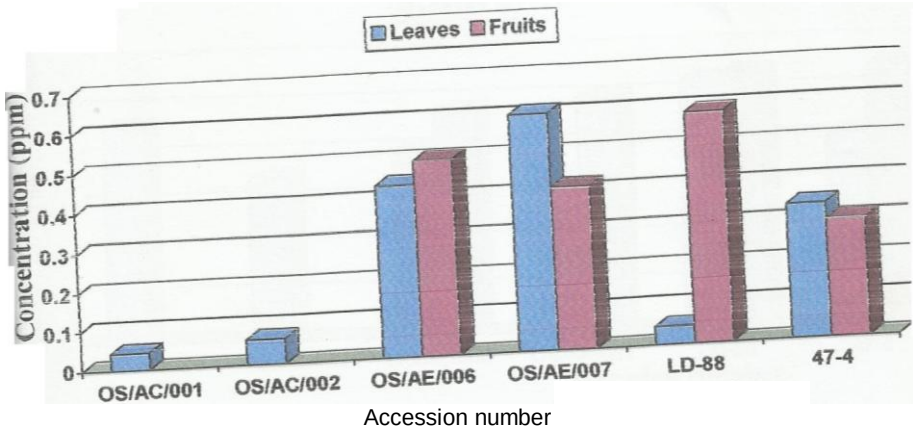

Figure 6. Concentration of zinc in the leaves and fruits of the six accessions grown in the contaminated soil sample.

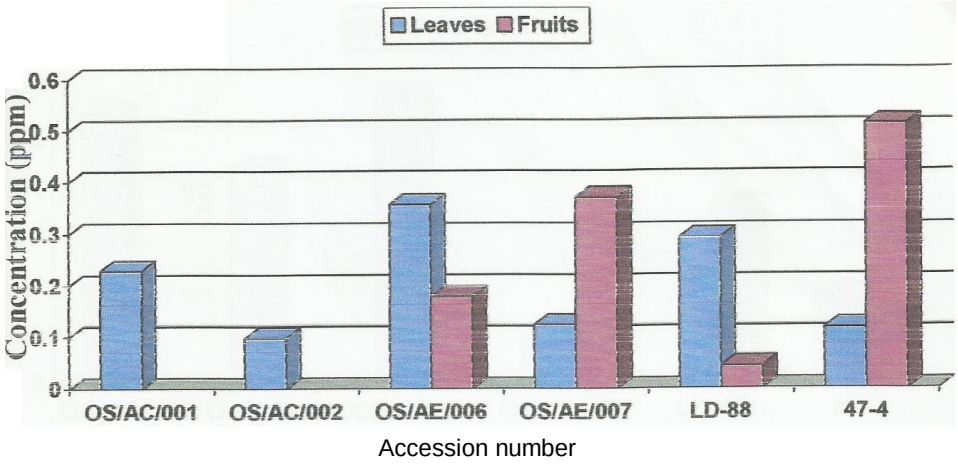

Figure 7. Concentration of lead in the leaves and fruits of the six accessions grown in the control soil sample.

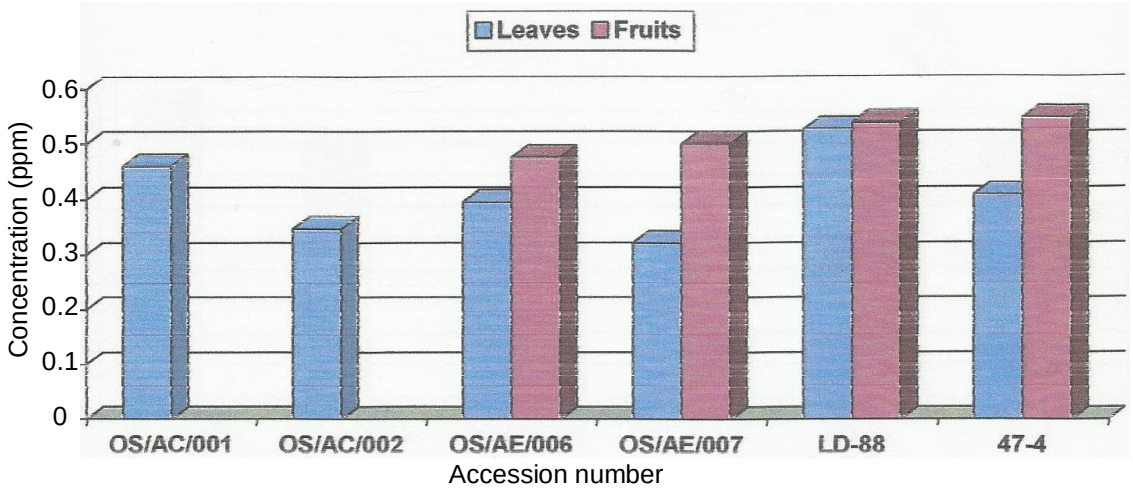

Figure 8. Concentration of lead in the leaves and fruits of the six accessions in contaminated soil sample.

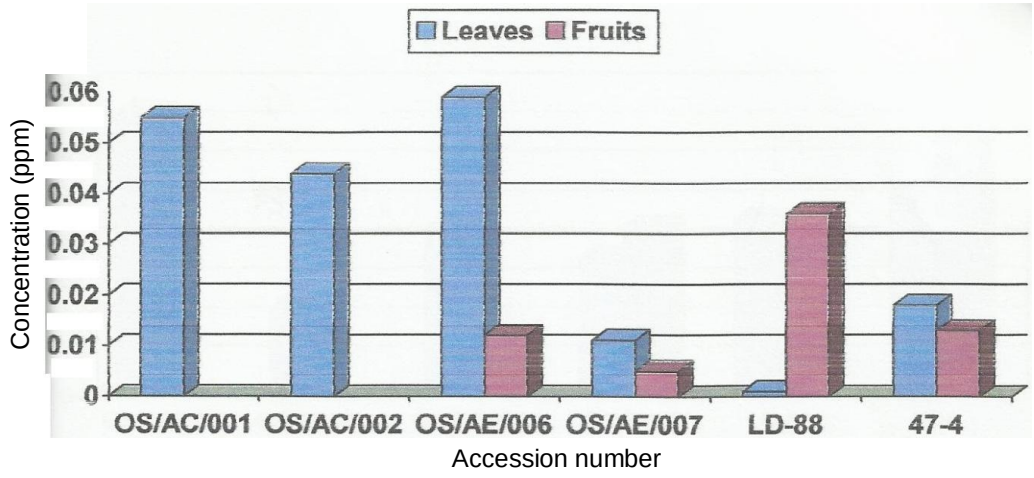

Figure 9. Concentration of cadmium in the leaves and fruits of the six accessions grown in control soil sample.

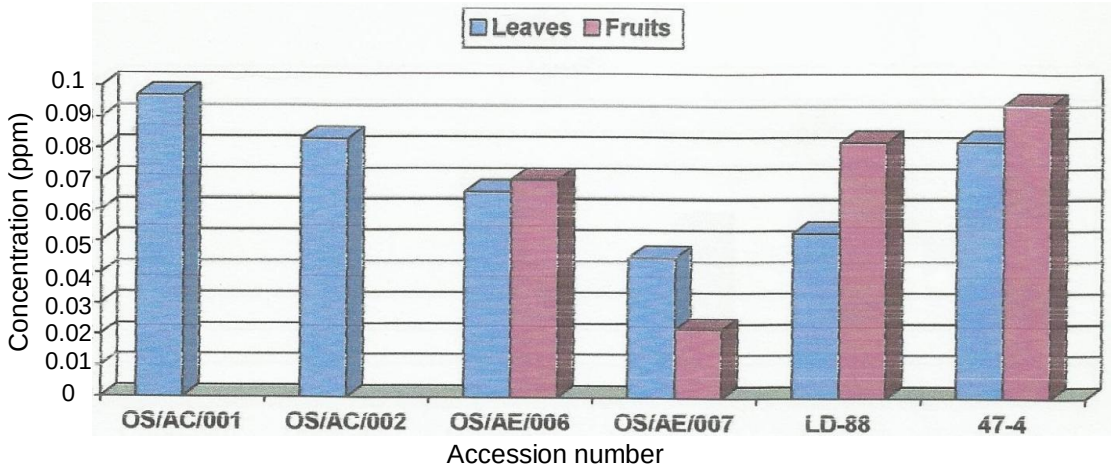

Figure 10. Concentration of cadmium in the leaves and fruits of the six accessions in contaminated soil sample.

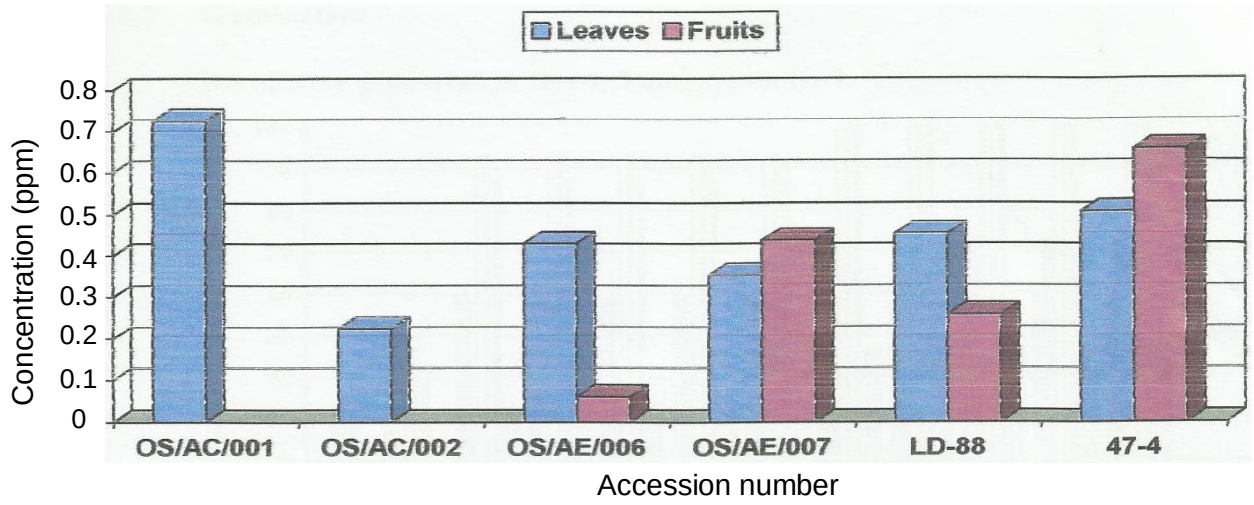

Figure 11. Concentration of manganese in the leaves and fruits of the six accessions grown in control soil sample.

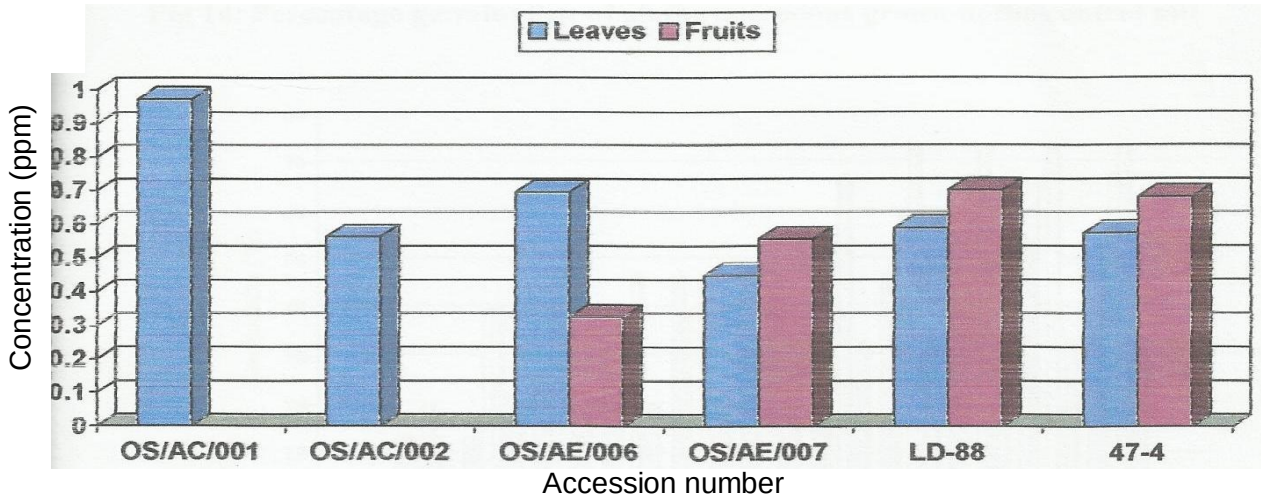

Figure 12. Concentration of manganese in the leaves and fruits of the six accessions grown in contaminated soil sample

However, accession LD-88 and 47-4 had little or no variation in germination growth and development in both soil samples. The analysis of the soil sample used in this study showed that the chemical elements Na, N and the ion H^+ in both control and the contaminated soil samples had no significant different. The concentration of C, P and K was observed to be higher in the control soil than in the contaminated soil. Ca, Mg, Mn, Pb, Fe, Zn, Cr, Cd and Cu concentrations were higher in the contaminated soil compared to the control (uncontaminated) soil. Ni and Al^{3+} were not detected in both soils (Table 2).

The soil pH was alkaline, 7.7 in control and 7.6 in contaminated soil. It can be suggested that contamination of alkaline soils tends to reduce the soil pH to a less alkaline or a neutral one. This is in agreement with the report of Vwioko et al. (2008) which states that crude oil contamination of soil increases the pH of the soil from acidic to neutral. Similarly, the particle size analysis of the two soil samples showed that crude oil contamination of soils may be responsible for the difference in the soil silt content.

Germination was initiated in the third day by the six accessions in the control soil sample with a gradual increase of percentage germination recorded day by day. Accessions OS/AC/001, OS/AE/006 and OS/AE/007 had 60 and above germination percentage on the fifth day which is in accordance with FAO (1998) recommended seed standard for agronomic practice.

The reverse was observed for accessions OS/AC/002 which had above 50 on the eleventh day LD-88 and 47-4 which did not have up to 50% germination percentage eve on day 14. In the contaminated soil, germination

Table 4. Fresh and dry weight of harvested plant organs from six accessions of okra grown in crude oil contaminated and control soil.

Accession number	Treatment	Fruit		Leaves	
		Fresh (g)	dry (g)	Fresh (g)	dry (g)
OS/AC/001	Control	-	-	2.70	0.77
	Contaminated	-	-	2.95	0.45
OS/AC/002	Control	-	-	3.00	1.00
	Contaminated	-	-	2.10	0.58
OS/AE/006	Control	6.54	1.00	3.20	0.82
	Contaminated	4.90	0.29	3.00	0.22
OS/AE/007	Control	6.50	0.89	3.00	0.54
	Contaminated	4.73	0.41	3.22	0.39
LD-88	Control	6.52	1.00	2.00	0.89
	Contaminated	5.55	1.00	2.00	0.65
47-4	Control	6.00	1.00	2.00	0.41
	Contaminated	5.00	0.87	2.06	0.40

was imitated on the fourth day in the six accessions. It took accessions OS/AE/006 and OS/AE/007 twice the number of days that was needed for them to attain 50% germination in the contaminated soil. OS/AC/002 had above 50% on the fourteenth day, OS/AC/001 was unable to reach 50% germination on the fourteenth day while accessions LD-88 and 47-4 never up to 50% germination, the same percentage germination was maintained with that of the control soil sample. The relatively low germination percentage of LD-88 and 47-4 in both soil samples may be due to the revival of the seeds from the storage bank of NIHORT two months before planting. During this period, the seeds were stored at a room temperature which may have induced shock on them. This may have resulted in low percentage germination. The fresh weights of fruits and leaves of the four accessions of *A. esculentus* exhibited a strong response to the crude oil contaminations. Among these accessions, the fruits and leaves of OS/AE/006 and OS/AE/007 grown in control soil gave weight values that were considerably higher than those from plants in the contaminated soil. The harvested plant parts of accessions LD-88 and 47-4 showed a little variation in weight for parts grown in both soil samples. Higher moisture content of parts was recorded for the control plants than in the contamination soil.

The contaminants in the soil may be responsible for the water content of plant parts hamster plant grown in the contaminated soil. There were observable differences on the dry weights of harvested plant parts in both soils are plants harvested from the control soil gave higher values. The leaves of both *A. caillei* accessions had higher dry weight values when compared with the four accessions of *A. esculentus* growth in both soils; highest values were recorded with the accessions in the control soil.

In conclusion, the environment is constantly affected by human activities. By-products and products of these activities especially from mining and oil exploration are deleterious to man and other biodiversity especially when released into the environment. They serve as a cycling matter in the ecosystem. Okra as a plant biodiversity has been shown to be vulnerable to crude oil in this study and when grown in soil polluted with crude oil, are likely to be contaminated with heavy metals and unsafe for human consumption due to the storage of these heavy metal on the edible parts.

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Full Length Research Paper

Effect of incorporation of cauliflower leaf powder on sensory and nutritional composition of malted wheat biscuits

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Cauliflower leaves are rich in β-carotene and iron and has highest waste index. Therefore, an attempt was made to utilize its leaves in value added product, thus reducing the wastage. The malted wheat flour was blended with cauliflower leaf powder in the ratios of 10, 20 and 30% for the development of biscuits. The developed products were stored for 90 days to ascertain the changes in proximate composition and sensory characteristics. The highest moisture, crude protein, crude fibre and ash content of 1.68, 9.49, 13.32 and 1.49% were recorded in biscuits prepared from 70:30: malted wheat flour: cauliflower leaf powder, respectively. However, 100:00: whole wheat flour: cauliflower leaf powder recorded highest value of crude fat (21.96%). On the basis of sensory evaluation, biscuits prepared from 90:10: malted wheat flour: cauliflower leaf powder was adjudged the best with regard to their acceptability and storability.

Key words: Cauliflower leaves, biscuits, malted wheat, β-carotene, iron, protein, fibre.

INTRODUCTION

The food processing industry produces large quantities of waste products. Over one million tonnes of vegetable trimmings from the vegetable processing industry are produced every year which can be used for value addition. They are inexpensively available in large quantities characterized by a high dietary fibre content resulting with high water binding capacity and relatively low enzyme digestible organic matter (Serena and Bach-Knudsen, 2007). However, studies on the use of vegetable fibre are scanty. Among vegetables, cauliflower (*Brassica oleraceae* var. *Botrytis*) is the most popular cole vegetable grown extensively in India. It belongs to family Brassicaceae. It is rich in nutrients but has highest waste index. The edible portion of cauliflower is curd (head), whereas, its leaves which are generally thrown away as waste are also rich source of iron and β-carotene and thus can be utilized in various value added products (Kowsalya and Sangeetha, 1999). The leaves contribute about 50% of the total production of cauliflower. The leaves of cauliflower are available only for a short period but these can be dried or stored for use during lean season (Singh et al., 2005). Dehydrated leaves are also rich source of β-carotene and iron which can be used in sparce season (Rao, 1993). Pilot studies have indicated that this effort is feasible and these dried vegetables are of good nutritive value and are acceptable (Gopalan, 1996). Wheat (*Triticum* spp.) on the other hand, is a worldwide cultivated cereal from the Fertile Crescent of the East. Wheat grain is a staple food and forms the major source of proteins and calories for large

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section of Indian population. Malting is a process of germination and subsequent drying of a grain. As a result, the complex proteins are degraded to readily available lower molecular weight fractions. Increases in protein and starch digestibility have been reported in germinated grains. These grains have been used in the formulation of low viscosity weaning supplementary and breakfast foods. Very few studies indicate the use of malted grains in snacks and biscuits (Goyle and Gujral, 1998).

Keeping in view the aforementioned facts, the present investigation were undertaken to examine the effect of blending cauliflower leaf powder and malted wheat on proximate composition of biscuits during storage.

MATERIALS AND METHODS

Cauliflower leaf powder

Cauliflower (*Brassica oleracea*) leaves were obtained in a single lot from local market. The leaves were separated from their stalks, washed under running tap water and were blanched for 10 to 15 s. After blanching the leaves were dried at room temperature for 1 to 2 h by spreading on filter paper followed by drying in hot air oven at 40°C for 4 to 6 h. The dried leaves were ground to fine powder, passed through 20 mesh seive and packed in air tight containers for further use.

Wheat

Wheat was procured in a single lot from the Division of Plant Breeding and Genetics, SKUAST-Jammu. The cleaned and dried wheat were divided into 3 lots. One lot was milled into flour and was treated as whole wheat flour.

Malted wheat flour

Malted wheat flour was prepared by soaking wheat for 12 h in water twice their volume. After that, the water was drained off, the soaked grains were wrapped in a moist muslin cloth and kept for germination for a period of 48 h at room temperature. The germinated wheat having an average root length of 1.6 to 3.3 cm, respectively were oven dried at 70 ± 5°C and milled (Table 1) (Goyle and Gujral, 1998).

Preparation of biscuits (sweet 'n' salty)

The process for preparation of sweet 'n' salty biscuits using flour of wheat was standardized using creaming method (Figure 1) (Singh et al., 2005).

Storage

The treatment combinations of wheat-cauliflower leaf powder products (in triplicate) namely, biscuits were packed in polythene pouches (Gauge 300) and then stored for a period of 90 days at room temperature. The stored products were analyzed for physico-chemical changes and sensory characteristics at an interval of 30 days.

Proximate analysis of wheat-cauliflower leaf powder biscuits

Moisture, ash and crude fibre were determined according to AOAC (1995). Crude protein was estimated by using micro-kjeldahl method, AOAC (1995) using the factor 6.25 for converting nitrogen content into crude protein. For fat content of noodles, 5 g sample was placed in Soxhlet extraction apparatus and subjected to extraction for 6 h using petroleum ether as solvent and percent fat content of noodle samples were calculated on a weight basis. β-carotene was determined by soaking 5 g sample in 15 ml of AR grade acetone for 2 h at room temperature under dark condition in order to get complete carotene extraction. The carotene layer was separated using petroleum ether through separating funnel. The volume was made up to 100 ml with petroleum ether and then this layer was again passed through sodium sulphate over the funnel in order to remove moisture from the layer. The optical density of the layer was measured at 452 nm using petroleum ether as blank (Srivastava and Kumar, 2002). Iron was determined by dissolving ash sample in 0.1 N (3 ml) hydrochloric acid in a crucible. It was then kept on water bath at 100°C and evaporated to dryness. 0.1 N (4 ml) hydrochloric acid and 2 ml distilled water were added and the dissolved fraction was filtered, made to 50 ml with distilled water and compared with iron standard curve at 535 nm OD (Thimmaiah, 1999).

The samples were evaluated for overall acceptability by semi-trained panel of 7 to 8 judges by using 9 point hedonic scale assigning scores 9- like extremely to 1- dislike extremely. A score of 5.5 and above was considered acceptable (Amerine et al., 1965).

Statistical analysis

The data obtained (in triplicate) were evaluated statistically with OPSTAT package program (OPSTAT software for Windows) by variance analysis. When variance analysis showed significant difference ($p<0.05$) among the means, the least difference test was used to evaluate means.

RESULTS AND DISCUSSION

Moisture

Biscuits prepared by using different ratios of wheat and cauliflower leaf powder revealed that there was significant increase in mean moisture content from 1.63 to 1.68% during 90 days of storage period (Table 2). The highest moisture content of 1.68% was recorded in T_8 (70:30:: malted wheat flour: cauliflower leaf powder) and the lowest of 1.63% in T_4 (70:30:: roasted wheat flour: cauliflower leaves powder). The increase in moisture content of biscuits might be due to hygroscopic nature of cauliflower leaf powder and wheat flour. Similar results were reported by Kumar and Barmanray (2007) in button mushroom fortified biscuits.

Crude protein

At 0 day storage, treatment T_8 (70: 30: malted wheat flour: cauliflower leaves powder) recorded highest protein content of 9.51% followed by T_7 (80:20: malted wheat flour: cauliflower leaves powder), having value 9.44% and T_6 (90:10: malted wheat flour: cauliflower leaves powder)

Table 1. Composition of different treatments used for the study.

Treatment	Details
T_1	100:00:: Whole Wheat flour: Cauliflower leaves
T_2	90:10:: Whole Wheat flour: Cauliflower leaves
T_3	80:20:: Whole Wheat flour: Cauliflower leaves
T_4	70:30:: Whole Wheat flour: Cauliflower leaves
T_5	100:00:: Malted Wheat flour: Cauliflower leaves
T_6	90:10:: Malted Wheat flour: Cauliflower leaves
T_7	80:20:: Malted Wheat flour: Cauliflower leaves
T_8	70:30:: Malted Wheat flour: Cauliflower leaves

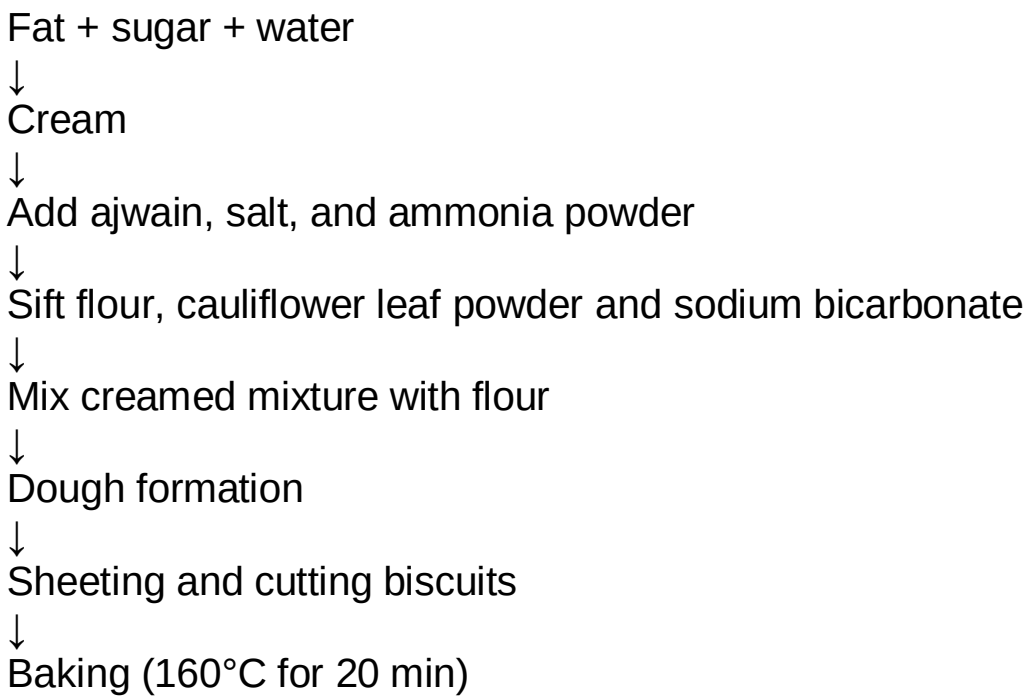

Figure 1. Flow chart of preparation of sweet 'n' salty biscuits.

having value 9.43% (Table 3). However, after 90 days of storage treatment, T_2 (90:10: whole wheat flour: cauliflower leaves powder) recorded the lowest value of 7.29% and highest value of 9.46% was observed in T_8 (70:30: malted wheat flour: cauliflower leaves powder). It was observed that with the passage of time during storage, crude protein content decreased significantly in all treatments. It decreased from its mean value of 8.41 to 8.36% during storage of 90 days. The decrease in protein content during storage might be due to hydrolysis of peptide bonds by the help of protease enzyme that cause splitting of protein molecules during storage. Similar decrease of protein content with storage period was reported by Kanchana et al. (2008) in value added single cell protein biscuits and Nwabueze and Atuonwu (2007) in African bread fruit seeds incorporated biscuits.

Crude fat

Data in Table 4 shows that the crude fat content of biscuits was influenced significantly by different treatments. The highest crude fat content (22.62%) was recorded in treatment T_1 (100:00: whole wheat flour: cauliflower leaves powder) at 0 day storage followed by 22.29% in T_2 (90:10: whole wheat flour: cauliflower leaves powder). The treatment T_8 (70:30: malted wheat flour: cauliflower leaves powder) recorded the lowest value of 19.35% at 90 days storage followed by T_5 (100:00: malted wheat flour: cauliflower leaves powder) having value of 19.90%. The reduction of fat content in malted samples might be due to sprouting which increases the activity of lipase enzyme leading to decreased fat content. These findings are in accordance

Table 2. Effect of treatments and storage period on moisture (percent) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	1.64	1.65	1.66	1.68	1.66
T_2	1.62	1.63	1.65	1.66	1.64
T_3	1.61	1.64	1.66	1.68	1.65
T_4	1.60	1.62	1.64	1.66	1.63
T_5	1.62	1.64	1.65	1.66	1.64
T_6	1.64	1.66	1.68	1.69	1.67
T_7	1.64	1.65	1.67	1.68	1.66
T_8	1.66	1.67	1.68	1.70	1.68
Mean	1.63	1.65	1.66	1.68	

Effects of C.D. ($p = 0.05$), Treatment, 0.01; Storage, 0.01; Treatment × storage, NS.

Table 3. Effect of treatments and storage period on crude protein (percent) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	7.36	7.34	7.34	7.33	7.34
T_2	7.37	7.36	7.35	7.29	7.34
T_3	7.38	7.36	7.34	7.32	7.35
T_4	7.38	7.38	7.36	7.35	7.37
T_5	9.42	9.41	9.40	9.37	9.40
T_6	9.43	9.43	9.41	9.38	9.41
T_7	9.44	9.43	9.42	9.39	9.42
T_8	9.51	9.5	9.48	9.46	9.49
Mean	8.41	8.40	8.39	8.36	

Effects C.D. ($P = 0.05$); Treatment, 0.01; Storage, 0.02; Treatment × storage, NS.

Table 4. Effect of treatments and storage period on crude fat (percent) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	22.62	22.07	21.97	21.18	21.96
T_2	22.29	21.96	21.57	20.88	21.67
T_3	22.24	22.07	21.51	20.63	21.61
T_4	21.97	21.71	21.07	20.14	21.22
T_5	20.98	20.67	20.15	19.90	20.43
T_6	20.83	20.49	20.21	20.07	20.40
T_7	20.64	20.06	19.78	19.44	19.98
T_8	20.12	19.75	19.56	19.35	19.70
Mean	21.46	21.09	20.73	20.20	

Effects C.D. ($P = 0.05$); Treatment, 0.02; Storage, 0.09; Treatment × storage, 0.18.

with the findings of Singh et al. (2008) which reported that crude fat decreased with storage in biscuits supplemented with various levels of jaggery.

Crude fibre

The data pertaining to crude fibre content of different

Table 5. Effect of treatments and storage period on crude fiber (percent) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	6.12	6.05	5.89	5.65	5.93
T_2	6.82	6.73	6.62	6.43	6.65
T_3	7.57	7.38	7.34	7.10	7.35
T_4	8.39	8.32	8.14	7.84	8.17
T_5	10.72	10.60	10.40	10.30	10.50
T_6	11.50	11.34	11.26	11.17	11.32
T_7	12.53	12.36	12.24	12.19	12.33
T_8	13.62	13.35	13.23	13.07	13.32
Mean	9.66	9.52	9.39	9.22	

Effects C.D. (P = 0.05);treatment, 0.02; Storage 0.03; treatment × storage, 0.07.

Table 6. Effect of treatments and storage period on ash (percent) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	1.10	1.07	1.04	1.03	1.06
T_2	1.13	1.11	1.08	1.04	1.09
T_3	1.14	1.11	1.09	1.06	1.10
T_4	1.20	1.18	1.12	1.10	1.15
T_5	1.33	1.27	1.21	1.16	1.24
T_6	1.46	1.39	1.31	1.25	1.35
T_7	1.43	1.38	1.32	1.26	1.35
T_8	1.59	1.49	1.46	1.42	1.49
Mean	1.30	1.25	1.20	1.17	

Effects C.D. (P = 0.05); treatment, 0.02; Storage, 0.01; treatment × storage, NS.

treatments in Table 5 depicts a significant decrease in crude fibre content for storage duration is concerned. However, with the incorporation of cauliflower leaf powder, the fibre content increased. The mean value of crude fibre content decreased from the initial level of 9.62 to 9.22% after 90 days of storage. At the beginning, the highest crude fibre content of 13.62% was recorded in T_8 (70:30: malted wheat flour: cauliflower leaves powder); whereas, the lowest crude fibre content of 6.12% was recorded in T_1 (100:00: whole wheat flour: cauliflower leaves powder). After 90 days of storage, the maximum crude fibre of 13.07% were recorded in T_8 (70:30: malted wheat flour: cauliflower leaf powder); whereas, the minimum crude fibre of 5.65% were recorded in T_1 (100:00: whole wheat flour: cauliflower leaf powder). The decrease in crude fibre might be due to the degradation of hemicelluloses and other structural polysaccharides during storage. Also, heat and moisture solubilizers degrade pectic substances leading to the decrease in the fibre content (Sharon and Usha, 2006). Similar decline in crude fibre content was reported by Singh et al. (2006) in pearl millet cake.

Ash

A perusal of data in Table 6 revealed that the effect of treatments on ash content (percent) of biscuits was significant. The treatment T_8 (70:30:: malted wheat flour: cauliflower leaf powder) recorded the highest value of 1.59% at 0 day storage; whereas, treatment T_1 (100:00: whole wheat flour: cauliflower leaf powder) recorded lowest ash content value of 1.03% after 90 days of storage followed by 1.04% in T_2 (90:10: whole wheat flour: cauliflower leaves powder) and 1.06% in T_3 (80:20: whole wheat flour: cauliflower leaves powder). The highest mean ash content of 1.49% was recorded in case of treatment T_8 (70:30: malted wheat flour: cauliflower leaves powder) which might be due to soaking of grains in tap water during sprouting. Since the water is good source of minerals, as a result through osmosis, ash content of malted grains increased. These results were supported by the findings of Eyidemir and Hayta (2009) in

Table 7. Effect of treatments and storage period on iron (mg/100 g) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	5.10	5.07	5.04	5.03	5.06
T_2	5.13	5.11	5.08	5.04	5.09
T_3	5.14	5.11	5.09	5.06	5.10
T_4	5.20	5.18	5.12	5.10	5.15
T_5	5.33	5.27	5.21	5.16	5.24
T_6	5.43	5.39	5.31	5.25	5.35
T_7	5.46	5.38	5.32	5.26	5.35
T_8	5.59	5.49	5.46	5.42	5.49
Mean	5.30	5.25	5.20	5.17	

Effects C.D. (P = 0.05); treatment, 0.03; storage, 0.03; treatment × storage, 0.04.

Table 8. Effect of treatments and storage period on β-carotene (mg/100 g) of wheat - cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	2.76	2.64	2.27	1.96	2.41
T_2	2.78	2.74	2.32	1.97	2.45
T_3	2.80	2.74	2.35	2.00	2.47
T_4	2.86	2.76	2.38	2.03	2.51
T_5	2.91	2.83	2.48	2.13	2.59
T_6	2.99	2.92	2.49	2.13	2.63
T_7	3.07	3.01	2.58	2.18	2.71
T_8	3.13	3.05	2.72	2.32	2.81
Mean	2.92	2.84	2.45	2.09	

Effects C.D. (P = 0.05); treatment, 0.02; storage, 0.05; treatment × storage, 0.08.

noodles supplemented with apricot kernel flour.

Iron

The effect of various treatments and storage on iron content of biscuits is represented in Table 7. The data revealed that the iron content of biscuits was significantly influenced by different treatments and increased with the increase in the percentage of incorporation of cauliflower leaf powder. Among various treatments, T_8 (70:30:: malted wheat flour: cauliflower leaves powder) recorded the highest iron content of 5.59 (mg/100 g) followed by T_7 (80:20:: malted wheat flour: cauliflower leaves (powder form) having an iron content of 5.46 (mg/100 g) at 0 day storage. However, after 90 days storage, lowest iron content of 5.03 (mg/100 g) was recorded by treatment T_1 (100:00: whole wheat flour: cauliflower leaves powder) followed by T_2 (90:10: whole wheat flour: cauliflower leaves powder) with iron content of 5.04 (mg/100 g). The highest mean iron content of 5.49 (mg/100 g) was recorded by treatment T_8 (70:30: malted wheat flour: cauliflower leaves powder) and the lowest mean iron content of 5.06 (mg/100 g) was recorded by treatment T_1 (100:00: whole wheat flour: cauliflower leaves powder). Similar results have been reported by Sikandra and Boora (2009).

β-Carotene

A glance of data in Table 8 reveals that with the incorporation of cauliflower leaves, the β-carotene content increased. At the beginning, treatment T_8 (70:30: malted wheat flour: cauliflower leaves powder) recorded highest β-carotene content of 3.13 (mg/100 g) followed by T_7 (80:20: malted wheat flour: cauliflower leaf powder) with β-carotene content of 3.07 (mg/100 g). On the other hand, after 90 days of storage, treatment T_1 (100:00: whole wheat flour: cauliflower leaf powder) recorded the

Table 9. Effect of treatments and storage period on mean score evaluation of overall acceptability of wheat-cauliflower leaf powder blended biscuits.

Treatment	Storage period (days)				
	0	30	60	90	Mean
T_1	7.73	7.7	7.65	7.59	7.67
T_2	7.79	7.55	7.34	7.21	7.47
T_3	6.10	5.97	5.85	5.66	5.90
T_4	5.58	5.38	5.16	5.04	5.29
T_5	7.49	7.28	7.22	7.38	7.34
T_6	8.20	8.02	7.91	7.67	7.95
T_7	6.57	6.41	6.27	6.15	6.35
T_8	6.32	6.18	6.04	5.94	6.12
Mean	6.97	6.81	6.68	6.58	

Effects C.D. (P = 0.05): treatment, 0.02; storage, 0.03; treatment × storage, 0.09.

lowest value of 1.96 (mg/100 g) followed by T_2 (90:10: whole wheat flour: cauliflower leaf powder) having β-carotene content of 1.97 (mg/100 g). Highest mean β-carotene content of 2.81 (mg/100 g) was recorded in treatment T_8 (70:30:: malted wheat flour: cauliflower leaves powder); whereas, the lowest β-carotene content of 2.41 (mg/100 g) was recorded by treatment T_1 (100:00:: whole wheat flour: cauliflower leaf powder).

The decrease in β-carotene content during storage might be due to the oxidative degradation of colour pigment. Potter (1987) also reported that carotenoids are very sensitive to oxidation which results in loss of colour. However, with the increasing level of incorporation of cauliflower leaf powder, the β-carotene content increased which might be due to higher concentration of β-carotene in leaves. The findings of Bhavani and Kamini (1997) in extruded maize products also support the same results.

Overall acceptability

Table 9 pertaining to the mean score awarded to the quality attributes of overall acceptability of biscuits reveals that at the beginning of storage, the maximum score of 8.20 was recorded in T_6 (90:10: malted wheat flour: cauliflower leaves powder); whereas, the minimum score of 5.58 was recorded in T_4 (70:30: whole wheat flour: cauliflower leaves powder). As the storage period advanced, there was decrease in overall acceptability score. After 90 days of storage, the highest score of 7.67 was recorded in T_6 (90:10:: malted wheat flour: cauliflower leaves powder). The mean score for overall acceptability attribute decreased from 6.97 to 6.58 during 90 days of storage. Similar results have been reported by Stojceska et al. (2008) in cereal based ready to eat expanded snacks using cauliflower by products.

Conclusion

Therefore, it can be concluded that incorporation of cauliflower leaf powder in biscuits up to 10% along with malted wheat flour not only improves the texture, taste and overall acceptability but also improves the nutritive value of these products without adding much to the cost of the product and cauliflower leaves, which are generally thrown away can be utilized in a better way, thus reducing wastage.

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Full Length Research Paper

Thidiazuron-induced shoot organogenesis of *Cleome viscosa* (L) through cotyledonary explants culture

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A reproducible protocol for direct shoot organogenesis of *Cleome viscosa,* an important medicinal weed herb was developed. The seed explants were collected primarily from field grown mature plants for *in vitro* germination on different strength of MS basal medium with or without selection of GA_3 at various concentration (0.1 to 1.0 mg/L). The highest rate of seed germination (55.3%) was noticed on full strength MS basal medium fortified with 0.5 mg/L GA_3 after 30 days of culture. The excised 7 to 10 days old cotyledonary leaf, cotyledonary node and hypocotyls explants cultured on MS medium fortified with different concentration of individual cytokinin (BA/KIN/TDZ) alone or BA+KIN or TDZ+KIN or TDZ in combinations with different auxins (IBA/NAA/IAA) influenced the frequency of adventitious microshoot formation. The rate of shoot multiplication was greatest (100%) in cotyledonary leaf explants cultured on 3.0 mg/L TDZ and 0.3 mg/L IBA tested medium after 45 days of culture. The individual microshoots were elongated well in 0.3 mg/L TDZ and 0.1 mg/L GA_3 treated medium, but more number of adventitious micro roots were developed on half strength MS medium fortified with 0.1 mg/L NAA. The regenerated healthy plants were hardened in pots containing soil mix and well established into complete state similar to that of field grown plants under greenhouse condition.

Key words: *Cleome viscosa* L, medicinal weed plant, shoot multiplication, direct organogenesis.

INTRODUCTION

Cleome viscosa L. (Capparidaceae) is commonly known as “wild or dog mustard,” found as a medicinal weed all over the plains of tropical and subtropical regions of southern, western and central parts of the India (Mali, 2010; Wake et al., 2011). The plants have devised a useful strategy of producing flowers simultaneously with maturation of fruits and seed dispersal also overlap. The events of floral biology ensure auto pollen deposition of plants being self- pollinated (Saroop and Kaul, 2011). The various constituents of these plants have shown profound therapeutic and prophylactic activities. The seeds, leaves and roots of the plant are widely used in traditional folk medicine as an antiscorbutic, analgesic, anthelmintic, antiseptic, cardiac stimulant, carminative, febrifuge, sudorific, anti-inflammatory, antimicrobial, antipyretic, hepatoprotective and immunomodulatory activities (Mali, 2010), anticonvulsant (Shah et al., 1983), antidiarrheal (Malhotra and Moorthy, 1973; Sharma et al.,

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Abbreviations: Kin, Kinetin; **TDZ,** thidiazuron; **BA,** 6-benzylaminopurine; **NAA,** naphthaleneacetic acid; **IBA,** indole-3-butyric acid; **GA_3,** gibberellic acid; **MS,** Murashige and Skoog basal medium.

1979), skin diseases (Purohit et al., 1985), itching, ulcers, leprosy, and malarial fevers (Chatterjee and Prakashi, 1991; Kirtikar and Basu, 1935; Nadkarni, 1982), antiemetic activity (Ahmed et al., 2011) and wound healing property (Panduraju et al., 2011). The seeds contain 18.3% oil, especially a mixture of amino acids, fatty acids and sucrose (Rukmini and Doesthale, 1979; Rukmini, 1978; Afaq et al., 1984; Deora et al., 2003) and minerals of potassium, phosphorus, iron, calcium, magnesium, copper in high concentration as a good source of essential nutrients required for the well being of human body for therapeutic purposes (Lavate et al., 2011).

Recently, the demands for the elite plant material in various countries are continuously increasing for medicinal purpose, because the exploitation coupled with increasing urbanization lead to a steady erosion and loss of diversity from the natural habitats of plants. *In vitro* clonal propagation is only the alternative way for the regeneration and expedites release of large number of *C. viscosa* plants under aseptic culture conditions. Earlier, there have been few reports on establishment of *in vitro* propagation through callus culture of *C. viscosa* (Anburaj et al., 2011a,b), micropropagation and shoot organogenesis from different explants of *Cleome spinosa* (Simões et al., 2004; Albarello et al., 2006; Qin et al., 2012; Albarello et al., 2013) and *Cleome gynanadra* (Rathore et al., 2013), somatic embryogenesis (Simões et al., 2010) cell suspension (Simões et al., 2012) and root culture with cryopreservation of *C. rosea* (Cordeiro et al., 2012). To develop improved methods of plant regeneration of *C. viscose*, the effect of urea-type cytokinin, and thidiazuron (TDZ) was investigated to overcome the unsatisfactory earlier findings. Some studies showed that TDZ have higher cytokinin activity to induce organogenesis in several plant species (Zhang et al., 2001; Oluk and Orhan, 2010). TDZ-induced organogenesis comprises a metabolic cataract including primary signaling event, storage, passage of endogenous plant signals and iron in plant cell, a system of secondary messengers and a simultaneous stress response (Guo et al., 2011). Therefore, TDZ emerged as an effective bioregulant in cell and tissue cultures in wide array of plant species (Li et al., 2000; Matand and Prakash, 2007). The present study was carried out to standardize a new protocol for *in vitro* seed germination combined with an efficient regeneration of *C. viscosa* from cotyledonary explants culture in optimal concentration of TDZ to formulate the strategies for the conservation of these invaluable plants in natural habitat.

MATERIALS AND METHODS

Plant material

The mature fruits of *C. viscosa* were collected from College Campus, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu, India. The fruits were dried under laboratory conditions for few days. The seeds were carefully removed mechanically from the fruits and washed under running tap water for 2 to 3 min, dried and stored at 4°C for four to five months.

Disinfection and *in vitro* seed germination

Seeds were initially sterilized with 10% *Teepol* (v/v) solution (commercial bleach solution) for 10 to 15 s and kept under running tap water for 20 min to remove dirty particles and detergents. Further processes were carried out under aseptic conditions by treating with 70% ethanol (v/v) for 1 min and rinsed with sterile distilled water for 2 min followed by 0.2% $HgCl_2$ (mercuric chloride) for 2.5 min. The surface sterilized seeds were finally washed thoroughly with sterile distilled water twice. Then, the seeds were implanted on 25×150 mm culture tube containing quarter, half and full strength MS basal salts (Murashige and Skoog, 1962), 100 mg/L myoinositol, 150 mg/L L-glutamine, 75 mg/L thiamine HCl, 100 mg/L pyridoxine, 3.0% sucrose (w/v) and 0.8% agar (w/v) (Hi-media laboratories limited, Mumbai, India) medium supplemented with or without addition of GA_3 (0.1 to 1.0 mg/L). All the cultures were initially incubated in darkness for 10 days at 25±2ºC and later transferred to 16/8 h light/dark conditions at a light intensity of 15 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Philips, India). Data on percent of seed germination was noticed after 5 to 30 days of culture.

Culture condition and direct organogenesis

Cotyledonary node, cotyledonary leaf and hypocotyl explants were isolated from 7 to 10 days old *in vitro* young seedlings of *C. viscosa* and cut into 0.5 to 1.0 cm length. The excised cotyledonary leaf and hypocotyl explants were wounded with sterile surgical blade and placed horizontally, cotyledonary node inserted vertically on MS basal salts, 100 mg/L myoinositol, 150 mg/L L-glutamine, 75 mg/L thiamine HCl, 100 mg/L pyridoxine, 3.0% sucrose (w/v) and 0.8% agar (w/v) medium fortified with different concentration of BA (3.0 to 7.0 mg/L) or KIN (0.5 to 1.5 mg/L) or TDZ (1.0 to 5.0 mg/L) or optimum concentration of BA (5.0 mg/L) and TDZ (3.0 mg/L) individually selected with various level of KIN (0.1 to 0.5 mg/L) in the first set of experiment. Further, TDZ (3.0 mg/L) alone was tested with different auxins (NAA/IAA/IBA) at various concentrations (0.1 to 0.9 mg/L). The pH of the medium was adjusted to 5.7 prior to autoclaving at 15 psi for 20 min. All cultures were incubated under 15 µmol $m^{-2}s^{-1}$ in 16 h photoperiod provided by cool white fluorescent tubes at 25±2ºC. Subcultures were done at two weeks of interval for adventitious microshoot induction. Data on percentage of response with total number of shoots per explants was recorded after 45 days of culture.

Shoot elongation, rooting and acclimatization

Individual microshoots (0.2 to 0.5 cm in length) were carefully excised from shoot clumps and transferred to MS basal medium supplemented with various concentrations of TDZ (0.0 to 0.5 mg/L) in combinations with GA_3 (0.1 mg/L) for frequency of shoot elongation after 2 weeks of culture. After shoot elongation, the individual green healthy shoots (<1.0 cm long) were carefully removed from the culture vessel and transferred to half strength MS basal salts, 1.5% sucrose and 0.8% agar medium supplemented with various concentrations of NAA or IBA or IAA (0.1 to 0.3 mg/L) for frequency of rooting after two to three weeks of culture. Data on shoot elongation, root induction frequency with total number of roots per plantlet was recorded after two to three weeks of culture. The rooted plantlets were removed carefully from the culture vessels and washed under running tap water to remove agar gels

Table 1. *In vitro* seed germination of *cleome viscosa* L.

Medium composition	*In vitro* seed germination frequency (%)			
	After 5 days	After 10 days	After 15 days	After 30 days
¼ strength MS basal salts	-	10^{d}	20^{d}	33.2^{c}
½ strength MS basal salts	-	19.1^{c}	26^{c}	37^{bc}
Full strength MS basal salts	10^{ab}	25^{b}	31.5^{b}	40^{b}
MS basal salts + 0.5 mg/L GA_3	12.2^{a}	28^{a}	47^{a}	55.3^{a}

25 seeds were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$)

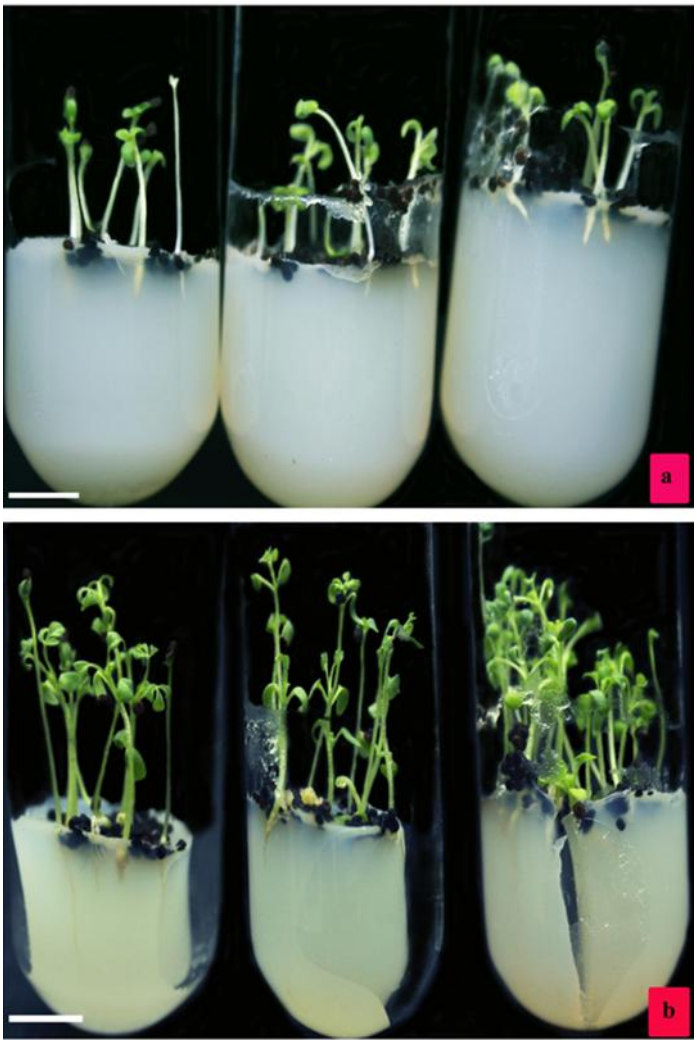

Figure 1. Effect of MS basal medium strength (a) and and GA, (b) on seed germination of *C. viscosa* L.

from the root base. The rooted plants were successfully transplanted into 6.0 cm diameter plastic pots mixed with sterile red soil, garden soil and sand in a ratio of 1:2:1. Each pot was enclosed in a clean polythene bag and maintained under a 16 h photoperiod at a light intensity of 15 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tube (Philips, India) to control high humidity. The plants were initially irrigated with autoclaved half strength MS liquid medium without sucrose at two days interval. Then, the plantlets were exposed 2 to 4 h daily to the conditions for natural humidity after five days of transfer. The well established plants were finally transferred to earthen pots containing soil mix and maintained under greenhouse condition. The rate of plant survival was observed after 2 to 4 weeks.

Statistical analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. All experiments were conducted with complete randomized block design (CRD) and factorial with different growth regulators as independent variables. Each experiment was repeated thrice. Percent of seed germination, plant regeneration and the number of shoots obtained during initial culture and subsequent transfers were tabulated. The data pertaining to frequencies of seed germination, shoot induction, shoot elongation, root induction and numbers of shoots per cultures were subjected to ANOVA test. Mean separation was carried out using Duncan's Multiple Range Test (DMRT) by SPSS (version 12.0) software package for expressing statistical significance in all culture practice.

RESULTS

In vitro seed germination response

In vitro surface sterilized seeds cultured on quarter, half and full strength of MS basal salts, 0.3% sucrose and 0.8% agar with or without addition of GA_3 at different concentrations induced germination. The seed germination was initiated under dark field for first 10 d. The onset of mean germination percentage was increased frequently in GA_3 tested medium. Although, the maximum value of seed germination (55.3%) was observed on full strength MS basal medium fortified with 0.5 mg/L GA_3 after 30 days of sowing under 16/8 h light/dark conditions about 33.2 and 37% seed germination was noticed from quarter and half strength MS basal medium, respectively (Table 1 and Figure 1a and b). The combined effect of GA_3 and dark field for the first 10 days overcame the dormancy of seeds and influenced germination significantly ($P<0.05$). The

germinated cotyledonary plants were converted into complete plants under light field.

Shoot induction and multiplication response

In the current study, cotyledonary leaf, cotyledonary node and hypocotyl explants were swelled after two to three days of culture in response to various form of plant growth regulators. Shoot bud initiation was started at axillary region of cotyledonary node and wounding sites of cotyledonary leaf and hypocotyl explants. The microshoot buds grew into tiny visible shoots in culture medium containing growth regulators. Thus, genotype dependant response with genetic variability of microshoot development was minimized based on the hormonal signal in explants. The maintenance of culture regime in subculture medium ensured continuous shoot multiplication. The present study shows that the individual or combinational usage of cytokinins, BA/KIN/TDZ stimulated microshoot induction and proliferation from three different explants of *C. viscosa*. Here, the healthy hypocotyl explants incubated on MS salts with 3.0% sucrose (w/v) and 0.8% agar (w/v) medium supplemented with BA (5.0 mg/L) influenced 57% shooting response with an increase in microshoot bud formation (10.5±0.3) and decrease in shoot height. The shoot length was decreased as the level of cytokinins increased in all culture condition. BA at 5.0 mg/L resulted to 50% shooting response with only 5.7±2.0 number of microshoots from cotyledonary node explants while cotyledonary leaf explants showed 61% shoot induction response with more number of microshoots (15.2±1.0).

Addition of KIN did not show better results for frequency of shooting in all three explants culture. Although, TDZ at 3.0 mg/L stimulated maximum of 99.1% shooting response with an average of 21.2±0.5 number of microshoots in cotyledonary leaf than cotyledonary node and hypocotyls explants culture. BA at 5.0 mg/L in combinations with 0.3 mg/L KIN influenced 73 and 53% shooting response with total of 13.5±1.9 and 7.2±1.0 microshoots from hypocotyls and cotyledonary node explants, respectively. Although, cotyledonary leaf explants showed maximum of 79.5% shooting response with an average of 17±3.7 number of microshoots in similar concentration of BA and KIN tested medium.

In other hand, cotyledonary leaf explants exhibited better shooting response (100%) with an average of 24.5±1.1 number of microshoots on 3.0 mg/L TDZ and 0.5 mg/L KIN tested medium. Hypocotyls tested medium showed moderate shooting response (96%) with total of 18±0.3 number of microshoots whereas shoot induction percentage from cotyledonary node explants was quite low (72%) with total of 11.1±1.0 number of microshoots on the above tested level of TDZ and KIN (Table 2). The shoot induction response could be dependent on their developmental state and gene expression pattern of the explants. Further, cotyledonary leaf, cotyledonary node and hypocotyl explants were subsequently tested with optimum level of TDZ along with various levels of auxins, IAA/NAA/IBA for frequency of shoot multiplication. In this case, the addition of TDZ (3.0 mg/L) and IAA (0.7 mg/L) to the medium also induced 100% shooting response with maximum of 24.9±1.0 numbers of microshoots with small amount of light green mucilaginous type of basal calli observed from cotyledonary leaf explants culture. NAA (0.5 mg/L) supported with optimum level of TDZ (3.0 mg/L) influenced minimum of 22.2±1.2 numbers of microshoots with low amount of light yellow brown calli formation in cotyledonary leaf. Hypocotyls incubated on 3.0 mg/L TDZ along with 0.5 mg/L NAA or IAA or IBA supplemented medium showed 82.2 (20±0.2), 99 (22±0.5) and 100% (24±2.0) shooting response, respectively. Cotyledonary node showed slow response to influence microshoots connection with small or less amount of basal callus formation on TDZ in combinations with IBA or NAA or IBA tested medium.

Nevertheless, cotyledonary leaf explants cultured on 3.0 mg/L TDZ in combinations with 0.3 mg/L IBA attained significant results (P <0.05) in shoot multiplication (100%) with maximum of 29.1±3.5 numbers of microshoots after 45 d of culture. Little amount of white mucilaginous basal callus was developed on medium touched portion of microshoots due to long time exposure of culture to same medium (Table 3 and Figure 2a and b). The addition of individual auxins (IBA/NAA/IAA) to the cytokinins (TDZ/BA/Zeatin/2-ip/CPPU) supplemented medium might be influenced by microshoot proliferation from basal callus of different explants culture (unpublished data). One way ANOVA analysis at the 95% confidence interval showed significant difference ($P<0.05$) in microshoot organogenesis in all cultures. Adventitious root formation was identified occasionally with few numbers of plantlets in the shoot clumps.

Elongation, rooting and acclimatization response

The combinations of hormones have some effects on shoot elongation, but they were different based on the morphological nature of microshoots. The isolated microshoots (0.2 to 0.5 cm length) placed directly on shoot elongation medium fortified with 0.3 mg/L TDZ and 0.1 mg/L GA_3 influenced maximum of 40% elongation after two weeks of culture (Figures 2c and 3). The abnormal microshoots with swollen leaf did not elongate into normal shoots. The elongated shoots were subjected to root induction medium for frequency of rooting. The reducing MS salt strength to one half normally enhanced rooting frequency but also reduced basal callus formation. Here, the individual excised plantlets subcultured on half strength MS medium supplemented with 1.5% sucrose along with different concentrations of IAA or NAA or IBA influenced adventitious root formation on stem

Table 2. Effects of cytokinins on microshoot regeneration from different explants of *Cleome viscosa* L.

MS medium composition (mg/L)	Cotyledonary leaf			Cotyledonary node			Hypocotyl		
	% Response	Mean no. of microshoots / explants	Mean shoot length (cm)	% Response	Mean no. of microshoots / explants	Mean shoot length (cm)	% Response	Mean no. of microshoots/ explants	Mean shoot length (cm)
BA									
3.0	42±2.2^{g}	7.3±1.4^{f}	0.5±2.4^{a}	21±3.5^{f}	1.0±3.3^{de}	0.5±4.0^{a}	38.2±2.5^{g}	3.0±2.7^{f}	0.6±4.0^{a}
5.0	61±1.5^{bc}	15.2±1.0^{cd}	0.4±0.7^{ab}	50±2.0^{cd}	5.7±2.0^{d}	0.4±1.0^{ab}	57±1.0^{e}	10.5±0.3^{e}	0.5±2.0^{ab}
7.0	50±0.4^{d}	15±0.7^{cd}	0.2±2.0^{bc}	40±1.0^{de}	5.0±2.6^{d}	0.2±1.6^{bc}	45±1.4^{f}	9.0±0.5^{e}	0.5±0.4^{ab}
KIN									
0.5	-	-	-	-	-	-	-	-	-
1.0	23±3.1^{h}	1.3±1.9^{g}	0.5±1.8^{a}	10±1.5^{g}	0.2±1.0e	0.1±0.5^{c}	15±1.6^{h}	0.4±0.4^{g}	0.4±2.2^{b}
1.5	14±1.0^{i}	0.5±2.0^{h}	0.5±1.3^{a}	-	-	-	9.0±2.8^{i}	0.3±1.5^{gh}	0.3±0.3^{bc}
TDZ									
1.0	80±0.5^{c}	15±1.4^{cd}	0.4±1.7^{ab}	37±0.7^{e}	7.5±1.8^{c}	0.3±1.5^{b}	62±4.0^{d}	13±1.0^{cd}	0.3±2.9^{bc}
3.0	99.1±0.4^{ab}	21.2±0.5^{b}	0.3±0.5^{b}	56.2±1.2^{c}	9±0.5^{b}	0.3±2.0^{b}	77±2.1^{c}	15±3.0^{bc}	0.2±4.2^{c}
5.0	98±1.3^{b}	20±0.7^{bc}	0.3±1.0^{b}	50±1.3^{cd}	8.8±0.1^{bc}	0.2±2.2^{bc}	76±0.2^{c}	13.8±1.5^{c}	0.2±2.0^{c}
BA + KIN									
5.0 + 0.1	63±.2.0^{f}	11.7±2.2^{e}	0.4±1.4^{ab}	25±2.5^{ef}	6±0.4^{cd}	0.4±4.4^{ab}	59.9±0.7^{de}	11±2.6^{d}	0.5±2.0^{ab}
5.0 + 0.3	79.5±0.6^{cd}	17±3.7^{c}	0.3±2.0^{b}	53±1.0^{c}	7.2±1.0^{c}	0.5±1.2^{a}	73±1.0^{cd}	13.5±1.9^{c}	0.4±0.5^{b}
5.0 + 0.5	70±1.5^{e}	15±1.5^{cd}	0.2±1.8^{bc}	48.7±1.8^{d}	7±0.7^{c}	0.3±0.7^{b}	70±1.6^{d}	9±1.7^{e}	0.4±0.4^{b}
TDZ + KIN									
3.0 + 0.1	99.5±3.6^{ab}	22±3.0^{b}	0.4±3.0^{ab}	60±1.0^{bc}	9.4±1.0^{b}	0.3±2.2^{b}	80±1.1^{bc}	15.8±2.2^{bc}	0.3±1.7^{bc}
3.0 + 0.3	100±2.0^{a}	24±2.8^{ab}	0.4±1.6^{ab}	66±1.6^{b}	10±0.5^{ab}	0.3±1.0^{b}	84±0.5^{b}	16.5±2.5^{b}	0.3±2.9^{bc}
3.0 + 0.5	100±0.9^{a}	24.5±1.1^{a}	0.3±2.5^{b}	72±0.9^{a}	11.1±1.0^{a}	0.2±1.1^{bc}	96±1.4^{a}	18±0.3^{a}	0.3±3.1^{bc}

-, No response. 45 explants were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

base of the plantlets. In this case, about 63.3 and 90% rooting response with small amount of callus interspersion was observed in half strength MS medium comprising of 0.1 mg/L IAA and IBA, respectively.

However, the plantlets on NAA (0.1 mg/L) supplemented medium exhibited significant factor ($P<0.05$) in determining 97% rooting with decreasing the level of callus interspersion after 3 weeks of culture (Table 4 and Figure 2d). For acclimatization, the rooted plants as well as the germinated plants from *in vitro* culture were transplanted into potting mix (Figure 2e) and covered with clean polythene bags to reduce 80% humidity. There was no detectable variation

Table 3. Optimum level of TDZ along with different auxins on microshoot induction in various explants of *Cleome viscosa* L.

Explants	Medium composition	% of response	Mean no. of microshoots/ explants	Shoot length (cm)	Nature of basal callus formation
Cotyledonary leaf	3.0 mg/L TDZ + 0.3 mg/L IBA	100±2.0^{a}	29.1±3.5^{a}	0.5±1.6^{a}	White mucilaginous calli
Cotyledonary node	3.0 mg/L TDZ + 0.5 mg/L IBA	98.3±1.2^{ab}	14.3±1.9^{cd}	0.4±0.7^{ab}	White mucilaginous calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L IBA	100±1.0^{a}	24±2.0^{b}	0.4±0.5^{ab}	White mucilaginous calli
Cotyledonary leaf	3.0 mg/L TDZ + 0.5 mg/L NAA	95±0.3^{b}	22.2±1.2^{bc}	0.4±1.0^{ab}	Light yellow brown calli
Cotyledonary node	3.0 mg/L TDZ + 0.1 mg/L NAA	67.5±1.7^{d}	11.4±0.2^{e}	0.3±1.4^{b}	Light yellow brown calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L NAA	82.2±1.5^{c}	20±0.2^{c}	0.3±3.0^{b}	Light yellow brown calli
Cotyledonary leaf	3.0 mg/L TDZ + 0.7 mg/L IAA	100±4.0^{a}	24.9±1.0^{b}	0.5±1.1^{a}	Light green mucilaginous calli
Cotyledonary node	3.0 mg/L TDZ + 0.5 mg/L IAA	90±2.7^{bc}	13±1.4^{d}	0.5±0.5^{a}	Light green mucilaginous calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L IAA	99±0.9ab	22±0.5^{bc}	0.4±2.0^{ab}	Light green mucilaginous calli

45 explants were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

among the acclimatized plants with respect to morphological and growth characteristics (Figure 2f). The plants were then transferred to earthen pots containing sterile soil mix in green house and maintained under natural conditions of day length photoperiod, temperature and humidity. The survival of *in vitro* raised plants from both seed and other explants culture were differed based on the composition and strength of medium with or without addition of growth regulators.

Although, the rate of plants survival was decreased from 99 to 95% via seed culture, but cotyledonary node, cotyledonary leaf and hypocotyl explants derived plants exhibited minimum survival rate (97 to 92.7%) for 2 to 4 weeks after transfer to green house condition (Table 5).

DISCUSSION

Effect of medium strength and GA_3 on seed germination

In general, the seed and vegetable parts of plants may pose challenges at all stages of culture. The seed coat covered with chemical inhibitors and mechanical resistance proved to be growth limiting factors to achieve better germination (Thokozani et al., 2011). The dormant seeds of various species subjected to different environmental conditions delayed germination with increasing mortality, growth reduction and low or unsuccessful reproduction (Kevin and Andrew, 2001). Thus, *in vitro* seed germination can be employed as potential method for propagation of medicinally valuable plants under aseptic condition. The seed germination rate is the "speed or velocity" of germination and can be expressed as the suitable exposure time for a defined percentage of seed to germinate (Niedz, 2008).

The present study was carried out to assess the rate of *in vitro* seed germination and conversion of normal plants of *C. viscosa* in different strength of MS basal medium with or without addition of GA_3. Seed germination frequency decreased in quarter and half strength MS medium. However, the surface sterilized seeds cultured on full strength MS basal medium fortified with 0.5 mg/L GA_3 found to be greatest for influencing 55.3% germination when compare to quarter and half strength of media tested after 30 d of culture. Similarly, Padilla and Encina (2003) reported that the various concentration of GA_3 promotes seed germination of *Annona cherimola* plants.

Influence of growth regulators on microshoot induction and proliferation

During the onset of experiment, the juvenile explants such as cotyledonary leaf, cotyledonary node and hypocotyl explants of *C. viscosa* were excised from *in vitro* young seedling and cultured on plant growth regulators supplemented medium for propagation. The regeneration via organogenesis is controlled primarily by the interaction of plant hormones, specifically cytokinins and auxins in the culture medium (Evans et al., 1981). In many cases, the hormone concentrations can-

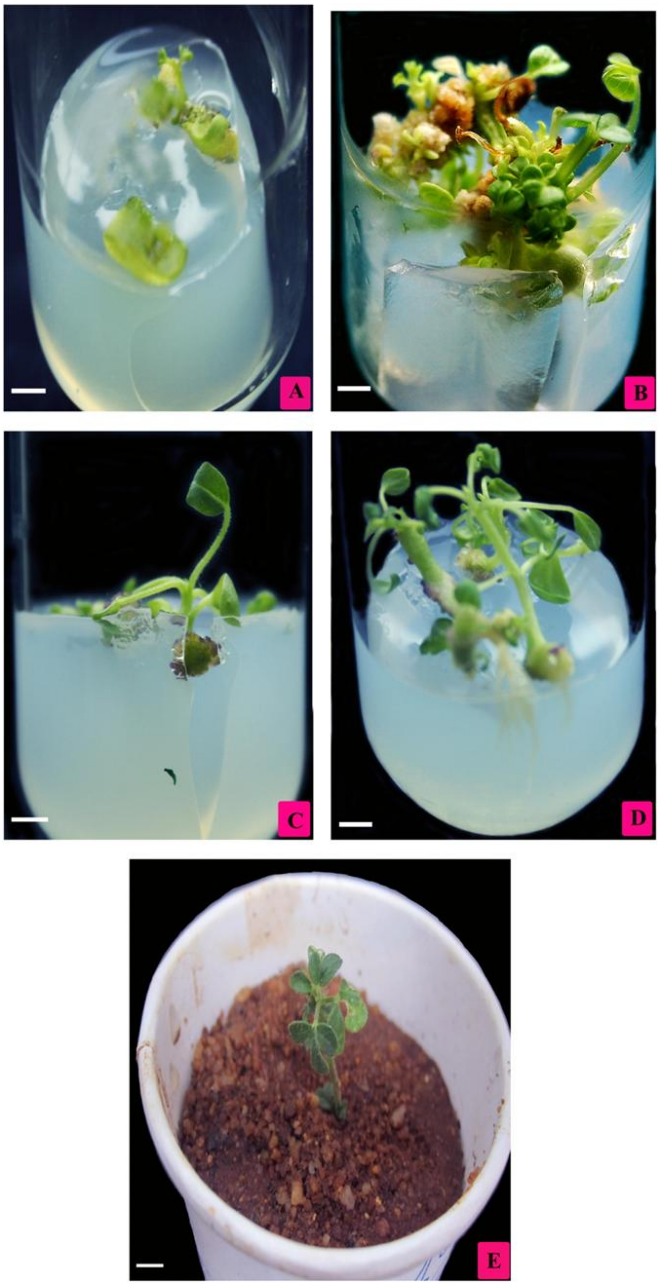

Figure 2. Effect of TDZ on efficient regeneration from cotyledonary leaf explants of *C. viscosa* L.

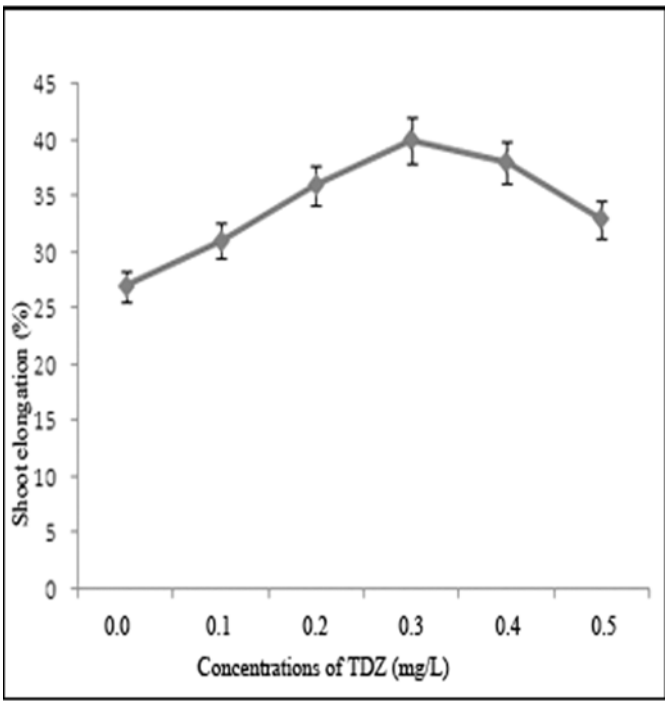

Figure 3. Effect of TDZ along with 0.1 mg/GA, on elongation.

not be the sole mechanism controlling *in vitro* developmental processes. It may be related to differences in tissue and cell differentiation and organization in all explant culture.

In the present study, cotyledonary leaf, cotyledonary node and hypocotyl explants were subjected to individual cytokinins; BA/KIN/TDZ treatment on medium showed varied response for microshoot regeneration although, cotyledonary leaf explants incubated on MS basal medium supplemented with 3.0 mg/L TDZ attained maximum of 99.1% microshoot induction response than cotyledonary node and hypocotyls explants after 45 days of culture. BA or KIN did not prove critical for high frequency microshoot induction in all three explants culture. Further, we found that no single plant growth regulator alone stimulated high frequency shoot regeneration. The enhancement of shoot induction process with subsequent development was significantly affected by combinational usage of growth regulators (BA+KIN/TDZ+KIN/TDZ+IBA/TDZ+NAA/TDZ+IAA).

Among them, TDZ at 3.0 mg/L and 0.5 mg/L KIN supple-mented medium showed 100% shooting response from cotyledonary leaf than hypocotyls and cotyledonary node explants culture. Percentage of microshoot induction per cotyledonary leaf, hypocotyls and cotyledonary node explants was decreased at optimum levels of BA and KIN treatment. In addition, optimum level of TDZ with various form of auxins (IBA or IAA or NAA) were also tested to enhance the rate of shoot multiplication from three different explants culture. In this case, the exposure of cotyledonary leaf explants on TDZ (3.0 mg/L) along with IBA (0.3 mg/L) or IAA (0.7 mg/L) and hypocotyl explants on 3.0 mg/L TDZ and 0.5 mg/L IBA treatments during induction phase have led to influence 100% microshoot growth than cotyledonary node explants. TDZ with NAA treatment did not exhibit more response in shoot multiplication from all these three explants culture.

However, cotyledonary leaf incubated on TDZ and IBA was found to be superior to influence 100% microshoots and produced maximum of 29.1±3.5 number of micro-

Table 4. Effects of auxins on adventitious rooting of *Cleome viscosa* L.

MS medium composition (mg/L)	% of response	Mean no. of roots/ plantlets	Shoot elongation (cm)	Basal callus formation
IBA				
0.1	90±1.0^{b}	2.7±0.3^{b}	1.2±0.5^{ab}	-
0.2	75±1.5^{c}	2.0±1.0^{bc}	0.9±0.1^{b}	+
0.3	60.2±0.6^{d}	1.4±1.2^{c}	0.7±1.0^{bc}	+
NAA				
0.1	97±0.7^{a}	3.8±2.0^{a}	1.8±1.5^{a}	-
0.2	95±1.0^{ab}	3.2±1.2^{ab}	1.2±2.0^{ab}	-
0.3	88±2.0^{bc}	2.6±1.0^{b}	1.0±0.3^{ab}	+
IAA				
0.1	63.3±0.9^{d}	1.4±0.5^{c}	0.7±1.0^{bc}	+
0.2	13.5±1.0^{f}	1±0.7^{cd}	0.5±0.6^{c}	+
0.3	25±3.0^{e}	0.5±1.0^{d}	0.5±1.2^{c}	++

-, Less amount basal callus; +, low amount of basal callus; ++, more amount of basal callus; 30 plantlets were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

shoots significantly after 45 days of culture. The long time exposure of culture (more than 2 weeks) to optimum level of TDZ in combinations with individual auxins (IBA/NAA/IAA) supplemented medium without subculturing converted the medium touched portions of explants along with microshoots to basal callus. The frequent subculture might control basal callus formation around shoot clumps in explants. The development of little amount of basal callus in regular subculture was not affected on the overall development of intact microshoots. The irregular shape of the leaves was not developed from the intact shoots or callus tissues in all cultures.

Thus, the small amount of basal callus formed around cotyledonary leaf, cotyledonary node and hypocotyl explants cultured on fresh medium influenced monopolar structure of microshoots formation (unpublished data). Earlier, Anburaj et al. (2011b) reported plant regeneration from leaf derived callus of *C. viscosa* in BAP and KIN tested medium. However, the shoot regenerative processes in explant cultures were provoked by TDZ alone and in collaboration with other plant growth regulators (Guo et al., 2011). Our results are in accordance with similar findings on efficient shoot regeneration of apple in TDZ with IBA or IAA supplemented medium (Yancheva et al., 2003).

Plant regeneration, hardening and acclimatization

The individual microshoots were excised from cotyledonary leaf, cotyledonary node and hypocotyls explants and placed on MS basal medium fortified with various concentration of TDZ and GA_3 influenced high rate of elongation of shoots. The inclusion of GA_3 alone at different concentrations had no effect on elongation of microshoots, but the addition of low level of TDZ along with GA_3 gave high response in shoot elongation after 2 weeks of culture. In similar fashion, Bhatt and Dhar (2000) reported that the supplementation of TDZ, KIN and GA_3 to the medium was generally beneficial in promoting shoot elongation of *Bauhinia vahlii* plants. Further, the elongated shoots were cultured on half strength MS basal medium comprised of 1.5% sucrose and 0.8% agar with different concentrations of individual auxins (IAA/NAA/IBA) for frequency of adventitious root formation. The rhizogenic basal calli were also observed from stem base of shoots connection with full strength MS basal medium supplemented with auxins.

Although, we have found that the half strength MS basal medium supplemented with NAA showed highest rooting response (75%) with an average of 3.8±2.0 number of adventitious microroot formation after 3 weeks of culture. Basal callus interspersion was almost controlled on half strength rooting medium with NAA. Previous study also revealed that the low level of NAA tested medium exhibited better rooting in shoot system of *C. viscosa* (Anburaj et al., 2011b). The medium devoid of growth regulators failed to develop frequency of rooting in this species. In contrast, Rathore et al. (2013) reported efficient rooting of in vitro propagated *C. gynandra* plants on MS medium with IBA, NOA (2-naphthoxyacetic acid) and activated charcoal while Albarello et al. (2006) achieved adventitious rooting of shoots from *C. spinosa* plants on MS0 medium without addition of plant growth regulators.

Table 5. The survival rate of *in vitro* raised plants in pots containing soil mixture.

Propagation	Mean no. of plants	Survival weeks after transfer to soil (%)		
		2nd week	3rd week	4th week
Seed culture	38.7±1.0[a]	99±0.9	9 7.1±0.4	95±2.0
CL/CN/HC explants culture	30±0.5[b]	97±2.0	94±1.7	92.7±1.6

CL, Cotyledonary leaf; CN, cotyledonary node; HC, hypocotyls. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

After successful root establishment, *in vitro* rooted shoots were transplanted in plastic cups containing soil mix. *In vitro* raised plants survived well and substantially grow into normal plants under greenhouse condition. They did not show any morphological abnormalities compared to donor plant during the maturation period. Therefore, the protocol developed is superior to earlier findings for high rate of multiplication of *C. viscosa* plants. This protocol could be used for the mass multiplication of this very important medicinal weed plants in a short period of time. The conservation of this plant species will cater for the growing need of pharmaceutical and biotechnological industries.

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Full Length Research Paper

Assessment of allelopathic potential of *Cassia sophera* L. on seedling growth and physiological basis of weed plants

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Allelopathy is described as both beneficial and deleterious biochemical interaction between plant and weeds, and/or plant and microorganisms through the production of chemical compounds that escape into the environment and subsequently influence the growth and development of neighbouring plants. The present laboratory experimental study was conducted to evaluate the allelopathic effect of *Cassia sophera* (L.) on three weed plants (*Chenopodium album* L., *Melilotus alba* Medik and *Nicotiana plumbaginifolia* Viv.). Aqueous extracts of *Cassia* at 0.5, 1.0, 2.0 and 4.0% concentrations were applied to determine their effect on seed germination, seedling growth, dry biomass, leaf area, relative water content, chlorophyll and protein content of test plants under laboratory conditions. The aqueous extracts had a significant retardatory effect on seed germination of test plants which varied among species and also with the different concentrations used. Root length and shoot length of weed species decreased significantly when plants were exposed to increasing aqueous concentration (0.5, 1, 2 and 4%). The noticed reduction in dry biomass, leaf area and relative water content were also significant. Physiological parameters (total chlorophyll content and protein content) in relation to three test species (*M. alba*, *C. album* and *N. plumbaginifolia*) were significantly reduced with the different concentrations of aqueous extract used. From this we can predict that cassia might possess allelochemicals that causes the suppressive ability.

Key words: Allelopathy, aqueous extract, *Melilotus alba*, *Chenopodium alba* and *Nicotiana plumbaginifolia.*

INTRODUCTION

Weeds have been a persistant problem for agriculture systems because it causes economic losses by reduction in crop yield, increase cost of crop production, and often cause total crop failure (Bhuler et al., 1998). Concerns about negative effects of herbicide use, such as environmental contamination, development of herbicide resistant weeds and human health problems, make it necessary to diversify or other weed management options (Holethi et al., 2008). The use of allelopathic behavior is one of the new options for sustainable weed management (Olofsdotter and Navarez, 1996). The invasive nature of weeds is due to the allelopathy which can be defined as the direct or indirect harmful or beneficial effects of one plant on another through the production of chemical compounds that escape into the environment. All these compounds usually are called

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Abbreviations: DAS, Day after sowing; **CRD,** completely randomized design; **AOSA,** Association of Official Seed Analysts; **DMSO,** di-methyl sulphoxide; **CSAE,** *C. sophera* aqueous extract; **GP,** germination percentage; **PL,** plumule length; **RL,** radicle length; **DW,** dry weight; **RWC,** relative water content.

secondary plant products or waste products of the main metabolic pathways in plants (Turk et al., 2003; Yokotani et al., 2003; Iqbal et al., 2006). The production of allelochemicals is widely influenced by genetics as well as environmental factors at different growth stages (Yu et al., 2003). They enter the environment through various routes such as leaching, volatilization, root exudation, seed-coat exudation after imbibition, and decomposition of diverse parts of the plant (Rice, 1984; Bertin et al., 2003; Higashinakasu et al., 2004). All plant parts of the weed including leaf, stem, root, and fruit depending upon plant species have allelopathic potential (Mahmood et al., 1999; Alam and Islam, 2002; Tinnin and Muller, 2006). Different groups of plants, crops and weeds have wide known allelopathic interactions (Inderjit and Dakshini, 1994; Ahmad et al., 2007; Uddin et al., 2007). There are several reports that some weed species have allelopathic effects on seed germination and seedlings growth of economically important crop plants (Mulatu et al., 2011; Shibu and Andrew, 1998; Rice, 1984; Delabays et al., 2004; Gulzar and Siddiqui, 2014; Sisodia and Siddiqui, 2012). The multiple effects resulting from allelochemicals include decreases in plant growth, absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turgor pressure, osmotic potential, dry matter production, leaf area expansion, stomatal aperture size, stomatal diffusive conductance, and photosynthesis (Booker et al., 1992; Chou and Lin, 1976; Einhellig et al., 1970; Einhellig and Kuan, 1971; Einhellig and Rasmussen, 1979; Einhellig et al., 1985; Gerald et al., 1992; Patterson, 1981; Weir et al., 2004).

The *Cassia sophera* belongs to the Leguminosae family and the subfamily of Caesalpinioideae, extending from Africa to India and South East Asia. *C. sophera* is believed to be native of South America. The species grows along roadsides and on waste ground and is reported by Mulay and Sharma (2012) to be a common weed in uncultivated lands. Its invasive nature is due to its fast growth rate, high reproductive and vegetative potential, adaptable to changing environmental conditions, wide ecological amplitude and allelopathy. Triterpenes from cassia are known for their allelopathic responses and great ecological significance with respect to invasion (Ghayal et al., 2007). The aqueous extract of the whole plant and leaves produces an inhibitory allelopathic effect on many weeds. The powdered leaves of *C. sophera* also affect the growth and metabolism of associated weeds. Recently, a study showed that the allelopathic effect of *Cassia occidentalis* L. causes the suppression of seed germination and growth in *Parthenium hysterophorus* L., a detrimental weed in India (Knox et al., 2011). Similarly, the allelopathic effect of *Cassia tora* on seed germination and growth of mustard has been elucidated by Sarkar et al. (2012). In ethno botanical literature, it is mentioned to be effective in the treatment of pityriasis, psoriasis, asthma, acute bronchitis, cough, diabetes and convulsions of children (Chopra et al., 1956; Agharkar, 1991; Dutt, 1995; Kirtikar and Basu, 2000). For this reason, the plants are not destroyed and instead allowed by the local people to grow near crop fields.

Therefore, in the present work, an attempt has been made to evaluate and compare the allelopathic potentiality of the common invasive weed (*C. sophera*) on the germination, seedling growth, dry biomass, leaf area and physiological parameters of three weed species (*M. alba*, *C. album* and *N. plumbaginifolia*).

MATERIALS AND METHODS

Collection of plant materials

The *C. sophera* was collected from the campus of Aligarh Muslim University, Aligarh (27°, 29 to 28°, 100 N.L and 77°, 29° to 78°, 38° E.L) where it was growing abundantly. The plants were uprooted at maturity. They were washed thoroughly with distilled water and air-dried at room temperature for 96 h. The aerial portions were separated, chopped into 1 cm long pieces, and ground into fine powder with mortar and pestle. Stock aqueous extract was obtained by soaking 4 g powder in 100 mL of cold distilled water (4% w/v) at room temperature (20±2°C) for 24 h with occasional shaking. The mixture was filtered through two layers of cheesecloth and centrifuged for 20 min to remove particulate material and the purified extract was adjusted to pH 6.8 with 1 M HCl. Different concentrations (0.5, 1 and 2%) were prepared from the stock solution in addition to the control (Singh et al., 1989). The aqueous extracts were individually bottled, tagged, and to maintain the efficacy of extracts, it is kept in refrigerator for further use.

Germination in Petri dishes

For growth studies, seeds of *C. album, M. alba* and *N. plumbaginifolia* were procured from National research centre for weed science, Jabalpur (M.P). Seeds of each test plants were first surface sterilized with 2% sodium hypochlorite solution for 2 min and washed thoroughly with distilled water. Next, sets of autoclaved Petri dishes were prepared, each containing a single layer of Whatman No. 1 filter paper and 5 mL of test extract for each concentration (0.5, 1, 2 and 4%) of aerial shoots. The Petri dishes treated with distilled water were taken as a control and considered to be set 0. The treatments were arranged in completely randomized design (CRD) with 10 replicates at room temperature on a laboratory bench with 12 h supply of fluorescent light during the night. The whole experiment was repeated once. The plants were sampled on 15th day after sowing (DAS) to record various observations.

Determination of germination percentage, root length, shoot length, dry biomass, leaf area and relative water content

Germinated seeds were counted daily according to the Association of Official Seed Analysts (AOSA) method (AOSA, 1990). The seeds were considered as germinated when the radical size was 2 mm. Fifteen (15) days after sowing, germination percentage was calculated using the formula: germination percentage = (germinated seed/total seed × 100) for each replication of the treatment followed by seedling root length (cm), shoot length (cm) and dry weight (mg) determination. The root and shoot length were measured by using a

meter scale; while, the dry weight was measured with the help of four digital balance of Scientech, Model ZSA 120, Colorado (USA). The leaf area was measured manually by using a graph sheet, where the squares covered by the fresh leaf were counted to calculate the leaf area. Using the equation of Deef and Abd El-Fattah (2008), the relative water content (RWC) was evaluated as RWC% = (FW - DW) / FW × 100.

Protein determination

The method as given by Lowry et al. (1951) was adopted for this purpose.

Chlorophyll determination

The total chlorophyll content from leaves of treated or control plants were extracted in di-methyl sulphoxide (DMSO) following the method of Hiscox and Israelstam (1979). Finely cut uniform discs (100 mg fresh weight) were made from fully expanded leaves of test plants. Dry weight equivalents of each of the treated samples were determined by keeping 100 mg fresh weight discs in an oven. The weighted material (100 mg fresh weight leaf disc) was suspended in 10 ml of di-methyl sulphoxide (DMSO) incubated at 65°C for 1 h (the period of incubation was found sufficient for the complete extraction of chlorophyll). The DMSO was recovered by thorough decantation. The final volume was corrected to 10 ml with fresh DMSO. The extinction of chlorophyll thus, recovered in DMSO was measured at dual wavelength of 645 and 663 nm on spectro-photometer against DMSO as blank. The extinction values were read and the amount of chlorophyll was calculated according to the equation given by Arnon (1949), with modification by Hiscox and Israelstan (1979).

Statistical analysis

Using standard procedures of statistical data analysis (including the software BioStat 2009, version 5.7.8.1, and the inbuilt mathematical functions of sigma plot version 10, the effects of different concentrations of *C. sophera* were correlated with the rate of germination, root length, shoot length, leaf area, DW, RWC, chlorophyll content and protein content of test species. Figures 1 to 4 show change in these parameters (the bars represent the standard deviation of measurement)

RESULTS

Germination

The germination percentage (GP) of *M. alba* was significantly affected by the increase in concentration of *C. sophera* aqueous extract (CSAE) (Figure 1). In control and 0.5% CSAE, GP values were 100 and 98%. The percentage was reduced to 57% at 1% and 55.6% at 2% CSAE concentration levels and to 30% at 4% CSAE concentration. Generally, GP of *C. album* seeds varied with CSAE concentrations (Figure 1) and it is supported statistically. In the control series, GP values were 80% but decreased upon applying 0.5 and 1% CSAE concentrations (67 and 70%, respectively). However, the reduction goes to a markedly lower level at 2 and 4% concentrations (47 and 50%, respectively). Figure 1 shows a great variation in the calculated values of GP of *N. plumbaginifolia* seeds. The GP was significantly affected by the increase in CSAE concentrations. In control, 0.5 and 1% CSAE, GP values were 100, 95 and 92%. The percentage was reduced at 2 and 4% CSAE concentration levels.

Seedling growth

The results of plumule length (PL) of *M. alba* imply that allelopathic substances affect negatively the seedling stage (Figure 2). PL was significantly reduced at different concentrations of treatments given. The value of PL was 20.45 cm at control level, but reduced to 18.20 cm at 0.5% CSAE concentration. The retardatory allelopathic action was recorded in 1, 2 and 4% CSAE concentrations, which significantly reduced PL. Allelopathic effect of CSAE concentration on PL of *C. album* is given in Figure 2. The plumule elongation was not completely inhibited by the extract, but the length was reduced at higher concentration levels. Obviously, all allelopathic concentrations have reduced PL. In control series, PL of *C. album* was 19 cm. In 0.5, 1, 2 and 4% concentrations, inhibition was observed and the values were 17.71, 17.77, 15.81 and 15 cm, respectively (Figure 2). PL of *N. plumbaginifolia* was significantly reduced in each treatment (Figure 2). PL was 18.17 cm in control series but was reduced to 17 cm at 0.5% CSAE concentration. The maximum allelopathic action of 1, 2 and 4% CSAE concentrations was observed in the form of reduction as 16, 14.90 and 14 cm, respectively.

A slight difference was observed among *M. alba* RL (Figure 1). The control value was 12.48 cm. Elevated CSAE concentrations had significant retardatory effect on radical growth. At 0.5% CSAE concentration, it was 11.48 cm. Upon applying highest CSAE concentration (4%), it was reduced to 5.99 mm. A gradual decrease in RL of *C. album* was observed with gradual increase in CSAE concentrations. RL was significantly affected by the treatment at 4% concentration. In the control, values of RL were 10 cm, but at higher concentrations of CSAE radicle emergence were clearly affected. At 0.5 and 1% concentrations, RL decreased to 9.50 and 8.31 cm; till it attained a value of ~6.39 and 4 cm at 2 and 4% concentrations. In the control series RL of *N. plumbaginifolia* was 9.00 cm. High CSAE concentrations had significant retardatory effect on radical growth (Figure 1). At 0.5% CSAE concentration it was 8.01 cm. Upon applying the highest CSAE concentration (4%), it was reduced to 3.99 cm and at 1 and 0.5% CSAE, the values were 7.11 and 8.01 cm, respectively.

Dry biomass, leaf area and relative water content

As per the dry biomass of test species affected by CSAE at 4% concentration, the percentage reduction observed was 34, 40 and 44% in *M. alba*, *C. album* and *Nicotiana plumbaginifolia* over the control (Figure 2). In contrast to

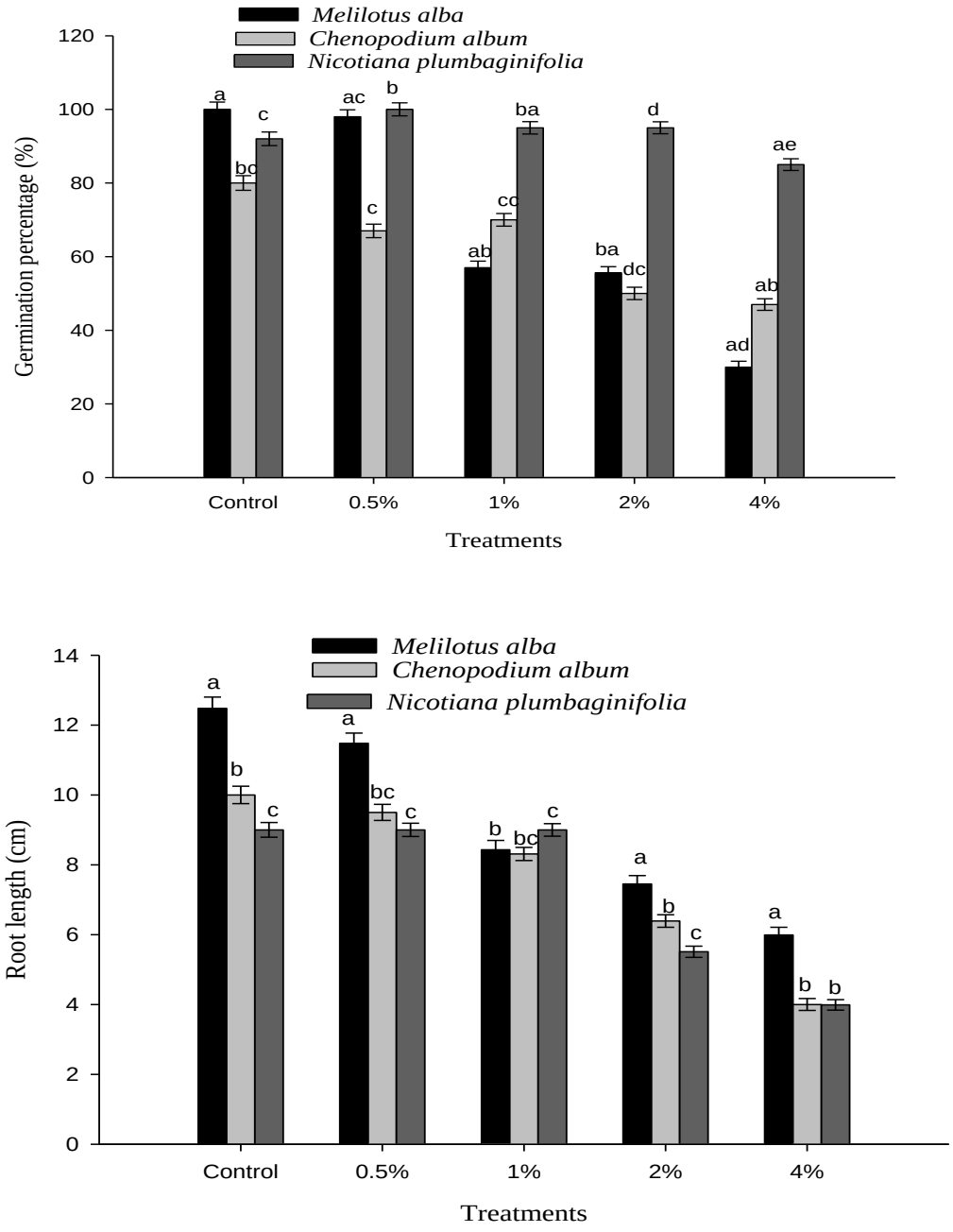

Figure 1. The effects of increasing concentrations of *Cassia sophera* on germination percentage (%) and root length (cm) of test species (n = 10).The bars indicate standard deviation. Different superscript symbols within each column represent significant difference among themselves at P<0.05 applying DMRT.

control, leaf area shows decrease with increase in extract concentration, reduced by 16, 10 and 19.65% in *C. album*, *M. alba* and *N. plumbiginifolia* at 4% CSAE concentration (Figure 3). Leaf relative water content (RWC) of the stressed plants showed significantly lower values at higher CSAE concentration than their control plants and that were 26.6% in *N. plumbiginifolia* and 14.28% in *M. alba* (Figure 3).

Protein and chlorophyll content

The protein and chlorophyll content of test species were affected significantly when treated with the different concentrations of CSAE. Although, treatment with 0.5% CSAE had very little impact on the protein and chlorophyll content of test seedlings, higher concentrations (4%) reduced the protein contents by 41.3, 20 and 33% in *M.*

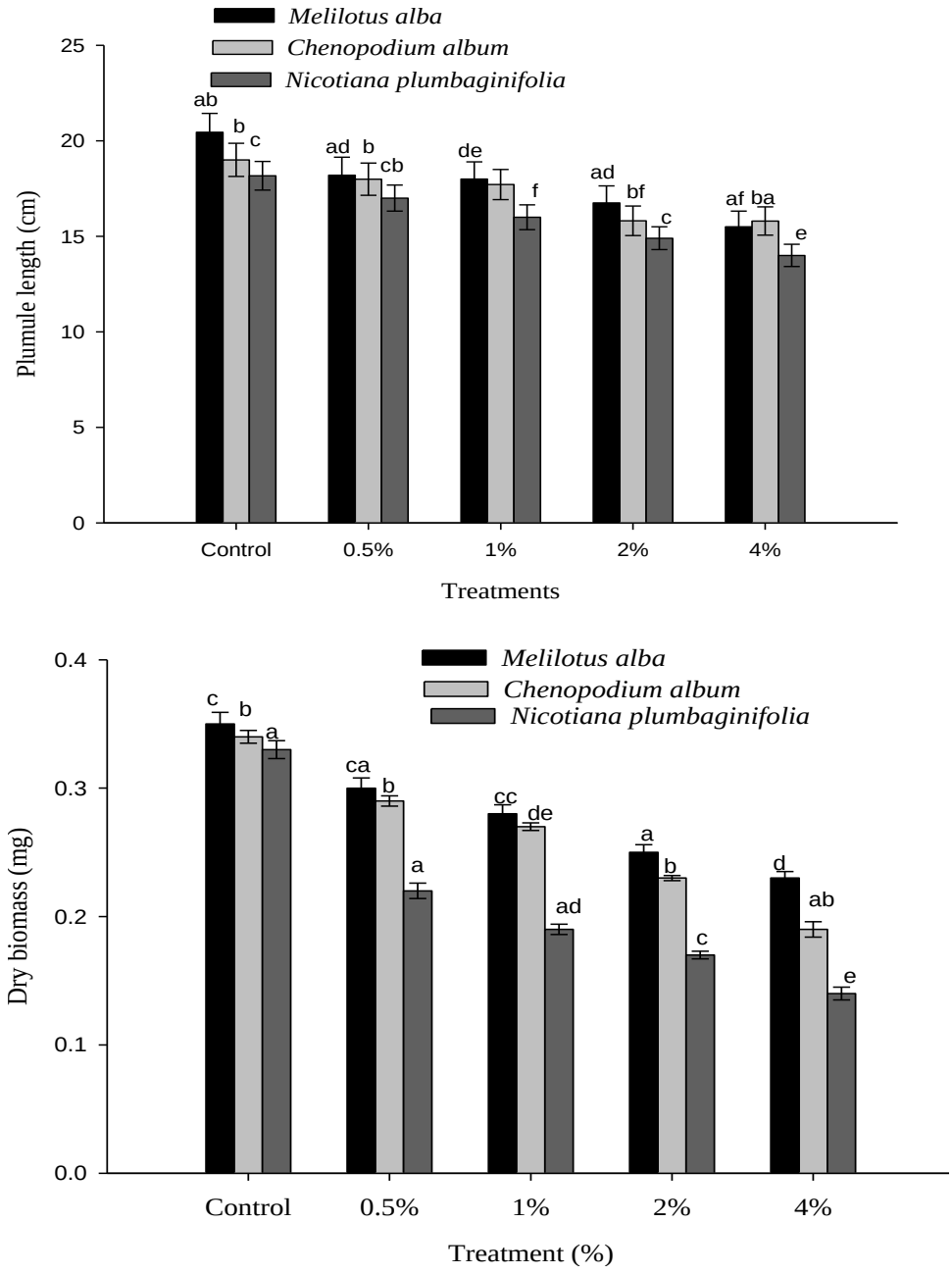

Figure 2. The effects of increasing concentrations of *Cassia sophera* on plumule length (cm) and dry biomass (mg) of test species (n = 10). The bars indicate standard deviation. Different superscript symbols within each column represent significant difference among themselves at P<0.05 applying DMRT.

alba, *C. album* and *N. plumbiginifolia* as compared to the control (Figure 4). The chlorophyll content was similarly affected resulting to more reduction at higher concentration (4%) in comparison to control (Figure 4).

DISCUSSION

Treatment by aqueous extract resulted in delayed germination and low germination rate of the weeds. Through delayed germination, lowered seed germination rate and reduced seedling growth, reduced root-shoot ratio, the treatments lowered dry matter. The inhibitory effects were increased with increasing concentrations. This study shows that different concentrations of leachate showed distinct allelopathic inhibitory effects on the weed species; lower concentrations (1 and 0.5%) showed weak inhibitory or even positive effects, whereas higher

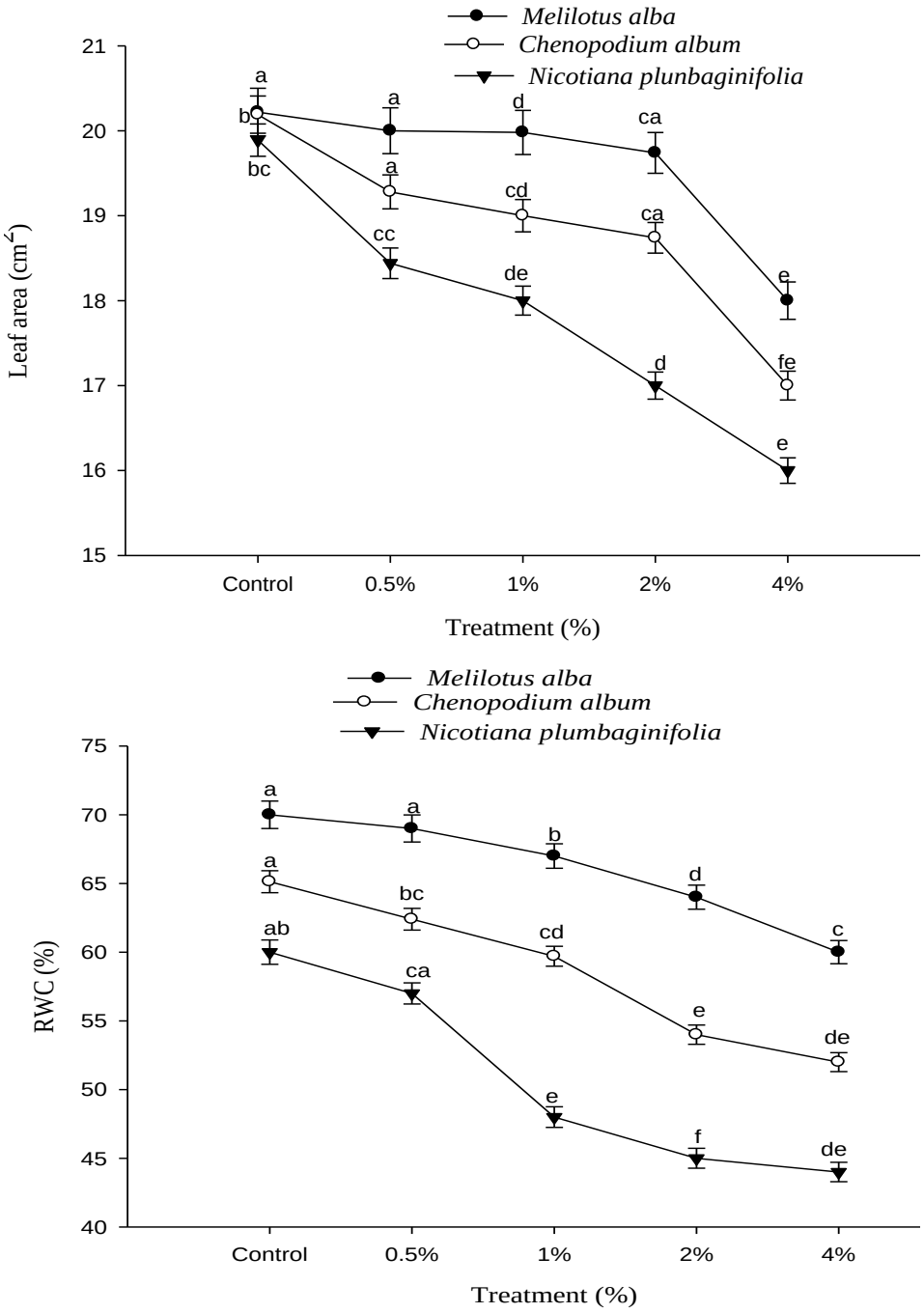

Figure 3. The effects of increasing concentrations of *Cassia sophera* on leaf area (cm^2) and Relative water content (%) of test species (n = 10). The bars indicate standard deviation. Different superscript symbols along each represent significant difference among themselves at P<0.05 applying DMRT.

concentrations (4 and 2%) showed stronger inhibitory effects. Therefore, considering that the plant allelochemicals could have beneficial or inhibitory effect, in order to control.

As observed in many studies (Hong et al., 2003; 2004; Mulatu et al., 2011; Lungu et al., 2011; Shapla et al., 2011), allelochemicals extracted from Melia (extracted with ethanol and water) inhibited germination and growth of receiver plants are in line with our studies. Similar observation was also concluded by Bogatek and Gniazdowska (2007) and Javed (2011) in radish germination inhibition due to the result of induction of

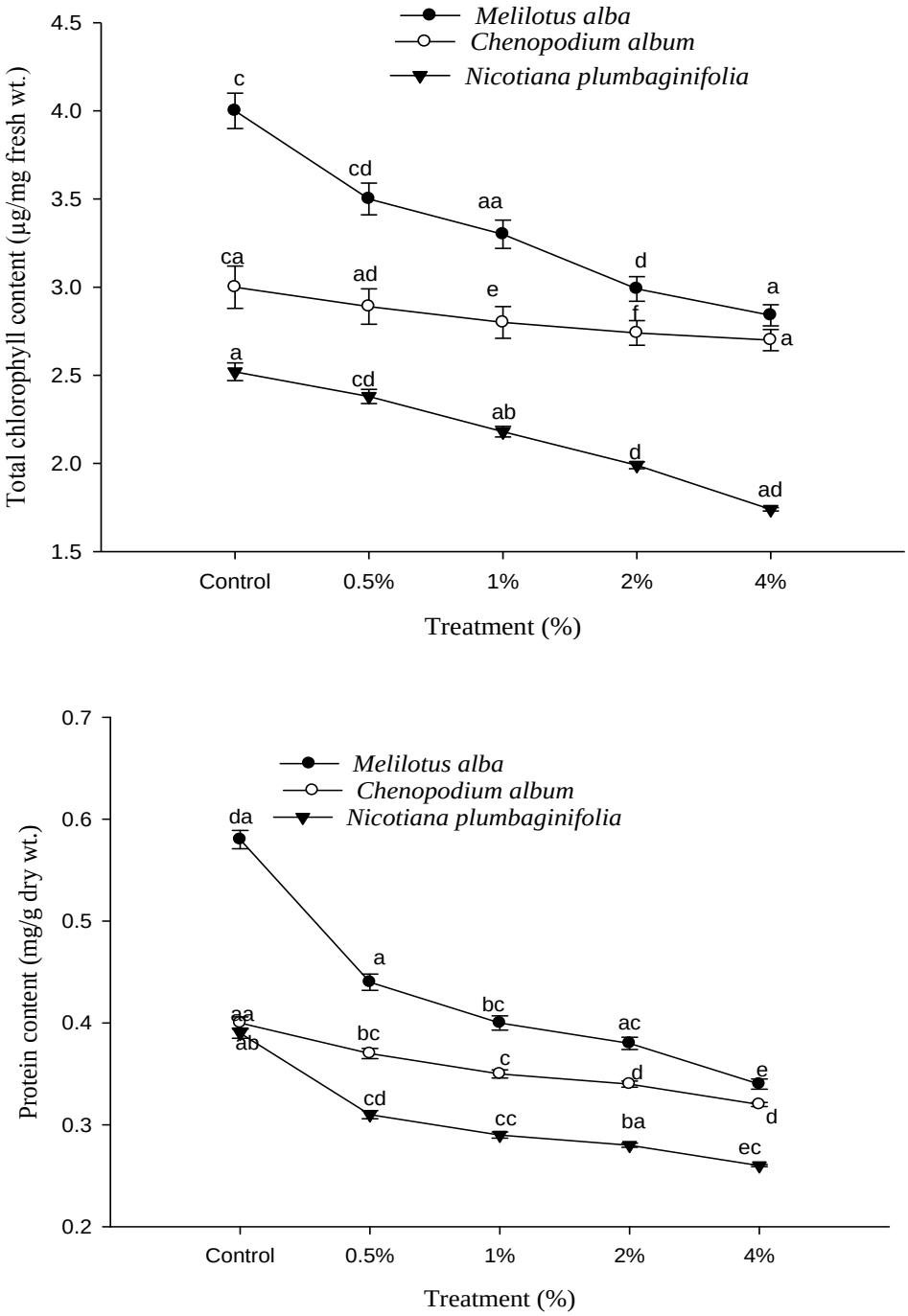

Figure 4. The effects of increasing concentrations of *Cassia sophera* on chlorophyll content (mg/g fr. Wt.) and protein content (mg/g dry weight) of test species (n = 10).The bars indicate standard deviation. Different superscript symbols along each curve represent significant difference among themselves at P<0.05 applying DMRT.

oxidative stress. Suggested mechanism for the inhibition of seed germination is the disruption of 'dark' or mitochondrial respiration and by this, a possible disruption of the activity of metabolic enzymes involved in glycolysis and oxidative pentose phosphate pathway (OPPP), which takes substrates from glycolysis and feeds its products back into glycolysis as observed in *Pinus laricio* seeds germination grown in the soils around *P. laricio* and *Fagus sylvatica* trees (Muscolo et al., 2001). In general, the negative effect increased with the concentration of the extract and biological activities of receiver plants to allelochemicals which are known to be

concentration dependent as noticed by Zhang et al. (2007), Singh et al. (2006) and Peng et al. (2004), which also is in line with our study.

The root length decreased as the concentration of extract increased and the greatest inhibition was observed at 4% concentration. As root membranes are a primary site of action for phenolics. The contact of phenolic acids with the root cell membrane leads to depolarization, an efflux of ions, and a reduction of hydrolic conductivity, water uptake and net nutrient uptake (Baziramakenga et al., 1995; Lehman and Blum, 1999). Root growth is characterized by high metabolic rates and, for this reason, roots are highly susceptible to environmental stresses such as allelochemicals in soils (Cruz-Ortega et al., 1998). Similarly, Baerson et al. (2005) reported that BOA impaired root system development, resulting in reduced root lengths and a complete absence of lateral root formation in 10-day-old *A. thaliana* seedlings by 50% (I_{50}) and 80% (I_{80}) at 540 µM and µM. In the present study, aqueous extract lowered relative water contents in leaves of all target species. Apparently, the three target species responded differently to the phytotoxic compounds, thereby contributing to species selectivity. Some other phenolic acids such as p-coumaric, caffeic, ferulic, and salicylic acids also cause water stress in plants (Einhellig, 1995; Barkosky and Einhellig, 2003). Sanchez-Moreiras and Reigosa (2005) reported that a 10% decrease in relative water content was correlated with a decline in leaf water potential in BOA-exposed lettuce plants concentration, respectively. From our results, it is clear that decreases in the relative water content of leaves in these plant species initially induce stomatal closure, imposing a decrease in the supply of CO_2 to the mesophyll cells and, consequently, photosynthesis could be lowered resulting in the decrease in chlorophyll content (Hussain and Reigosa, 2011). The significant reduction of chlorophyll content seen with all concentrations may be due to the inhibition of chlorophyll biosynthesis, the stimulation of chlorophyll-degrading substances, or both (Yang et al., 2007; Patterson, 1981). One of the best-characterized phytotoxic mechanisms induced by allelochemicals is the inhibition of photosynthesis and oxygen evolution through interactions with components of photosystem II (PSII) (Einhelling, 1995). Higher concentrations of extracts were found to cause mosaic chlorosis, resulting in the yellowing of leaves and thereby causing the reduction in the chlorophyll content. A significant downgrade in leaf protein contents in all three species due to aqueous extract of different concentration is supported by findings of Baziramakenga et al. (1997); Mersie and Singh (1993); Hussain et al. (2010) that many phenolic acids reduced the incorporation of certain amino acid into proteins and thus reduced the rate of protein synthesis.

The dry mass reduction is supported by previous study of Terzi and Kocacaliskan (2010), where the elongation and DW of barley and wheat seedlings were reported to be reduced by the walnut allelochemical juglone (5-hydroxy-1,4-naphthoquinone) in a similar pattern. Macro- and micronutrient absorption and IAA oxidase in plant root cells is inhibited by various allelochemicals (Yang et al., 2004), which may lead to the observed reductions in DW and RWC of germinating mustard seedling.

Conclusion

The allelopathic effect from aqueous extracts *C. sophera* showed a significant retardatory effect on all the above mentioned parameters of *M. alba, C. album, N. plumbaginifolia*. Hence, the allelochemicals extracted from aqueous extract of *Cassia* can be employed for the natural control of the tested weeds, thus achieving the aim of environmental safety. There is need for further study to be carried out on identifying the inhibiting allelochemical in the parts investigated. By delaying germination, lowering the germination rate of the weeds and inhibiting seedling growth, leachate from *C. sophera* could provide an effective way of controlling the weeds.

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Full Length Research Paper

Xylanase from *Fusarium heterosporum*: Properties and influence of thiol compounds on xylanase activity

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The properties of xylanase purified from *Fusarium heterosporum* that was grown in barley-brewing residue under solid-state fermentation and the effects of thiol compounds on the reactivation of the metal ion-inhibited xylanase were investigated. Xylanase was purified to homogeneity by ion exchange chromatography, and its molecular mass was estimated to be 19.5 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH for the xylanase was 5.0, and it was stable in acidic pH (4.5 to 5.5), where it retained more than 87% of its activity after 24 h. The optimum temperature was 50°C, and it had a half-life of 53 min at 45°C. The apparent Km and Vmax values for the xylanase were 5.63 mg/ml and 800 µmol/mg/min, respectively. Ba^{2+}, Ca^{2+}, Mg^{2+} and the thiol compounds β-mercaptoethanol and dithiothreitol (DTT) enhanced xylanase activity, while Hg^{2+}, Pb^{2+} and Zn^{2+} strongly inhibited enzyme activity. Furthermore, this xylanase had an alternative mode of regulation in the presence of thiol compounds because the enzyme was able to recover its catalytic activity after inhibition by heavy metal ions.

Key words: Hemicellulase, fungus, solid-state fermentation, barley brewing residue, thiol compounds.

INTRODUCTION

Xylan is the major component of hemicellulose, which is abundant in the cell walls of monocot plants and hardwoods. This heteropolymer is composed of xylose units that are connected by a β -1, 4 linkages in the backbone and can be found in the side chains of glucuronic acid, α-arabinose, acetyl, feruloyl and p-coumaroyl residues (Dutta et al., 2007). Due to the structural complexity of xylan, its complete hydrolysis requires the synergistic action of several enzymes, including the endo-1, 4-β-D-xylanases (EC 3.2.1.8), which are important for cleavage of the glycosidic β-1, 4 bonds of the backbone of xylan to produce short-chain xylooligosaccharides of various lengths; β-xylosidase, α-arabinofuranosidase, α-methylglucuronidase and acetyl xylan esterase (Collins et al., 2005; Lafond et al., 2011). These xylanases are classified as Glycosyl Hydrolases

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Abbreviations: DTT, Dithiothreitol; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis.

(GH) based on their amino acid sequences (http://www.cazy.org) and belong to families 5, 7, 8, 10, 11 and 43 (Cai et al., 2011). Xylanase is an industrially important enzyme with many applications, such as the bioconversion of lignocellulosic material or agro-wastes to sugar, bio-bleaching in the paper industry, the improvement of texture and loaf volume of bread (Dobrev et al., 2007), the clarification of juice and wine, the improvement of the nutritional value of animal feed stock, and the extraction of plant oil, coffee and starch (Ahmed et al., 2012).

Filamentous fungi secrete higher levels of xylanase than bacteria and yeasts (Polizeli et al., 2005), and xylanases are produced by several fungi including *Aspergillus*, *Trichoderma*, *Penicillium*, *Aureobasidium*, *Fusarium*, *Chaetomium*, *Phanerochaete*, *Rhizomucor*, *Humicola* and *Talaromyces* (Kheng and Omar, 2005). Likewise, a large number of fungal xylanases have been studied and purified from organisms such as those of the *Trichoderma* sp. (Xiong et al., 2004; Zhou et al., 2011), *Aspergillus carneus* M34 (Fang et al., 2008) and some species of *Fusarium* (for example, *F. verticillioides* and *F. proliferatum*) (Saha, 2001, 2002). *Fusarium* fungi show a cosmopolitan distribution in soil and are associated with plants, as described by Nees in 1832 (Feldman et al., 2008). Some *Fusarium* species are considered plant pathogens [for example, *Fusarium solani* (Bogale et al., 2009) and *Fusarium graminearum* (Kikot et al., 2009)]; however, a few studies have reported the use of *Fusarium* species in the production of hemicellulases under solid-state fermentation.

Recently, a novel isolate of *Fusarium heterosporum* was obtained from local soil and showed the potential to produce xylanase using barley-brewing residue under solid-state fermentation. To our knowledge, no report in the literature concerning xylanase production by *F. heterosporum* exists. However, lipase production by this fungus has been widely studied. Some fungal xylanases have been reported to enhance their activity in the presence of thiol compounds such as dithiothreitol (DTT) and β-mercaptoethanol; however, these enzymes are strongly inhibited by certain heavy metal ions. Thus, in this study, the influence of thiol compounds on xylanase from *F. heterosporum* that had been inhibited by heavy-metal ions and the recovery of the catalytic activity of metal-ion-inhibited xylanase is reported.

MATERIALS AND METHODS

Fungal strain and culture conditions

The fungus *F. heterosporum* was newly isolated from local soil from Cascavel, Paraná state, Brazil and identified at the Instituto de Botânica (Institute of Botany), São Paulo, Brazil. The fungus strain was cultivated in potato-dextrose-agar (PDA) at 28°C for 7 days, and after growth, its spores were harvested in sterile, distilled water. Solid-state fermentation (SSF) was carried out by seeding 10^5 spores ml^{-1} of *F. heterosporum* on 5 g of various carbon sources (agro-industrial residue) and moistened with distilled water (1:1 w/v). The cultures were incubated at 28°C for six days, and after incubation and growth, 50 ml of cold, sterile, distilled water was added and agitated in an orbital shaker (150 rpm) at 20°C for 60 min. The solid materials and fungal biomass were subsequently vacuum-filtered on filter paper, the filtrate was centrifuged at 5,000 x *g* for 10 min, and the clear supernatant was used to determine the enzymatic activity.

Enzymatic assay and protein quantification

The xylanase activity was assayed by analyzing the reducing sugars that were released after incubation in a properly diluted enzyme solution containing 1% (w/v) birchwood xylan in 50 mM acetate buffer (pH 5.0) at 50°C for 10 min. The amounts of reducing sugars were determined using the dinitrosalicylic acid (DNS) method by Miller (1959). One unit of xylanase was defined as the amount of enzyme that was capable of releasing 1 µmol of D-xylose and served as the standard under the assay conditions. The amount of protein was estimated by the Bradford method (1976) using bovine serum albumin as a standard, and an absorbance of 280 nm was used for monitoring the protein in the column eluates.

Purification of xylanase from *F. heterosporum*

The crude extract of *F. heterosporum* was cultured using barley-brewing residue under solid-state fermentation (fungal strain and culture conditions) after incubation for six days. Subsequently, the culture was filtered and centrifuged at 5000 *g* for 10 min at 4°C. The supernatant was then dialyzed using 25 mM sodium acetate buffer (pH 5.5), loaded onto a diethylaminoethyl cellulose (DEAE-Cellulose) chromatographic column (2.0 × 20 cm) and eluted using a linear gradient of NaCl (0 to 0.5 M) in the same buffer. 5 ml fractions were collected at a flow rate of 1.0 ml/min, and those with xylanase activity were pooled, dialyzed overnight using 25 mM sodium acetate buffer (pH 5.5) at 4°C, applied onto a carboxymethyl-cellulose (CM-cellulose) chromatographic column (2.0 × 20 cm), and eluted using a linear gradient of NaCl (0 to 0.5 M) in the same buffer. 3 ml fractions were collected at a flow rate of 0.5 ml/min, and those with the highest activity were pooled, lyophilized and used for biochemical characterization of the purified xylanase.

Effect of pH and temperature on enzyme activity and stability

The optimum pH for xylanase was determined to be 50°C using the McIlvaine buffer (1921) at pH values ranging from 2.2 to 8.0. The optimum temperature was determined by assaying for xylanase activity at temperatures ranging from 40 to 65°C. The thermal stability of xylanase was determined by pre-incubating the enzyme samples at 45, 50 and 55°C; aliquots were withdrawn at various time points, and the residual activity was measured under standard conditions.

Electrophoresis

Samples of purified enzyme were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The gels were silver-stained according to Blum et al. (1970), and the utilized molecular mass markers were as follows: phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.1 kDa) and α-lactalbumin (14.1 kDa).

Kinetic parameters

The kinetic parameters of the purified enzyme were determined

Table 1. Effect of carbon source on xylanase production by *F. heterosporum*.

Carbon source	Xylanase activity[a] (U/ml)
Sugar cane bagasse	0.12 ± 0.01
Barley-brewing residue	6.94 ± 0.21
Peanut husks	0.66 ± 0.02
Passion fruit husks	0.66 ± 0.05
Wheat bran	3.58 ± 0.27
Corn straw	0.82 ± 0.03

[a]Mean and standard deviation of three replications.

Table 2. Summary of the purification of xylanase from *F. heterosporum*.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (U/mg prot.)	Yield (% recovery)	Purification (fold)
Crude extract	45.11	2,526.55	56. 09	100	1
DEAE-Cellulose	18.91	1,781.20	94. 21	72.8	1.7
CM Cellulose	1.09	139.29	127.78	21.1	2.3

Total U: U ml^{-1} × volume of extract; total protein: mg ml^{-1} × volume of extract.

using birchwood xylan as the substrate for the xylanase assay, and the concentrations of xylan ranged from 1 to 20 mg/ml. The K_m and V_{max} values were determined using the Lineweaver-Burk plot (1934).

Effects of metal ions and other compounds on xylanase activity

Salts [$BaCl_2$, $CaCl_2$, $CoCl_2$, $HgCl_2$, KCl, $MgCl_2$, NaCl, $PbCl_2$, $ZnCl_2$ and $(NH_4)_2SO_4$] and other compounds [β-mercaptoethanol, dithiothreitol (DTT), cystine, L-cysteine, ethylenediaminetetraacetic acid (EDTA), iodoacetamide and sodium dodecyl sulfate (SDS)] were also tested. The metal ions and compounds (1 or 5 mM) were pre-incubated with the enzyme for 15 min. After incubation, an aliquot was withdrawn and chilled on ice, and the hydrolytic activity was determined by the standard assay with xylan as the substrate.

Influence of thiol compounds on the recovery of heavy metal ion-inhibited xylanase activity

Xylanase was pre-incubated for 10 min with heavy metal ions (Hg^{2+}, Pb^{2+} and Zn^{2+}) at 0.5 mM to promote enzymatic inhibition. Subsequently, the recovery of xylanase activity was assayed by incubating the enzyme with thiol compounds (DTT and β-mercaptoethanol; 0.5 mM) for 20 min and then measuring the enzyme activity under standard conditions. The protective effect of the thiol compounds on metal-inhibited xylanase was assayed by pre-incubating the enzyme with DTT and β-mercaptoethanol (0.5 mM) 15 min before the addition of heavy metal ions (Hg^{2+}, Pb^{2+} and Zn^{2+}). Then, the enzymatic activity was assayed under standard conditions as described in 'enzymatic assay and protein quantification'. The activity recovery of xylanase was observed against a control; that is, the absence of the heavy metal ions).

RESULTS AND DISCUSSION

Effect of an alternative carbon source on the production of xylanase

The ability of the new strain of soil *F. heterosporum* to produce xylanase under SSF was studied using a variety of carbon sources such as sugar cane bagasse, barley-brewing residue, peanut husks, passion fruit husks, wheat bran and corn straw. *F. heterosporum* was capable of growing and producing xylanase (6.94 U/ml) in a culture containing barley-brewing residue, which is an inexpensive substrate that is disposed of in large scale from the brewing industry in Brazil. This result indicates that a substrate such as barley-brewing residue, which is rich in cellulosic and non cellulosic polysaccharides, can be efficiently used to induce xylanase production by *F. heterosporum* (Table 1). Similarly, Silva et al. (2005) have obtained high yields of xylanase using different wastes such as corncob, green grass, dried grass, corn straw and wheat bran as raw materials for SSF using the thermophilic fungus *Thermoascus aurantiacus*.

Purification of xylanase

The xylanase from *F. heterosporum* was purified to apparent homogeneity by ion exchange chromatography, and a summary of the purification procedure is presented in Table 2. Xylanase was purified 2.3-fold with a recovery of 21.1% by two chromatographic, ion-exchange purification steps: a DEAE-cellulose chromatographic column

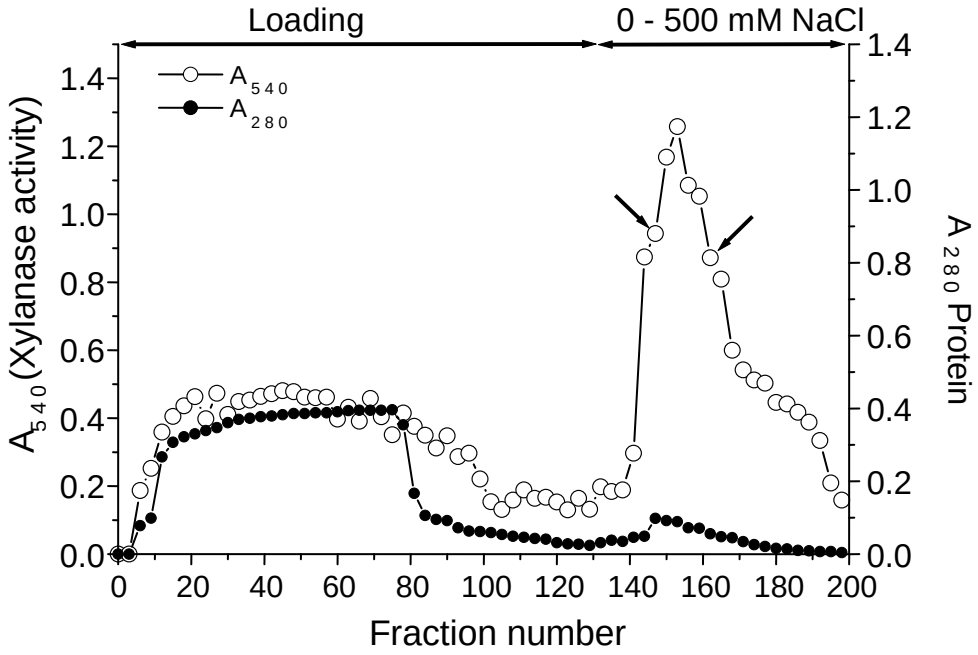

Figure 1. Chromatographic profiles of the xylanase that was produced by *F. heterosporum* using a CM-Cellulose column. The collected fractions are indicated by arrows (→).

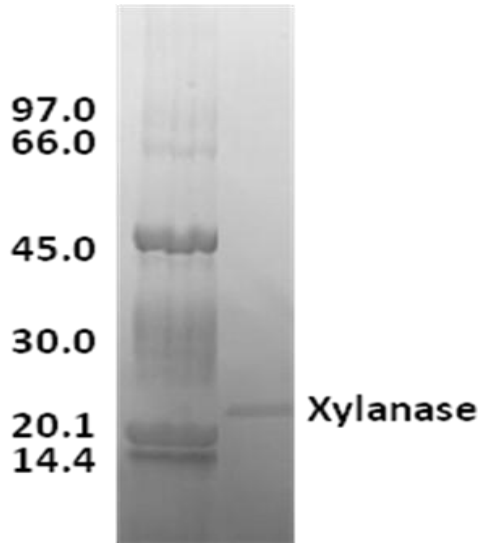

Figure 2. Polyacrylamide gel electrophoresis 10% SDS-PAGE. The xylanase that was produced by *F. heterosporum* is shown in lane B; and molecular mass markers are shown in lane A: phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.1 kDa) and α-lactalbumin (14.1 kDa).

followed by a CM-cellulose chromatographic column. The enzyme eluted as a single peak with 75 mM NaCl (Figure 1), and this peak showed a single band with an apparent molecular mass of 19.5 kDa by 10% SDS-PAGE (Figure 2). The molecular mass of this xylanase was lower than that of xylanases produced from *Thermomyces lanuginosus* SS-8 (23.79 kDa) (Shrivastava et al., 2011) and *Penicillium occitanis* Pol6 (30 kDa) (Driss et al., 2012).

Effect of pH on the activity and stability of xylanase

The optimum pH of the purified xylanase from *F. heterosporum* was 5.0 (Figure 3A). The enzyme was active within the acidic pH range of 4.5 to 5.5 and retained more than 87% of its activity after 24 h of incubation (Figure 4A). This optimum pH value was similar to that described for xylanase produced by *F. solani* SYRN7 (Arabi et al., 2011) and the optimum pH for xylanases of other *Fusarium* species has been reported to be within the range of 4.5 to 8.0; for example, *Fusarium oxysporum* f. sp. *lycopersici*, pH 4.5 (Ruiz et al., 1997) and *F. solani*, pH 8.0 (Bakri et al., 2013).

Effect of temperature on the activity and stability of xylanase

The xylanase exhibited optimum activity at a temperature of 50°C (Figure 3B), and the enzyme was stable within a temperature range of 50 to 55°C for 15 min. However, at 45°C, the half-life ($t_{½}$) of the xylanase was 53 min (Figure 4B). Likewise, xylanases from *Aspergillus niger* (Lopes et

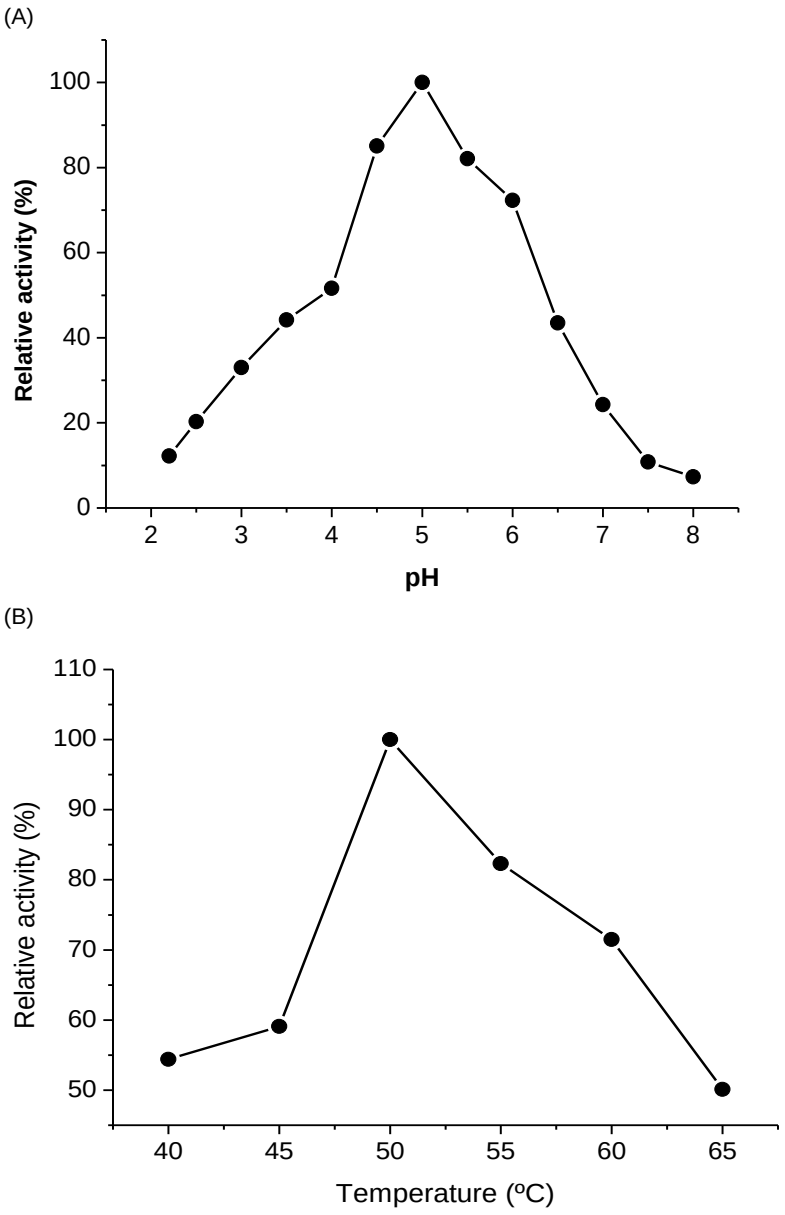

Figure 3. Effect of pH (A) and temperature (B) on the activity of xylanase from *F. heterosporum.*

al., 2013) and *A. carneus* M34 (Fang et al., 2008) showed optimum activity at 50°C. Interestingly, similar result regarding optimum temperature was obtained with xylanase from *F. verticillioides* NRRL 26518 (Saha, 2001). In the contrast, xylanase from *F. solani* showed optimum temperature at 25°C (Bakri et al., 2013).

Kinetic parameters

The apparent K_m and V_{max} values for this xylanase were found to be 5.63 mg/ml and 800 µmol/mg/min, respectively; when birchwood xylan was used as the substrate. The K_m value obtained for xylanase from *F. heterosporum* shows that the enzyme has a higher affinity for xylan than xylanases produced from *Streptomyces cyaneus* SN32 (K_m = 11.1 mg/ml) (Ninawe et al., 2008) and *Humicola grisea* var. *thermoidea* (K_m = 10.87 mg/ml) (Lucena-Neto and Ferreira, 2004). The products released after hydrolysis of birchwood xylan were analyzed by thin layer chromatography and were found to be xylooligosaccharides (X2 to X5) but no free xylose, indicating that this enzyme is typically an endo-xylanase (results not shown). Similar results have been obtained for xylanase from *F. oxysporum* f. sp. *ciceris* (Jorge et al., 2005).

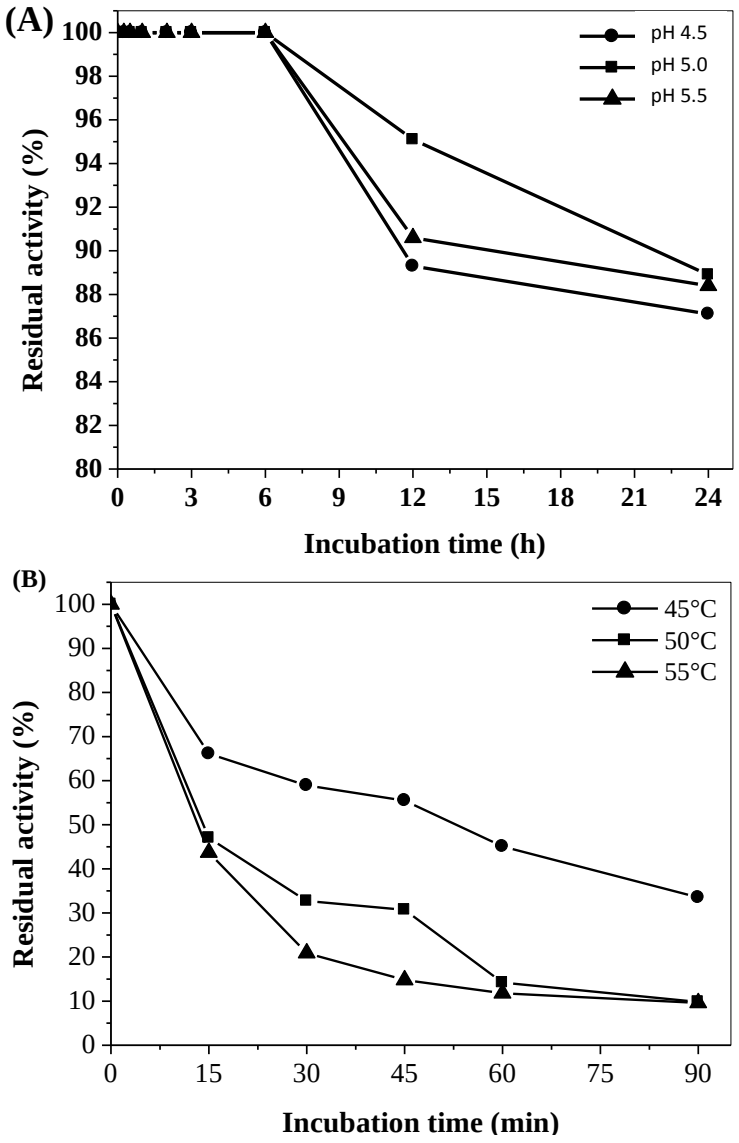

Figure 4. pH (A) and thermal stability (B) of xylanase from *F. heterosporum*.

Effect of metal ions and other compounds on xylanase activity

Metallic ions and some compounds influenced the xylanase activity of *F. heterosporum* (Table 3). Ca^{2+}, Ba^{2+} and Mg^{2+} (1 mM) increased the enzyme activity by 20, 28 and 38%, respectively. However, Zn^{2+}, Pb^{2+} and Hg^{2+} were the most effective inhibitors (27, 39 and 74% inhibition, respectively). This inhibition by heavy-metal ions may occur due to complex formation with reactive groups of the enzyme, for example, metals of group IIb exhibit high affinity for SH, $CONH_2$, NH_2, COOH, PO_4, and this effect is similar to that of mercaptides (Khasin et al., 1993). Furthermore, the heavy metal ions may bind non-specifically to regions other than the cysteine thiol group of the enzyme to induce inhibition (Krajewska, 2008). SDS also inhibited (70%) enzyme activity. Similarly, a total loss of xylanase activity from *Penicillium glabrum* was observed in the presence of SDS, indicating that hydrophobic interactions may be important in maintaining the structure of xylanase (Knob et al., 2013). Among the amino acids tested, L-cysteine and cystine did not increase enzyme activity.

Interestingly, compounds containing thiol groups such as β-mercaptoethanol and DTT enhanced the activity of xylanase from *F. heterosporum* by 55% and 54%, respectively, and a similar effect was observed for xylanase from *Termitomyces* spp. in the presence of reducing agents (Faulet et al., 2006). Likewise, crude xylanase from *T. longibrachiatum* and *A. niger* were activated by L-cysteine, DTT and β-mercaptoethanol (Medeiros et al., 2003). In contrast, DTT (5 mM) inhibited

Table 3. Effects of various compounds on the activity of xylanase from *F. heterosporum*.

Compound	Residual activity (%)	
	1 mM	5 mM
Control	100	100
$BaCl_2$	128 ± 3.1	132 ± 2.8
$CaCl_2$	120 ± 1.8	146 ± 1.4
$CoCl_2$	111 ± 0.2	112 ± 0.4
$HgCl_2$	26 ± 0.4	6 ± 0.3
KCl	112 ± 2.6	115 ± 2.3
$MgCl_2$	138 ± 3.3	148 ± 3.7
NaCl	110 ± 2.6	117 ± 2.4
$PbCl_2$	61 ± 0.4	52 ± 0.5
$ZnCl_2$	73 ± 2.0	62 ± 2.0
$(NH_4)_2SO_4$	118 ± 0.4	118 ± 0.4
β-Mercaptoethanol	133 ± 2.7	155 ± 2.6
DTT	141 ± 2.7	154 ± 2.3
Cystine	99 ± 2.7	82 ± 2.4
L-cysteine	97 ± 2.4	81 ± 2.5
Iodoacetamide	84 ± 1.9	82 ± 1.7
SDS	30 ± 1.5	12 ± 1.3
EDTA	91 ± 1.0	89 ± 1.5

Results are expressed as a percentage of the control, which is 100%. Xylanase used: 2 U ml^{-1}.
[a]Residual activity is expressed as a percentage of the control.

xylanase from *F. proliferatum* NRRL, 26517 by 23% (Saha, 2002).

Influence of thiol compounds on xylanase activity

In this study, we observed that the activity of xylanase from *F. heterosporum* was inhibited by metal ions (Hg^{2+}, Pb^{2+} and Zn^{2+}), but this activity was subsequently restored to control levels after exposure to DTT and β-mercaptoethanol (Figure 5A). Furthermore, our studies show the protective effect of thiol compounds on this enzyme when these compounds are previously pre-incubated with heavy metals (Figure 5B). These results suggest that compounds such as β-mercaptoethanol and DTT can interact with the enzyme at a higher affinity and prevent the formation of heavy metal - enzyme complexes. This behavior can be explained by a model proposing the non-essential enzyme activation of asparaginase from *Erwinia carotovora* (Warangkar and Khobragade, 2010). When β-mercaptoethanol and DTT are added, they bind to another site other than the site of the enzyme substrate, a conformational change in the enzyme results in decreased binding affinity for the heavy metal, and the enzyme can recover its catalytic activity. Therefore, the finding of a xylanase with an alternative form of regulation due to a higher affinity for thiol compounds will be particularly useful because of the potential to improve and promote the recovery of the catalytic efficiency of the enzyme after it has been inhibited by metallic ions.

Conclusion

The new isolate *F. heterosporum* proved to be a promising strain in producing xylanase using a low-cost, alternative substrate such as barley-brewing residue. Interestingly, the enzyme showed the versatility of interacting with thiol compounds and promoting the recovery of the catalytic efficiency of xylanase that had been inhibited by heavy metal ions, which thereby accentuates the biotechnological potential of this enzyme.

ACKNOWLEDGEMENTS

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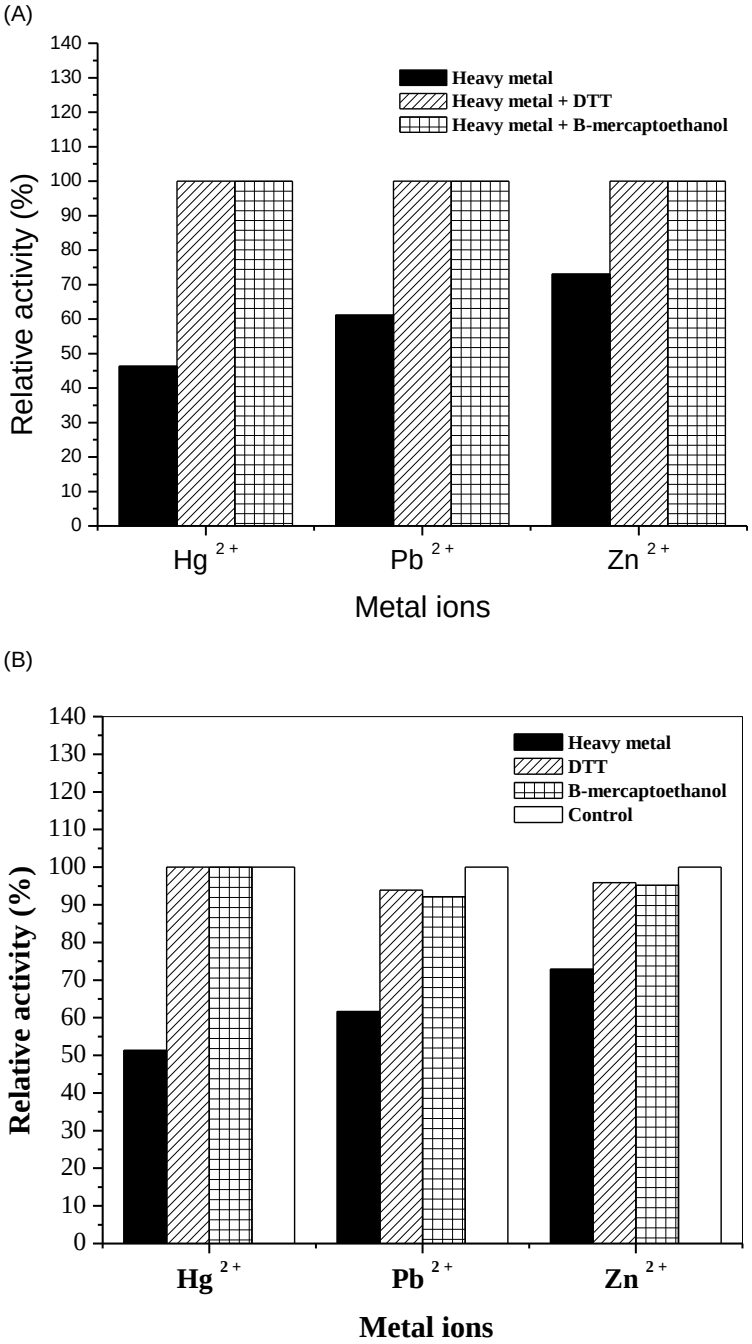

Figure 5. Recovery of heavy metal-inhibited xylanase activity by thiol compounds (A). Protective effect of the thiol compounds on heavy metal-inhibited xylanase (B). The control (□) is the xylanase activity in the absence of a heavy metal ion. [a]Relative activity is expressed as a percentage of the control.

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Full Length Research Paper

Infection potential of vegetative incompatible *Ganoderma boninense* isolates with known ligninolytic enzyme production

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Ganoderma boninense **produces ligninolytic enzymes namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase to degrade the lignin composition in plant cell walls. The present study aimed to evaluate the infection potential of vegetative incompatible isolates of *G. boninense* in causing Basal Stem Rot (BSR) disease in oil palm through the production of ligninolytic enzymes. Vegetative incompatibility test was carried out to test the antagonistic relationship of *G. boninense* isolates. *G. boninense* isolates with strong antagonistic reaction were selected for ligninolytic enzymes analyses. *In vitro* infection of oil palm seedlings and downstream analyses were then carried out. Control experiment was carried out with GBLS, a known *G. boninense* isolate. From this study, *G. boninense* isolates *a1* and *h2* showed the highest laccase (43.07 and 40.44 UI^{-1}) and MnP (14.80 and 16.21 UI^{-1}) enzymes production. Oil palm seedlings infected by isolates *a1* and *h2* resulted in relatively high percentage of disease severity index (DSI) (42.50 and 56.25% respectively). GBLS had lower laccase and MnP enzyme activities (24.31 and 9.27 UI^{-1} respectively) and obtained the lowest DSI value (29.55%). Overall, a direct relationship was observed between the production of ligninolytic enzymes and the infection potential in vegetative incompatible *G. boninense* isolates.**

Key words: Oil palm, Basal Stem Rot (BSR), vegetative incompatible, ligninolytic enzymes, *in vitro* infection.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an erect monoecious plant that belongs to *Elaeis* genus of Palmae family. It offers the highest yields of oil per hectare of land as compared to other oil producers up to date (Abdullah and Wahid, 2011). Palm oil is not only crucial as source of food and energy supplied to developing countries, but also scientifically proven to have nutritional values that benefit human health (Sundram et al., 2003). Before World War II, oil palms were largely free from serious diseases and disorders (Hartley, 1988). As the crop plantation area has expanded, there have been serious outbreak of oil palm diseases in different parts of the world. In the Far East severely affected areas, more than 50% of oil palms might surrender to *Ganoderma* spp. According to Su'ud et al. (2007), *Ganoderma boninense* can cause lethal effects in oil palm by degrading its xylem

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Abbreviation: BSR, Basal stem rot; **LiP,** Lignin peroxidase; **MnP,** Manganese peroxidase; **VP,** Versatile peroxidase; **CWDEs,** Cell wall degrading enzymes; **AAR,** Applied Agricultural Resources; **ABTS,** 3-ethylbenzothiazoline-6-sulphonic acid; **PDA,** Potato dextrose agar; **MS,** Murashige and Skoog; **MEB,** Malt extract broth; **DSI,** Disease severity index; **PCR,** Polymerase chain reaction; **ANOVA,** Analysis of variance; **SEM,** Standard error of mean; **ITS,** Internal transcribed spacer.

and affect the allocation of water and nutrients to other parts of palm trees. This disease is believed to be a major problem for oil palm cultivation because loss in crop yield can reach 80 % after constant planting cycles. Although different strategies and controls, such as cultural practices, mechanical and chemical treatments, have been applied on this fungus in order to inhibit its infection, the results were not satisfactory (Moller and Schultz, 1997). This may be due to poor understanding of the biology and behavior of *G. boninense* upon infecting host plants, the mechanisms of disease establishment, development and spread. Such understanding may include the potential of vegetative incompatible isolates of *G. boninense*in producing ligninolytic enzymes that break down the cell wall of the host plant.

Basal Stem Rot (BSR) infection in oil palm by *G. boninense* appeared to involve a few stages of developmental switches as described by Rees et al. (2009). The first stage involved the biotrophic phase in the infected root cortex or stem base where the intracellular layers of oil palm are greatly colonised by *G. boninense* hyphae. This phase of invasion mode will switch to an aggressive necrotropic phase which involves extensive oil palm cell wall degradation through the production of a series of cell wall degrading enzymes (CWDEs). Ligninolytic enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and laccase are examples of CWDEs used by *G. boninense* to degrade oil palm's lignin polymers that have high level of biodegradability (Polaina and MacCabe, 2007). Oil palm's lignin is comprised of a high percentage of aryl ether-linked syringyl (S) units (Paterson et al., 2009). In addition, significant amount of *p*-hydroxybenzoic acid and small amounts of syringic, vanillic acids and esterified *p*-hydroxybenzoic acid are also present in the lignin of oil palm (Suzuki et al., 1998). This composition is the main reason that oil palm's lignin is more vulnerable when compared with wood lignin that contains high guaiacyl (G) units (Paterson et al., 2009).

The compatibility relationship is a unique characteristic of basidiomycetes, including *G. boninense*. This relationship is related to the life cycle of these fungi and is often described as the ability between two isolates of single fungal species to exist concurrently without conflict. Pilotti et al. (2002) discovered that *G. boninense* is heterothallic with multiple alleles at both mating type loci and possess bifactorial incompatibility (tetrapolar) that favours out-crossing within a population. Hence, somatic incompatibility that allows sexual recombination to promote out-breeding amongst individuals is common in *G. boninense*. Vegetative incompatibility systems in *Ganoderma* are allelic in nature; they mainly act to limit the transfer of nuclear and cytoplasmic components when fusion (anastomosis) of two incompatible fungal hyphae takes place. Antagonistic interaction between somatically incompatible dikaryon isolates can be distinguished by the occurrence of thin line or pigmented clear zone where the mycelium of two cultures meet (Qi et al., 2003), and this solid demarcation line is called the "barrage" by Pilotti et al. (2002). According to Burgess et al. (2009), vegetative compatibility in fungi reveals the phenotypic differences among individual isolates from similar species.

It is believed that different isolates of a single fungal species may have different strength of compatibility to cause infection to a different degree of severity on similar host. It was hypothesized that the vegetative incompatible *G. boninense* isolates with higher amount of ligninolytic enzymes production are more aggressive and have a higher potential to cause BSR disease in oil palm. Thus, this study aimed to detemine the relationship between the ligninolytic enzymes activities of each *G. boninense* isolates and the degree of infection caused by particular isolates on the oil palm through artificial *in vitro* infection approaches to conclude the aggressiveness of incompatible *G. boninense* isolates in causing disease in oil palm.

MATERIALS AND METHODS

Sources of fungal isolates and growth

In this study, *Ganoderma boninense* isolate GBLS was served as the positive fungal control. This isolate was previously characterized by amplifying its DNA fragment with GbITS1 and GBITS4 primers and confirmed as *G. boninense* after sequencing (GenBank KF164430.1). The mycelium pure culture of this isolate was obtained from Plant Pathology Laboratory, School of Biosciences, University of Nottingham Malaysia Campus. Another six *G. boninense* isolates (*g1, a1, c3, d4, e2* and *h2*) in the form of pure culture mycelium were also initiated from a single basidiocarp (fruiting body), termed as T4G1 through sexual recombination of monokaryotic mycelium from previous study (Chai, 2011). These isolates were obtained from a basidiocarp on an infected 24 year-old oil palm within Plot No. PM86B of the Boustead Balau Estate, Semenyih, Selangor, Malaysia. Pure culture mycelium of *G. boninense* isolates were maintained by sub-culturing on fresh potato dextrose agar (PDA; Merck) at 25°C for every three weeks to provide optimum conditions for the fungal growth.

Oil palm seedlings

One month old oil palm seedlings used for *in vitro* infection studies in this paper were supplied by Applied Agricultural Resources (AAR) Sdn. Bhd. They were grown in 72 × 72 × 100 mm^3 sterilised Incu Tissue Culture Jars (SPL) containing 40 ml Murashige and Skoog (MS) medium (Duchefa), in a growth chamber (Conviron, CMP 6010) at 27°C, 16 h of light intensity and 50% relative humidity.

Vegetative incompatibility test

Vegetative incompatibility test among the *G. boninense* isolates was carried out as described by Pilotti et al. (2002). *G. boninense* isolates *g1, a1, c3, d4, e2* and *h2* were paired among each other, together with the GBLS isolate. Self-pairings of each isolate were served as controls in this test. Mycelial interactions between each culture were assessed and recorded at 7 days intervals (day 0, 7, 14, 21 and 28). The antagonistic relationship

Table 1. Scores of the symptoms of oil palm seedlings on a disease scale of 0-3.

Disease class	Symptoms of Infection
0	Healthy plants with green leaves without appearance of fungal mycelium on any part of plants
1	Appearance of white fungal mass on infected region (stem) without chlorotic leaves
2	Appearance of white fungal mass on infected region (stem) with chlorotic leaves (1-3 yellowing leaves)
3	Appearance of white fungal mass on infected region (stem) with chlorotic leaves (1-3 browning leaves)

between the isolates were categorised based on the level of antagonism (strong, medium, weak) as illustrated by Pilotti et al. (2002).

Quantification of *G. boninense* ligninolytic enzymes by colorimetric assays

Five mycelia discs (5 mm²) of each fungal strain (GBLS, *a1*, *e2* and *h2*) grown on PDA medium were transferred into 250 ml Erlenmeyer flasks containing 30 ml of malt extract broth (MEB; Merck) and 5.00 g of sterile rubber wood chips. The cultures were then agitated at 120 rpm for 7 days at room temperature for the induction of ligninolytic enzymes. After incubation period, 0.1 M of sodium acetate (SYSTERM®) was added to the liquid medium at a ratio volume of 1:1. The mixture solution was then agitated at 120 rpm for 4 to 5 h before incubating overnight at 4°C. Ligninolytic enzymes produced from each isolates were harvested after the incubation period by filtering through a Whatman Grade 4 filter paper. The enzymes solution was centrifuged (Eppendorf, 5810R) twice at 6000 rpm for 30 min, the supernatants were collected in new tubes as enzyme sources and stored at -20°C.

Laccase activity was verified by the oxidation rate of 0.03% (w/v) 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, ~98 %) as the substrate at 30 °C as described by Murugesan et al. (2007). The assay mixture (100 µl) contained 10 µl of 100 mM sodium acetate buffer (pH 5.0; SYSTERM®), 80 µl of 0.03% (w/v) ABTS (Sigma-Aldrich) and 10 µl of crude enzyme. Absorbance values of the assay mixture were read at 420 nm (E_{420} = 36.0 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

Lignin peroxidase (LiP) activity was quantified by monitoring the oxidation of veratryl alcohol (~96 %) as described by Tien and Kirk (1988). The reaction mixture (200 µl) comprised of 80 µl of 125 mM sodium tartarate buffer (pH 3.0; QRëC), 40 µl of 10 mM veratryl alcohol (Sigma-Aldrich) and 40 µl of crude enzyme. The mixture was incubated at 30°C for 2 min before adding 40 µl of 2 mM hydrogen peroxide (Ajax Finechem). Absorbance values of the reaction mixture were monitored at 310 nm (E_{310} = 9.3 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

Manganese peroxidase (MnP) activity was assayed by the oxidation of guaiacol as enzyme substrate (Patrick et al., 2011). The assay mixture (180 µl) consisted of 20 µl of 0.5 M sodium succinate buffer (pH 4.5; SYSTERM®), 20 µl of 4 mM of guaiacol (Acros Organics), 20 µl of 1 mM manganese (II) sulphate (SYSTERM®), 80 µl of distilled water and 20 µl of crude enzyme. The mixture was incubated at 30°C for 2 min before adding 20 µl of 1 mM hydrogen peroxide. Absorbance values of the reaction mixture were monitored at 465 nm (E_{465} = 12.1 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

For blank reaction mixture (control), equal volume of distilled water was used to replace crude enzyme in these three enzyme assays. Reaction mixtures for each assay were pipetted into individual wells of a 96-flat test plate in triplicates and each enzyme assays were repeated for three times. The absorbance values of these mixtures were measured using a 96-well microplate reader (Thermo Scientific, Varioskan Flash) at respective wavelengths. For all enzymes under evaluation, one activity unit (U) was defined as "the amount of enzyme necessary to produce 1 µmol of product per minute at 30°C" (Murugesan et al., 2007).

Artificial *in vitro* infection technique

The *in vitro* infection technique designed for inoculating oil palm stems with *G. boninense* isolates (GBLS, *a1, e2* and *h2*) in this study is the first report of its use in this plant-fungal interaction. Artificial wounds were first made on the stem regions of healthy oil palm seedlings at approximately 0.50 cm above the crown region by puncturing with sterilised needles (18G × 1.5", Terumo). Mycelium of each *G. boninense* isolate were scraped off the surface of 9 cm² Petri dish cultures and applied directly onto the wounded seedlings. A control treatment was comprised of wounded but non-inoculated seedlings. For treatments, *in vitro* infection of oil palm seedlings was conducted using four replicates with 3 units for each replicate. All treated seedlings were then replanted in growth medium and grown in the growth chamber for three weeks at 27°C, 16 h of light intensity and 50% relative humidity. Three weeks after inoculation, the infected oil palm seedlings were removed from the culture jars and the external *G. boninense* mycelium were washed away with 20% bleach solution, before proceeding with further analytical tests.

Disease severity scale

The symptoms developed from treated oil palm seedlings were observed and evaluated based on the formation of white mycelium and number of chlorotic leaves as derived from the disease severity scale presented by Izzati and Abdullah (2008). The scale comprises four categories, ranging from 0 to 3 as described in Table 1 and Figure 1. Photographs of the infected seedlings were taken using a camera (Panasonic, Lumix LX5) for later assessment on the disease severity index (DSI) of these seedlings. The degree of disease severity caused by *G. boninense* isolates on replicated oil palm seedlings was assessed by calculating the DSI value as shown in the formula below:

$$\text{Disease severity index (DSI)} = \frac{\sum(\text{A X B})\text{x } 100}{\sum \text{B x } 4}$$

Where, A is the disease class ranging from 0, 1, 2, and 3 according to the symptoms of infection (Table 1) and B is the number of plants showing that disease class per treatment (in this case, per isolate).

Microscopic observation of the internal tissues of treated oil palm seedlings

In order to observe the severity of internal tissue damage and the extent of decay on the site of infection on oil palm seedlings, the stem regions were cut into longitudinal slices. A drop of lactophenol

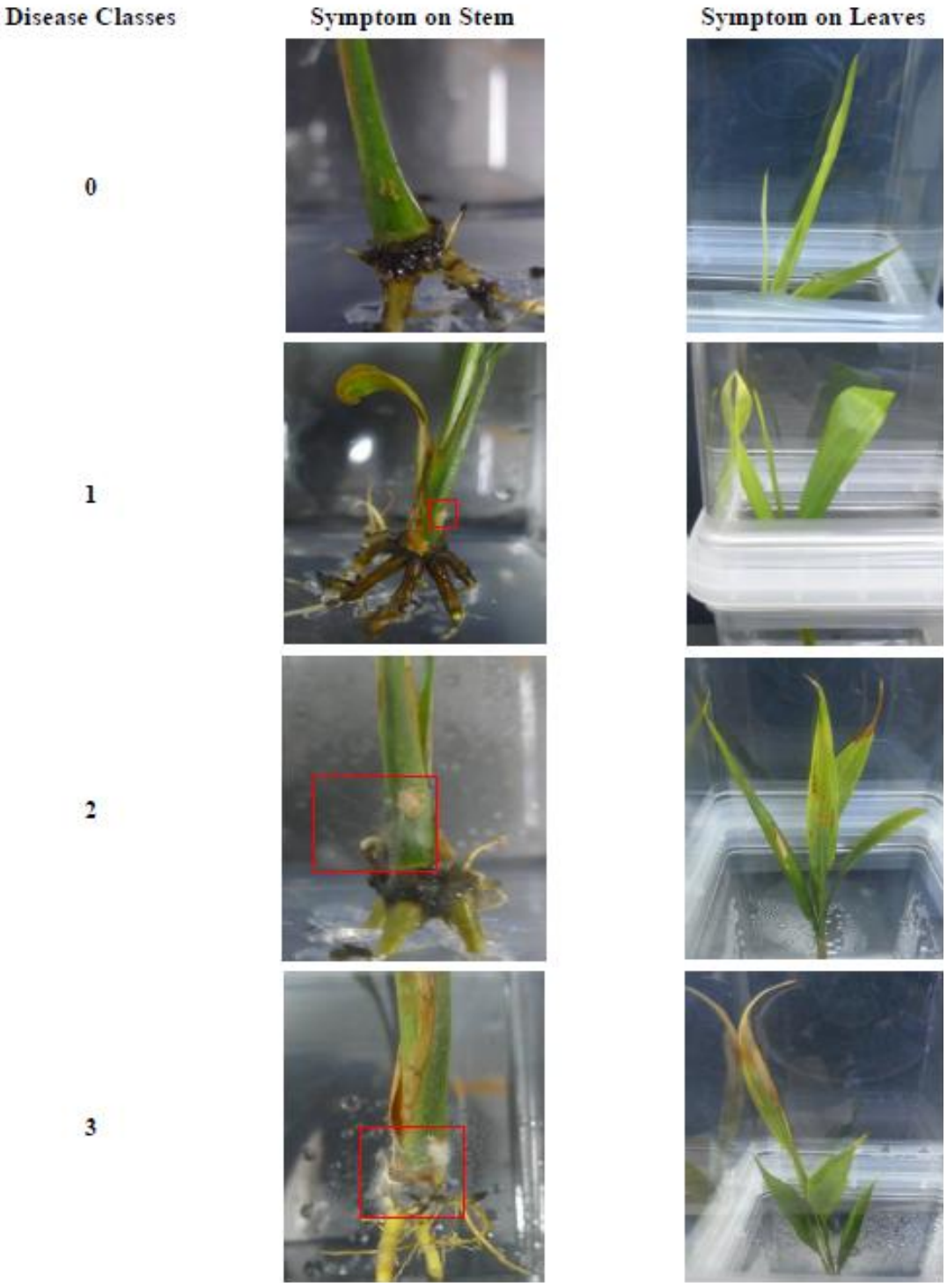

Figure 1. Illustration of the symptoms developed in treated oil palm seedlings of different disease classes.

blue dye was placed onto the samples to stain the *G. boninense* fungal cell walls. The stained stem regions were observed under a light microscope (Nikon, AZ100) using 1x objective lenses and the magnification was adjusted to 30x, 60x and 80x. Photographs for cross sections of seedlings'stem were captured using a camera system (Nikon, DS-Ri1 and NIS Element software).

***G. boninense* mycelia harvesting for DNA extraction and purification**

G. boninense isolates (GBLS, *a1*, *e2* and *h2*) were grown in MEB solution based on a technique modified from Idris et al. (2003). Five mycelial plugs of each *G. boninense* isolate were obtained from the

Table 2. Antagonistic relationships observed between *G. boninense* isolates at Day 21. (X indicated compatible interaction; W, M and S indicated weak, medium and strong incompatible interactions respectively).

Isolate	GBLS	*g1*	*a1*	*c3*	*d4*	*e2*	*h2*
GBLS	X	M	S	W	M	M	S
g1		X	M	W	M	M	X
a1			X	X	M	S	S
c3				X	M	W	M
d4					X	W	X
e2						X	S
h2							X

actively growing region using a sterilised cork borer. These plugs were then transferred into a 50 ml falcon tube containing 25 ml of MEB. The mycelium culture were incubated and remained agitated at 120 rpm for 7 days at room temperature. After incubation, the mycelium cultures were harvested through centrifugation at 10,000 rpm for 20 min. The supernatant was discarded in bleach solution and the pellets were air-dried before grinding with liquid nitrogen.

G. boninense and Oil Palm DNA extraction and purification

Extraction of *G. boninense* and oil palm DNA were carried out based on conventional methods described by Góes-Neto et al. (2005) and Moller et al. (1992) respectively. Before amplifying the extracted DNA from *G. boninense* mycelium and oil palm seedlings through polymerase chain reaction (PCR), the quantity and quality of extracted DNA were measured with a nanodrop spectrophotometer (Thermo Scientific, ND-1000). The quantity of DNA extracted was measured in ng/µl and the quality (purity) was determined by using the ratio of absorbance value at 260/280 nm.

Molecular diagnostic of DNA extracted

PCR amplification technique was carried out to increase the amount of extracted DNA from *G. boninense* mycelium and treated oil palm seedlings. According to Karthikeyan et al. (2006), two 18 mers were selected as the forward and reverse primers in this study to yield a DNA fragment at 167 bp.

DNA of *G. boninense* isolates (GBLS, *a1*, *e2* and *h2*) served as the positive control for oil palm seedlings infected by respective *G. boninense* and distilled water served as the negative control. A PCR thermocycler (G-storm, GS-1) was programmed as follows; 5 min of pre-heating at 95°C followed by 48 cycles consisting of denaturation at 94°C for 40 s, annealing at 45°C for 40 s and extension for 45 s at 72°C with a final extension for 72°C for 12 min.

After PCR reaction, PCR products were analysed by electrophoresis on a 1.5 % (w/v) agarose gel (1st Base) that stained with SyBr Safe (Invitrogen) in 1x TBE buffer (1st Base). GeneRuler 100 bp Plus DNA Ladder (Fermentas) was used as marker. The gel was run at 80 V (Biorad, PowerPad® Basic) for 1.5 h, after which the DNA bands were visualized and photographed under UV transilluminator (Biorad, Gel Documentation XR System).

The DNA bands were then excised and purified by using GF-1 nucleic acid extraction kit (Vivantis) as described in the manufacturer's instructions. Purified DNA samples and the forward primer were sent for single pass sequencing (1st Base) to identify the sequences of the amplified fragment.

Statistical analysis

Data of this study were statistically analysed by one-way analysis of variance (ANOVA), except for the percentage of RBBR dye decolourisation which were analysed by two-way ANOVA. The significant differences between isolates and treatments were detected by Tukey Multiple Comparison Test using GraphPad Prism programme version 5.02.

RESULTS

Vegetative incompatibility test

All self-pairing controls in this test showed compatible reactions (Table 2). Among the 28 antagonistic reactions of different isolates, three of them showed complete compatibilities, five showed strong incompatibilities, 9 showed medium incompatibilities and another 4 showed weak incompatibilities. The morphological illustrations of the compatible, weak, medium and strong antagonistic barrage formed on the plates between these isolates are shown in Figure 2.

Quantification of *G. boninense* ligninolytic enzymes by colorimetric assays

According to the laccase assay (Figure 3A), isolate *a1* possessed the highest level of laccase activity (43.07 UL^{-1}). In contrast, isolate *e2* had the lowest laccase activity (19.19 UL^{-1}). Statistically, the laccase activity between all isolates were significantly different ($P < 0.05$), except for laccase activity between isolates *a1* vs. *h2*.

In MnP assay (Figure 3B), all *G. boninense* isolates tested gave positive MnP results. Isolates *h2* had highest level of MnP activity (16.22 UL^{-1}), signifying that this isolate produced the highest amount of MnP enzyme. The MnP activity detected in isolate *e2* was the lowest. Statistically, the MnP activity between isolates GBLS vs. *e2* and *a1* vs. *h2* were not significantly different ($P < 0.05$) from one another.

Based on the LiP assay graph (Figure 3C), all *G.*

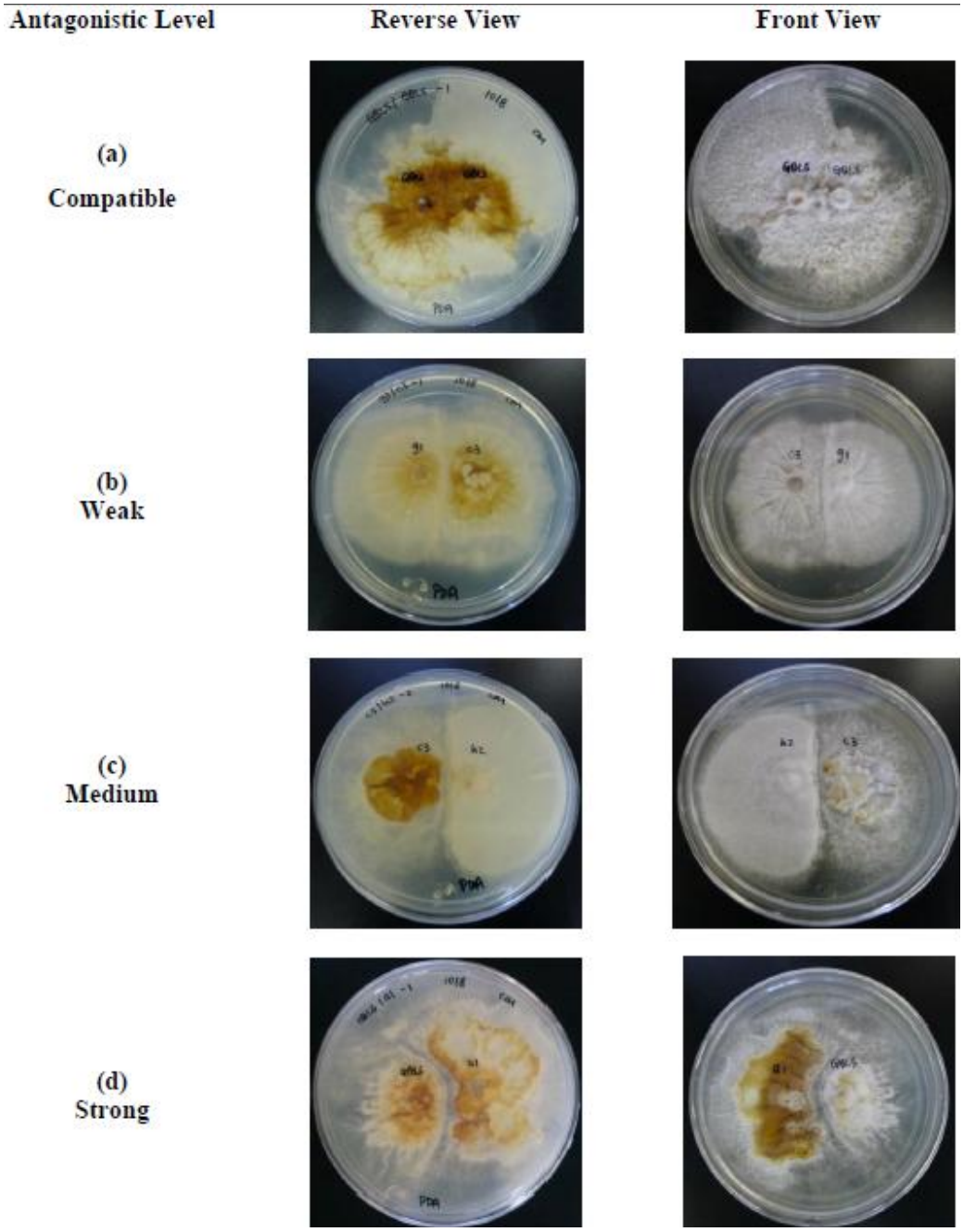

Figure 2. Front and reverse view of the (a) compatible, (b) weak, (c) medium, (d) strong antagonistic relationship among *G. boninense* isolates.

boninense isolates showed no lignin peroxidase activity, except for isolate *e2* that showed a relatively low level of activity (0.27 UL^{-1}), signifying that this isolate produced a low amount of lignin peroxidase enzyme.

Disease severity scale

Oil palm seedlings infected with *G. boninense* isolates showed different disease severity index (DSI) values (Table 3) after three weeks of inoculation. In contrast, non-infected oil palm seedlings which served as the control, remained healthy with green leaves and DSI value of 0.00%. The highest DSI value of 56.25% was in the oil palm seedlings infected with *h2* isolate, followed by *e2* and *a1* isolates. GBLS isolate, which served as the control *G. boninense* isolate, had the lowest value of DSI of 29.55%.

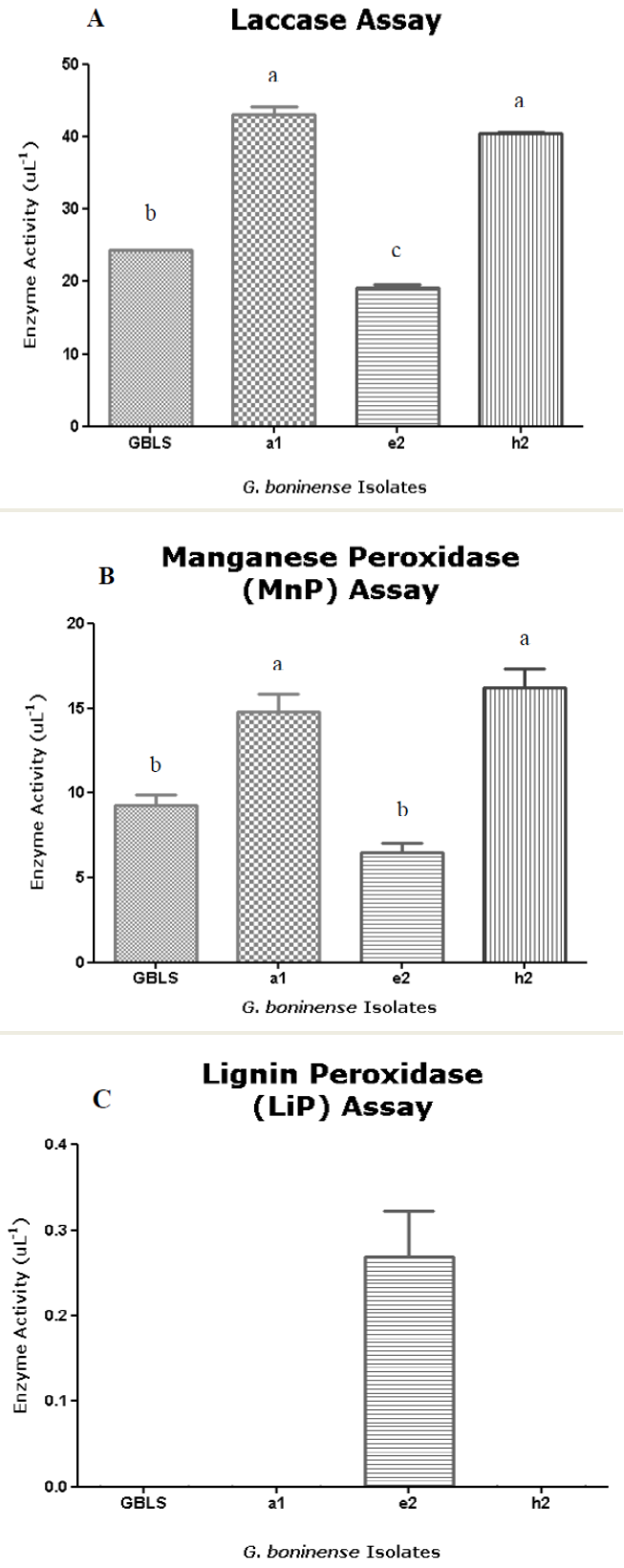

Figure 3. (A-C) Ligninolytic enzyme assays in *G. boninense* isolates. Bars indicated the standard error of mean (SEM) for triplicate culture plates. Means tagged with the different alphabetic letter are significantly different at $P< 0.05$.

Microscopic observation of the internal tissues of treated oil palm seedlings

Before staining with lactophenol blue dye, the cross-section of all oil palm seedlings stem, including control seedlings, showed a necrotic lesion at the wounded region (Figure 4). The necrotic lesions in all infected oil palm seedlings were more severe as compared with the mild lesion in control seedlings, which was believed to be due to the mechanical injury exerted on the stem region during the wounding process. After staining, intense blue mass of fungal mycelium was found around the wounded region of infected seedlings. In contrast, the wounded region of control seedling was unstained and showed no indication of fungal mycelium.

Molecular Assessment of G. boninense DNA in Treated Oil Palm Seedlings

All the DNA samples showed amplified bands at 167 bp (Figure 5), except for the DNA of control uninfected oil palm seedlings (lanes 2 to 4). There was no DNA band amplified in the negative control lane, indicating no contamination in PCR products during the PCR amplification process.

Sequencing confirmed that the isolates used in this study (GBLS, *a1*, *e2*, *h2*) as *G. boninense* after BLAST analysis using the NCBI database. The isolates' sequences recognized by the forward primer (GbF) are as followed:

5'_GATTTTTTCCATTTAGAAAATACTGCTCTCCACTCT
ACACCTGTGCACTTACTGTGGGTTATAGATCGTGTG
GAGCGAGCTCGTTCGTTTGACGAGTTCGCGAAGCGC
GTCTGTGCCTGCGTTTTATCACAAACACTATAAAGTA
TTAGAATGTGTATTGCGATGTAACGC_3'

From the results of BLAST analysis, all the isolates shared 99% similarity with the *G. boninense* sampled by Utomo et al. (2005) in Indonesia.

DISCUSSION

Based on the results obtained in vegetative incompatibility test, variations in antagonistic relationship can be explained by the degree of genetic heterogeneity which affects the reception or rejection of a nucleus within the interacting fungal hyphae of vegetative incompatible isolates (Kope, 1992). Since majority of the pairings of *G. boninense* isolates resulted in weak and medium incompatibility, it suggests that these isolates were genetically related. This assumption was supported in a study conducted by Pilotti et al. (2002), where increasing genetic relatedness of incompatible isolates was linked with a decrease in their somatic interactions. Nevertheless, strong antagonistic responses were still

Table 3. Number of treated oil palm seedlings in different disease classes and their disease severity index (%) after three weeks of inoculation.

Isolate	Disease class (No. of treated oil palm seedlings)					Disease Severity Index* (DSI; %)
	0	1	2	3	Contamination	
Control	12	0	0	0	0	0.00 ± 0.00^{c}
GBLS	3	3	5	0	1	29.55 ± 9.24^{b}
a1	0	4	5	1	2	42.50 ± 4.30^{ab}
e2	0	1	7	2	2	52.50 ± 4.26^{a}
h2	0	1	7	4	0	56.25 ± 3.99^{a}

All values of DSI represent mean ± standard error of mean (SEM) for 4 replicates. *Disease severity index with different alphabetic letters was significantly different at $P < 0.05$ by Tukey multiple comparison test.

found in a small portion of pairings in this experiment, indicating the presence of genetic diversity in *G. boninense* isolates within a single fruiting body. Apart from the self-pairing controls, three pairs of *G. boninense* isolates also showed complete compatible interaction. Lack of antagonism in these three pairings could be either due to non-self recognition in compatible mating, or "switching off" of putative incompatibility genes in these isolates after fusion of their hyphae by unknown underlying mechanisms (Pilotti et al., 2002). From these results, it can be concluded that *G. boninense* is highly heterozygous and the assumption of individualism among the isolates was confirmed.

In this study, laccase and MnP enzymes were produced in greater quantity in all *G. boninense* isolates, ranging from 19.09 UL^{-1} to 43.07 $UL^{-1}$and 6.52 UL^{-1} to 16.21 UL^{-1} respectively. In contrast, the LiP production was significantly lower or undetected in all *G. boninense* isolates. These results obtained from the colorimetric assays were complemented with the findings of Pelaez et al. (1996), where laccase and MnP enzymes were more broadly distributed in white rot fungi including *G. boninense* as compared to LiP. Insignificant amounts of LiP detected in this study may be due to the fact that LiP activity is often restricted to the culturing method utilised for the induction of ligninolytic enzymes (Reddy, 1993). Previous reports also stated that LiP was very difficult to be detected in lignocellulose extracts and only a few reports found the secretion of LiP in their natural substrates, such as wood and straw (Vares et al., 1995; Mester et al., 1998). Furthermore, it was suggested that the production of LiP and MnP enzymes are often inhibited by agitation of submerged fungal mycelium in liquid culture as their production are optimal at high oxygen tension, whereas laccase production can be enhanced by agitation (Wesenberg et al., 2003). Since the induction of ligninolytic enzymes in this experiment was done by agitating the mycelial culture in liquid broth, as predicted the laccase production was predominant in all *G. boninense* isolates.

In addition, negligible or low amount of LiP enzymes produced in all *G. boninense* could be also due to the timing of the onset of production of LiP enzyme (Lankinen, 2004). According to his study, there was no sign of LiP activity during the first 5 days of *Phlebia radiate*'s cultivation and often peaked on day 8 or 9 of growing period. Initial production of LiP in this fungus was most probably masked by interfering compounds in the crude liquid culture, which tend to compete with veratryl alcohol as the substrates and inhibit LiP activity. Hence, it was assumed that the LiP in *G. boninense* isolates after 7 days of cultivation in this study may not reach its peak of production. In fact, RBBR decolourisation test was initially used to perform an early detection and evaluation of ligninolytic enzymes production in *G. boninense* isolates GBLS, *a1*, *e2* and *h2.* However, the results for this experiment were not shown as they were contradicting with the main findings of colorimetric assays.

On the other hand*, in vitro* infection of oil palm seedlings showed that disease symptoms only developed in infected oil palm seedlings, but not in the control seedlings. Since the entire experiment was done in an *in vitro* approach and the treated oil palms seedling were incubated in similar controlled conditions, the effects of environmental factors and biotic stress agents such as insects were not considered (Kozai et al., 1997). Furthermore, there was very little possibility that these symptoms were due to wound-induced injuries as all the control seedlings (wounded but non-infected) remained healthy. Hence, it was deduced that the external symptoms found on the infected oil palm seedlings were solely based on the effects of *G. boninense* inocula. In addition, the time required for disease symptom development in this study was found to be much shorter (three weeks) as compared with some previous studies in which oil palms were artificially infected using rubber wood blocks *in vivo*. According to Izzati and Abdullah (2008), the first disease-induced symptom was found after 14 weeks with 12.50% DSI value. Similarly, another recent study done by Naher et al. (2012) showed that disease symptoms were only detected on the *G. boninense* infected oil palm after five weeks after

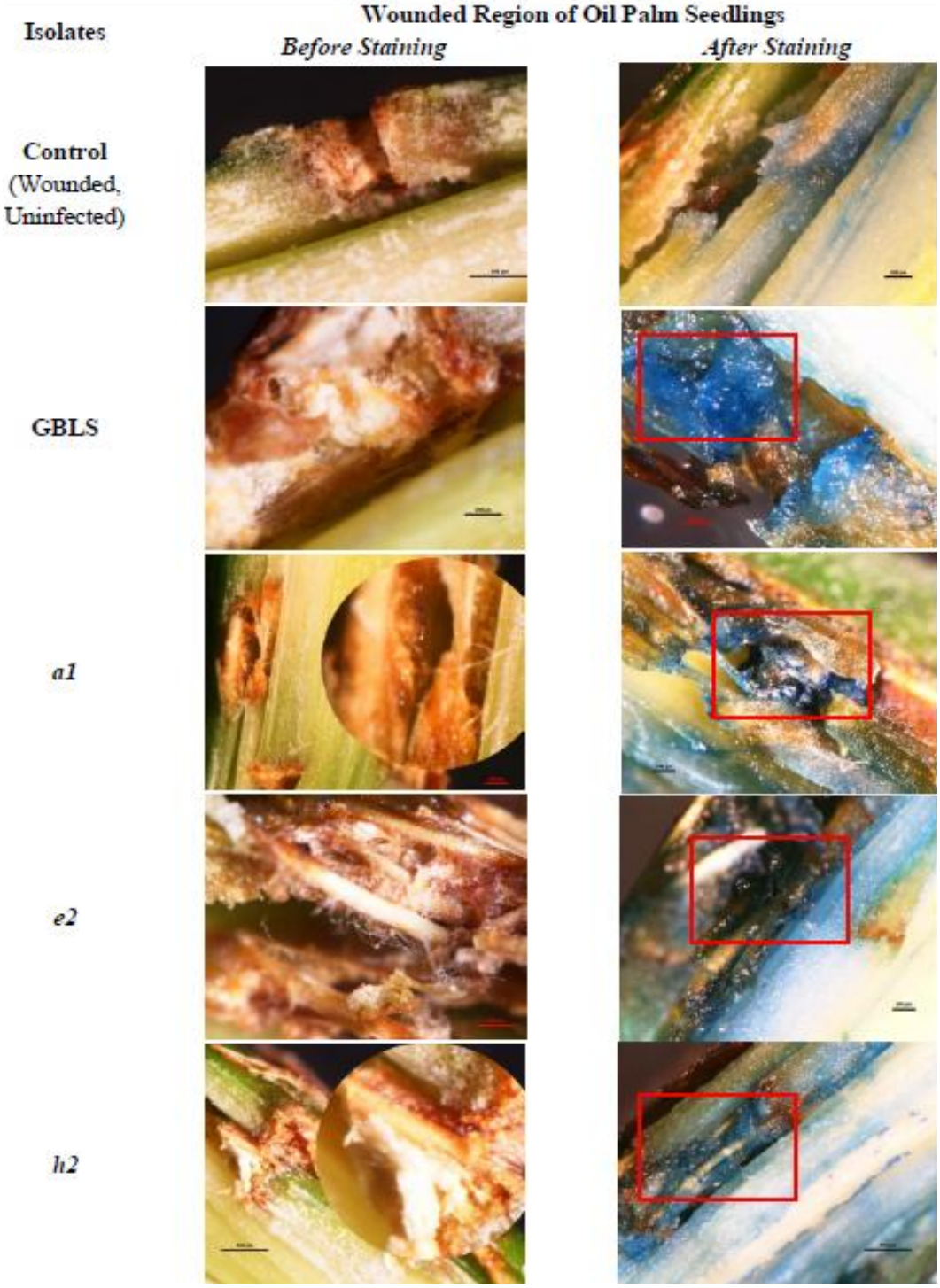

Figure 4. Microscopic observation of the infected region of oil palm seedlings before and after staining with lactophenol blue dye.

inoculation at 8.30% DSI value.

All *G. boninense*-infected oil palm samples and *G. boninense* mycelia DNA showed amplification at location 167 bp, indicating the presence of *Ganoderma* in all parts of infected oil palm seedlings (leaves, stems and roots). These results were complemented with previous findings by Karthikeyan et al. (2006; 2007) that they adopted similar primer sequences (Gan1 and Gan2) which amplified a DNA fragment of the size of 167 bp internal transcribe spacer (ITS) region when *Ganoderma* isolates

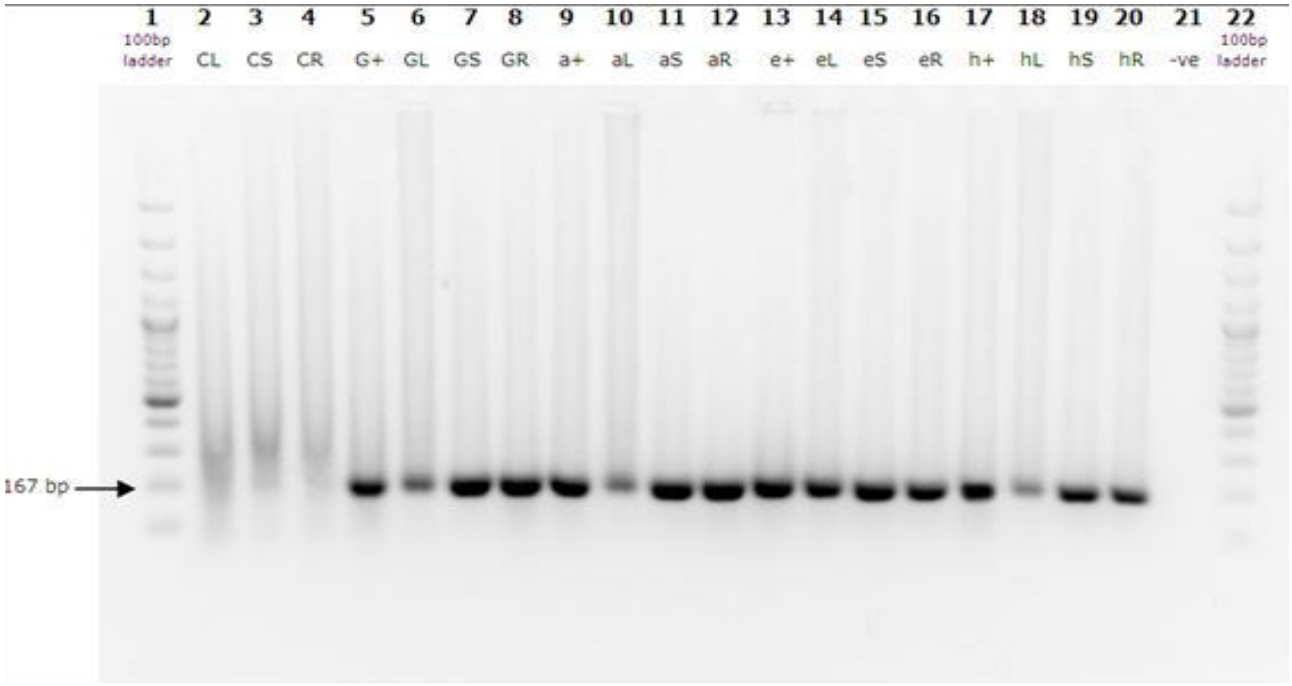

Figure 5. PCR amplification of the DNA of infected oil palm seedling with *G. boninense* isolates using GbF and GbR primers. Lane 1 and 22: 100 bp DNA ladders; Lane 5, 9, 13 and 17: Positive control of GBLS (G), *a1* (a), *e2* (e) and *h2* (h) fungal DNA; Lane 2-4: Leaves (L), stems (S) and roots (R) DNA samples of control uninfected oil palm seedlings; Lane 6-8, 10-12, 14-16 and 18-20: DNA samples of infected oil palm seedlings; Lane 21: Negative control.

were used for amplification.

From this study, all of the *G. boninense* isolates (GBLS, *a1*, *e2* and *h2*) were vegetatively incompatible with strong antagonistic relationships. Isolates *a1*, *e2* and *h2* originated from a similar fruiting body, whereas isolates GBLS was isolated from another fruiting body. According to the results of disease severity, the DSI values in oil palm seedlings infected with isolates *a1*, *e2* and *h2* were not significantly different ($P < 0.05$) from one other, but were significantly different ($P < 0.05$) from the seedlings infected with isolate GBLS. This suggested that *G. boninense* isolates originated from similar fruiting body had relatively similar level of infection potential, although they showed strong level of vegetative incompatibility. This phenomenon was probably because of isolates originated from a single fruiting body are genetically related (Pilotti et al., 2002).

Overall, *G. boninense* isolates (*a1* and *h2*) with high ligninolytic enzymes production resulted in relatively high DSI values (42.50 and 56.25%, respectively) when these isolated were used to infect oil palm seedlings through *in vitro* infection. In contrast, seedlings infected by isolate GBLS with lower laccase and MnP enzyme activities (24.31 and 9.27 UL^{-1}, respectively) obtained the lowest DSI value (29.55%) after the inoculation period. These results indicated that *G. boninense* isolates with higher laccase and MnP production had higher infection potential as they can cause more severity on the oil palm seedlings when these particular isolates were used to infect the seedlings *in vitro*. Hence, it was deduced that there was a direct relationship between the ligninolytic enzyme production and the infection potential in vegetative incompatible *G. boninense* isolates. This relationship is supported by Ali et al. (2004), who clearly indicated that pathogenic *G. boninense* produced lignin degrading enzymes (ligninolytic enzymes) and diminished the lignin content in dead oil palm samples *in vitro* when they were used to infect oil palm. Although a direct relationship between the ligninolytic enzyme production and infection potential cannot be drawn from the results for isolate *e2*, one explanation could be due to the present of LiP enzymes produced by this isolate. According to Paterson (2007), LiP is capable to attack wider range of linkages, including the oxidation in both phenolic and non-phenolic compounds, suggesting its strong impact in lignin degradation even if it is present at low level. Since LiP is also a type of ligninolytic enzyme, it was deduced that the ligninolytic enzymes in isolate *e2* directly affect its infection potential in oil palm seedlings as well.

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Comparison of humic acids production by *Trichoderma viride* and *Trichoderma reesei* using the submerged fermentation of oil palm empty fruit bunch

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The remarkable properties of humic acids have generated a broad spectrum of applications in pharmaceutical, cosmetic and agricultural fields, and encouraged fermentation studies focusing on humic acids production. This work compares the humic acids production of *Trichoderma* (*viride* and *reesei*) species using empty fruit bunch as the substrate during submerged fermentation. The performance of each species was compared by examining spore production in oat medium, and the significant medium components and fermentation conditions were identified using Plackett and Burman statistical design. For both *Trichoderma* species, the results indicated that humic acids production can be enhanced by increasing the temperature, empty fruit bunch and peptone concentrations and by decreasing the $(NH_4)_2SO_4$ concentration. *T. reesei* performed better than *T. viride*, generating 3-fold more of humic acids.

Key words: Humic acids, *Trichoderma reesei, Trichoderm aviride*, submerged fermentation, empty fruit bunch.

INTRODUCTION

Trichoderma is a genus of asexually reproducing fungi with a high level of genetic diversity (Harman et al., 2004). They are frequently found growing in soil as well as on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions (Druzhinina, 2011). These characteristics indicate that this genus could be used in many biotechnological applications (Esposito and Silva, 1998). The genus *Trichoderma* is widely used in industrial applications, because they produce extracellular lignocellulose-degrading hydrolases in large amounts (Cavaco-Paulo and Gubitz, 2003), which can be useful for recycling cellulosic waste materials as well as producing useful by-products (Samuels, 1996).

Humic acids (HA), a component of the organic matter in soil, are the soil fraction that is most resistant to microbial degradation. They are complex polymeric organic acids with a wide range of molecular weights and exist as heterogeneous mixtures of a variety of organic

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compounds, including aromatic, aliphatic, phenolic, and quinolic functional groups (Aiken, 1985). They are one of the most active fractions of organic matter and affect a variety of chemical, physical, and biological reactions. Previously recognized for agricultural applications, they have received growing attention from the biomedical field primarily due to their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities (Yamada et al., 1998), which are of great importance for pharmaceutical and biomedical applications (von Borstel et al., 1994). Traditionally, HA is extracted from lignite, brown coals and humified organic materials (Asing et al., 2011). However, harvesting HA from non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peat lands, which is associated by environmental problems (Joosten et al., 2012). Thus, it is desirable to use more ecologically sustainable precursors/feedstock for HA and to develop cheaper and cleaner methods for the extraction of this valuable product.

For economic reasons, industrial fermentation involves are complex, almost indefinable substrates that are often the by-products of other industries. Empty fruit bunch (EFB), a cellulosic material source containing 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin (Hamzah et al., 2011), is a strong candidate for use as a fermentation substrate. EFB is the product of oil palm processing and is produced in large quantities, but it is a waste product that has not been completely utilized as 23% of the fresh fruit bunch (Harun et al., 2013) used in oil palm production remains unused as EFB. Thus, there is a growing interest in making EFB useful, reducing the large volume of waste and its ensuing environmental problems (Oviasogie et al., 2010).

Although no quantitative studies have been performed previously, *Trichoderma* sp. is a suitable genus for the production of HA from EFB. These fungi have been recognized for their extreme facility in producing a large variety of extracellular enzymes and the degradation of lignocellulose (Kirk and Farrell, 1987). Furthermore, *T. viride* and *T. reesei* are the most extensively studied fungi in the field of cellulosic material degradation (Cullen and Kersten, 1992).

In our previous work, it was demonstrated that the production of HA from EFB is by using a *T. viride* strain (Motta and Santana, 2013). This work extends our previous findings by comparing the performance of *T. reesei* and *T. viride* in HA production from EFB, as well as evaluating medium components and fermentation conditions. Spores from both species were produced by submerged fermentation in oat medium as previously described for *T. viride* (Motta and Santana, 2012). The effects of medium components and fermentation conditions were compared using Plackett and Burman (PB) statistical design (Plackett and Burman, 1946).

MATERIALS AND METHODS

Microorganism maintenance

For storage, the *T. reesei* culture was grown on potato dextrose agar plates at 24°C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 ml cryotubes at -70°C.

Production of *T. reesei* spores in oat medium

The spore production and fermentation analysis for *T. reesei* were performed as previously described by Motta and Santana (2012) for *T. viride*. The medium was prepared by adding 30 g of oat meal (Quaker Oats Company®, thin flakes; Table 1 for detailed composition) into 1 L of distilled water and boiling the suspension for 90 min at 90°C with constant stirring. Immediately after heating, the suspension was filtered through a sieve (0.150 mm diameter holes). Distilled water was added to the filtrate to achieve a final volume of 1 L. Then, 5 g of potato peptone purchased from Sigma-Aldrich was added, and the media was adjusted to pH 6.0 (Al-Taweil et al., 2009) prior to autoclaving at 121°C for 15 min.

The cultures were grown in 500 mL Erlenmeyer flasks containing 300 mL of oat meal culture media inoculated with the spore suspension prepared as described in the item “microorganism maintenance”. To obtain the same initial concentration of spores for both species, 0.4 ml of the *T. reesei* spore suspension were used. The culture flasks were incubated at 24°C at 150 rpm. During 120 h of cultivation, samples were collected every 24 h for dry biomass estimation and cellular protein quantification, which are direct and indirect methods for fungal biomass determination, respectively. Image analysis was performed every 24 h, and spore counts were performed at 0 and 120 h of fermentation.

Dry weight biomass

Based on the methodology adapted by Szijártó et al. (2004), in which the optical density was used to evaluate *T. reesei* biomass behavior in delignified pine pulp, the dry biomass concentration of *T. reesei* was evaluated by reading the optical density at 600 nm in a spectrophotometer. The absorbance at 600 nm was correlated to the cell dry weight per culture volume. To determine the dry weight, the fungal biomass was extracted from the culture medium by heating an aliquot of known volume at 85°C under constant agitation for 10 min after dilution in distilled water at a ratio of 1:10. After heating, the aliquot was filtered through coffee filter paper, and the filtrate was collected. The solids were retained, heated and filtered as described above until the green color (typical of *Trichoderma* spores) disappeared. The total volume of the filtrate was centrifuged at 10,000 *g* at 5°C, and the supernatant was discarded. The precipitate was dried at 105°C to achieve a constant weight and placed in a desiccator until fungal mass was determined.

Cellular protein quantification

Indirect estimation of the fungal biomass was performed by determining the protein concentration in each sample. Protein quantification was performed according to the method adapted by Callow and Ju (2012) to quantify only the cellular proteins. Culture samples (3.0 mL) were collected and centrifuged at 10,000 *g* for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were resuspended and washed twice with

Table 1. Composition of oatmeal thin flakes (data collected from the manufacturer's product specification sheet, Quaker Oats Company®).

Constituent	Content (g per 100 g)
Carbohydrate	56.7
Protein	14.3
Total fat	7.3
Fibers	9.7
β-glucan	4.0

deionized water. After each wash step, the biomass was centrifuged and the water was discarded. To release intracellular proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100°C for 10 min. After cooling, the digested samples were centrifuged at 10,000 *g* for 10 min to remove the cell debris and other solids; the supernatants were then collected, and the protein concentrations were determined by the bicinchoninic acid assay (Smith et al., 1985) using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were generated with bovine serum albumin.

Spore counts

The spores were directly counted using a Neubauer chamber. After mycelia and conidia were observed with a Reichert-Jung Series 150 microscope (Reichert, USA), indicating that fungal sporulation had been completed at 120 h of fermentation, the spore count was performed at 0 and 120 h to compare the initial concentration of spores to the spore concentration at the end of fermentation.

Humic acids (HA) production

The HA production for both species was analyzed and compared according to the results obtained with the PB assay as well as the effect of medium components and fermentation conditions.

Submerged fermentation

EFB was provided by Oil Palm S/A - Agro-industrial OPALMA (Bahia, Brazil) and was milled to a standardized particle size between 125 and 500 µm (115 and 32 mesh in the Tyler series, respectively). The composition of the EFB particles was determined with an elemental analyzer CNH (Perkin Elmer Series II 2400, USA) which indicated that the elemental mass percentage is 48.0±0.7% carbon, 2.6±0.1% nitrogen and 6.1±0.2% hydrogen. These results are expressed as the mean of triplicates and the average deviation. Potato peptone was purchased from Fluka Analytical (France); $(NH4)_2SO_4$ and K_2HPO_4 were purchased from Ecibra (Brazil). *T. reesei* and *T. viride* were cultured in 500 mL Erlenmeyer flasks containing 270 mL of culture media inoculated with 30 mL of inoculum, which for both species consisted of spores produced in oat medium for both species (item "Production of *T. reesei* spores in oat medium"). The culture flasks were incubated at 150 rpm, and samples were withdrawn at 120 h of fermentation for HA quantification.

Humic acids (HA) quantification

According to the methods adapted by Badis (2010), the samples were centrifuged at 10,000 *g* for 15 min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant fractions were filtered using the microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted five-fold with 0.5 M NaOH solution, and the absorbance at 350 nm at pH 4.5±0.01 was measured. Standard curves were obtained from the absorbance at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in 0.5 M NaOH solution, pH 4.5±0.01.

Plackett and Burman (PB) design

The Plackett and Burman (PB) design was used for screening the selected variables, which had significant effects on HA production. Six variables were screened: EFB (g/L) as a carbon source; potato peptone (g/L) as an organic nitrogen source; pH; temperature (°C); and K_2HPO_4 (g/L) and $(NH_4)_2SO_4$ (g/L) as inorganic nitrogen sources (Table). Each factor in this experimental design was examined at three levels: low (−), high (+) and central (0) to evaluate the linear and curvature effects of the variables (Table). Table shows the design with 16 PB trials along the levels. This statistical design does not involve the interactions between the selected variables and follows a linear approach for screening the factors (Plackett and Burman, 1946).

RESULTS

Spores production in oatmeal medium

As shown in Figure 1, the biomass and protein concentration curves have the same behavior over 120 h of cultivation for both *T. viride* (Motta and Santana, 2012) and *T. reesei*. Moreover, it is possible to observe by both biomass estimation methods that there is effectively no fungal growth after 96 h of fermentation, with the largest growth rate observed between 24 and 72 h of fermentation. With regard to the relationship between *T. viride* and *T. reesei* biomass concentration and cellular protein concentration, as described by Equations 1 and 2, respectively, there is a strong correlation value (0.98) for both species.

$$\text{Biomass (g/L)} = 3.78 * \text{Protein (g/L)} + 0.11 \quad (1)$$

$$\text{Biomass (g/L)} = 2.33 * \text{Protein (g/L)} + 0.23 \quad (2)$$

Using image analysis with optical microscopy, it was observed that at 120 h of fermentation the *T. reesei* strain completed its sporulation phase, and only spores were present in the culture medium at 120 h, as was observed for the *T. viride* strain by Motta and Santana (2012). The initial concentration of spores in the culture medium was 4.54×10^4 spores/mL, and after 120 h of fermentation, the spore concentration found in the fermentation medium was 6.36×10^6 spores/mL.

Humic acids (HA) production

Six variables were screened using PB, as shown in Table

Table 2. Six variables screened using PB design at lower (-1), higher (+1) and central (0) levels.

Variable	Unit	Experimental value		
		-1	0	+1
EFB	g/L	10.0	20.0	30.0
Peptone	g/L	1.00	3.85	6.70
pH	-	4.0	6.0	8.0
Temperature	°C	25.0	30.0	35.0
K_2HPO_4	g/L	0.28	1.54	2.80
$(NH_4)_2SO_4$	g/L	0.24	0.77	1.30

Table 3. Experimental PB design used to screen six variables with real and code values (parentheses) for the response of HA production along with its observed values.

Trial	Experimental value						HA (mg/L)*	
	EFB (g/L)	Peptone (g/L)	pH	Temperature (°C)	K_2HPO_4 (g/L)	$(NH_4)_2SO_4$ (g/L)	Observed for *T. reesei*	Observed for *T. viride*
1	30.0 (+1)	1.00 (-1)	8.0 (+1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	128.1	12.6
2	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	0.28 (-1)	0.24 (-1)	318.7	85.2
3	10.0 (-1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	0.24 (-1)	112.6	48.9
4	30.0 (+1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	189.1	38.3
5	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	182.9	107.0
6	30.0 (+1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	159.8	44.3
7	10.0 (-1)	6.70 (+1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	113.7	46.9
8	10.0 (-1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	75.4	46.9
9	10.0 (-1)	1.00 (-1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	1.30 (+1)	27.1	10.3
10	30.0 (+1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	1.1	3.3
11	10.0 (-1)	6.70 (+1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	1.30 (+1)	85.5	47.6
12	10.0 (-1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	0.6	20.2
13	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	143.4	58.8
14	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	142.3	58.1
15	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	139.7	57.5
16	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	147.1	60.8

***** HA concentration obtained after 120 hours of fermentation.

3, which reports the HA concentration for each trial and for both species. Trials 13 to 16 consisted of the center points, which were the same conditions used in our previous work in which it demonstrated the production of HA using EFB and the *T. Viride* strain (Motta and Santana, 2013). According to the values obtained for these center points, the average production of HA was 143.1±3.0 mg/L and 58.8±1.4 mg/L for *T. reesei* and *T. viride*, respectively. The largest HA production by *T. reesei* was observed in Trial 2 (318.7 mg/L), which used the highest level for EFB, peptone and temperature, and the lowest level (-1) for pH, K_2HPO_4 and $(NH_4)_2SO_4$. For *T. viride*, Trial 5 generated the highest HA production (107.0 mg/L), which used the highest level for EFB, peptone, temperature and K_2HPO_4, and the lowest level (-1) for pH and $(NH_4)_2SO_4$.

From the summary of effects shown in Table and obtained using Statistica version 8.0 (Statsoft, Oklahoma, USA), the temperature and the concentration of EFB, peptone, $(NH_4)_2SO_4$ and K_2HPO_4 were the statistically significant variables with an effect on HA production in *T. reesei* (p-value<0.1). Among these five variables, $(NH_4)_2SO_4$ and K_2HPO_4 concentration had a negative effect on HA production, presenting similar estimated effect values (-40.3 and -46.1, respectively). The EFB concentration had the greatest positive effect on HA production, followed by peptone concentration and temperature. EFB, peptone and temperature have a highly significant effect on HA production, with a *p*-value much smaller than 0.1. For *T. viride*, four of the six

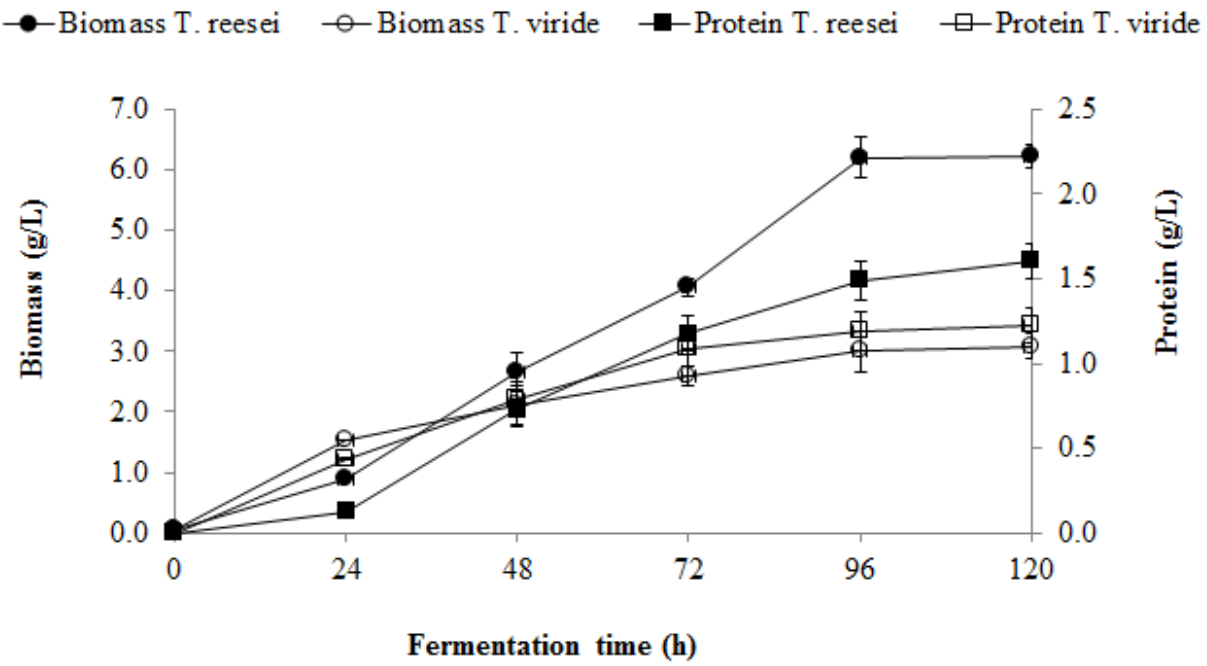

Figure 1. Biomass and cellular protein concentration over time during the submerged fermentation of oatmeal with *T. viride* (Motta and Santana, 2012) and *T. reesei.*

Table 4. Summary of estimated effects from the PB.

	T. reesei				*T. viride*			
Factor	**Estimated effect**	**Standard error**	**t(8)**	***p*-value**	**Estimated effect**	**Standard error**	**t(8)**	***p*-value**
Mean/Interc.	116.2	10.2	11.4	<0.0001	42.6	3.1	13.6	<0.0001
Curvature	53.8	40.7	1.3	0.2229	32.3	12.5	2.6	0.0323
EFB	94.1	20.3	4.6	0.0017	11.6	6.3	1.9	0.0998
Peptone	92.0	20.3	4.5	0.0019	41.4	6.3	6.6	0.0002
pH	27.1	20.3	1.3	0.2188	-6.0	6.3	-1.0	0.3671
Temperature	69.9	20.3	3.4	0.0089	26.3	6.3	4.2	0.0030
K_2HPO_4	-46.1	20.3	-2.3	0.0530	1.7	6.3	0.3	0.7984
$(NH_4)_2SO_4$	-40.3	20.3	-2.0	0.0828	-21.7	6.3	-3.5	0.0085

variables screened were significant, because unlike *T. reesei*, K_2HPO_4 levels were not statistically significant. For *T. viride*, $(NH_4)_2SO_4$ concentration had a negative effect on HA production (-21.7), and the peptone concentration had the greatest positive effect on HA production, followed by temperature.

Using the Pareto Chart for both *Trichoderma* species (Figure 2), it is possible to identify the variables that do or do not significantly affect HA production by comparing the value of the statistic (t_{cal}) for each variable, represented by the length of the bar, and the critical value (t_{crit} = 1.860), represented by the red line. Variables with $t_{cal}>t_{crit}$ have a statistically significant effect on HA production, and the variables with $t_{cal}<t_{crit}$ are not considered statistically significant. With respect to the curvature, it was not statistically significant for *T. reesei* (t_{calc} = 1.322 < t_{crit} = 1.860) but was statistically significant for *T. viride* (t_{calc} = 2.586 > t_{crit} = 1.860).

DISCUSSION

Biomass is a fundamental parameter in the characterization of microbial growth, and its measurement is essential for kinetic studies on fermentation. Complete recovery of fungal biomass from

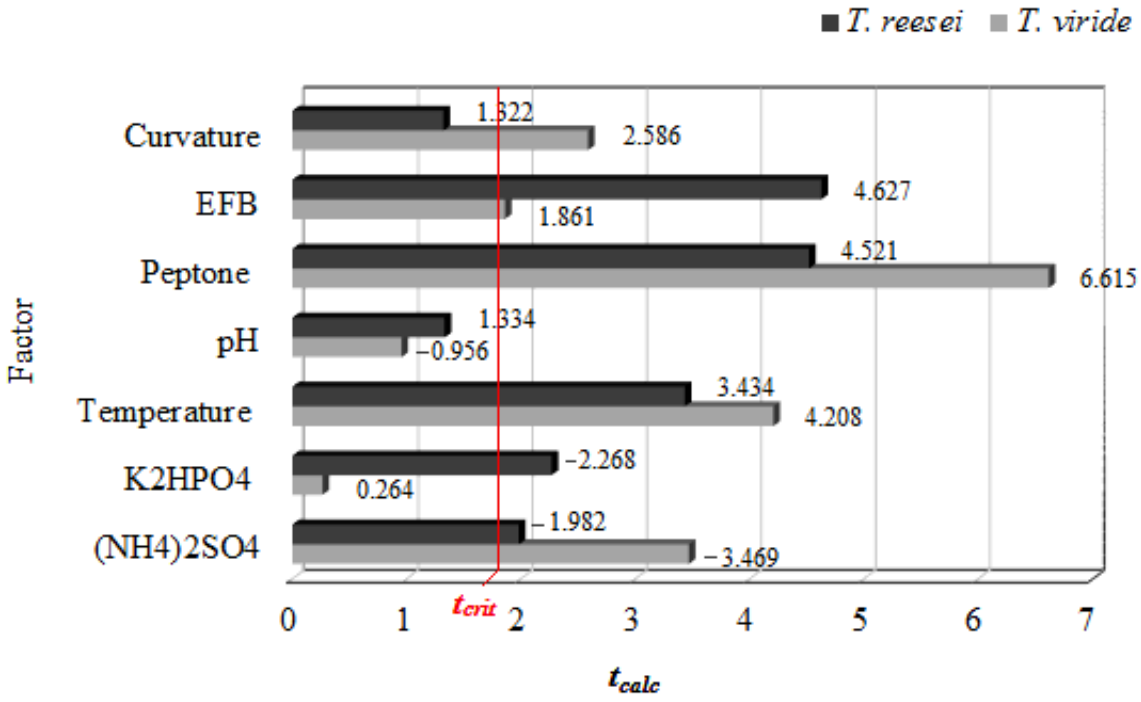

Figure 2. Pareto chart of the standardized effects of independent variables. Response HA mg/L and alpha = 0.10.

the substrate is very difficult when the fungal hyphae penetrates into and binds tightly to the solid substrate particles (Abd-Aziz et al., 2008). The present study adopted two methods for biomass quantification, one direct and one indirect, consisting of dry biomass and cellular proteins quantification, respectively. Christias et al. (1974) determined in their work the amount of protein present in the biomass of five different genera of fungi, and they obtained a range of 30 to 40%. By applying these results to the present study, it is possible to observe that the ratio between protein and biomass concentration is within the range obtained by Christias et al. (1974).

According to the optical Microscopic image analysis, after 120 h of fermentation, the sporulation phase is complete, and only spores are present in the culture medium. Therefore, the spore counts were performed at 0 and 120 h of fermentation to determine the increase in the fungal population using oat medium for submerged fermentation. The comparison between the initial concentration of spores in the culture and after 120 h of fermentation indicated that there were two phases of log growth during the 5 days of oatmeal-based fermentation.

Watanabe et al. (2006) used a culture medium containing soluble starch and soybean meal supplemented with KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ to produce *T. asperellum* spores by submerged fermentation. They obtained 7.8 x 10^8 spores/mL after 7 days of fermentation from a starting inoculum of 1.0 x 10^6 spores/mL. Jakubíková et al. (2006) optimized *T. atroviride* sporulation in submerged fermentation on cellobiose supplemented with $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, and $FeSO_4$. The culture medium was inoculated with 1 x 10^6 spores/mL, and the spore concentration reached a maximum level of 2.68 x 10^8 spores/mL after 4 days of fermentation. Comparing the results obtained in the present work to past results indicate that the submerged fermentation of oatmeal is a good alternative for the production of *T. reesei* spores. Moreover, the culture medium used in this study for spore production was supplemented only with peptone, a safe supplement for culture media that is animal-and endotoxin-free. These compounds can cause illness in humans and are considered as contaminants that must be avoided or minimized in the preparation of pharmaceutical products. Because both species were grown under identical conditions and are completely transformed into spores after 120 h of fermentation, it is possible to make a comparison between the two species regarding growth in oat medium. For *T. viride*, the initial concentration of spores (0 h) was 4.20 x 10^4 spores/mL, reaching 3.52 x 10^6 spores/mL at the end of the fermentation (120 h) (Motta and Santana, 2012). With respect to the *T. reesei* spore concentration, the initial and final values were 4.54 x 10^4 and 6.36 x 10^6, respectively. Therefore, it is possible to observe that

there was an 84-fold increase in the population of *T. viride* and a 140-fold increase in the population of *T. reesei* after 120 h of cultivation under the same conditions. The highest growth potential for *T. reesei* with respect to *T. viride* can also be observed by comparing the biomass concentration (g/L) throughout the fermentation for both species (Figure 1). *Trichoderma* species produces large quantities of hydrolytic enzymes (Papavizas, 1985) including chitinases, β-1,3-glucanases (Howell et al., 2000), cellulases, amylases and proteases (Bastos, 1996), which explains the high growth capability of each species. However, *T. reesei* is regarded in the literature as one of the main producers of endoglucanases, secreting at least five types of these enzymes and justifying the increased growth in culture compared to *T. viride*, because the oat media is comprised of β-glucans that can be hydrolyzed to glucose, providing the fungus with a readily usable carbon source for growth (Herpoël-Gimbert et al., 2008).

The *T. reesei* and *T. viride* spores were then used in the submerged fermentation of EFB for HA production. By performing the PB trials, the species were compared in terms of the HA produced as well as analyzed for the effect of fermentation conditions on the production of HA. By comparing the central points of the PB for both species, it is possible to observe that *T. reesei* HA production was 2.4-fold greater than *T. viride*. With respect to the highest HA production for each species (Trial 2 for *T. reesei* and Trial 5 for *T. viride*), this value increases to 3-fold. Although many species of *Trichoderma* have previously been used for industrial enzyme production and lignocellulosic degradation (Nevalainen et al., 1994), *T. reesei* is known to be the main producer of cellulases and hemicellulases acting in synergy to degrade lignocellulosic materials (Herpoël-Gimbert et al., 2008), which explains its increased production of HA. To understand the effect of the variables involved in the proposed process, PB statistical analysis was used, in which the response variable (HA concentration) was examined at 120 h, because HA accumulated in the culture medium, due to the resistance of HA to microbial degradation compared to other organic materials (Aiken, 1985). This idea was confirmed by the work of Motta and Santana (2013), in which the production of HA by submerged EFB fermentation was studied. The profile obtained for HA concentration increased as fermentation time increased, demonstrating accumulation in the media. The Pareto chart (Figure 2) distinguishes the statistically significant variables from the statistically insignificant among the factors studied, as previously discussed, by comparing the value of the statistic (t_{cal}) for each variable to the critical value (t_{crit} = 1.860). Center points were added in the statistical analysis, allowing the performance of explicit statistical significance tests of curvature. These values were not statistically significant for *T. reesei*, indicating that there is a linear relationship between the factors and the dependent variable. For *T. viride*, the curvature was statistically significant, meaning that at least one variable is involved at an order higher than one. Among the analyzed variables, only pH was considered to have no effect on HA production at the confidence level studied (90%) for both *Trichoderma* species. According to Bhattiprolu (2008), *T. viride* is able to grow in wide range of pH (4.0 to 9.0), which may explain the fact that this factor was not been statistically significant for either species in this work. Regarding the nitrogen sources, peptone (L) and $(NH_4)_2SO_4$ (L) concentration had a significant effect on HA production by both species, although the first had a large positive effect and the second a negative effect. Juwon and Emmanuel (2012) tested the influence of nitrogen sources on growth and enzyme production in *T. viride* and observed that organic nitrogen substrates, like peptone, supported increased biomass yield and enzyme activity of the fungus compared to the inorganic nitrogen substrates tested.

The variable that affected HA production is the least in *T. reesei* and K_2HPO_4 concentration, and its effect was not significant in *T. viride*. According to the average chemical formula elucidated by Schnitzer and Khan (1978) (C_{187} H_{186} O_{89} N_9 S_2), HA does not contain phosphorus and potassium, explaining the minimal influence of this compound on the production of HA. Temperature has a large effect on both species, likely because enzyme activity as well as regulation and transport systems are generally largely affected by temperature in microbial systems (Anastassiadis, 2006). The linear effect of EFB concentration was larger in *T. reesei* than in *T. viride* and had the largest effect on this species. Although this component is the main carbon source for HA production in the studied media for both species, its effect was greater for *T. reesei*, possibly because this species is capable of utilizing the best carbon and energy source available when exposed to a mixture of carbon sources and down regulates the expression of genes involved in the degradation of less favorable and complex carbon sources (Seiboth et al., 2011).

Conflict of Interests

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

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Full Length Research Paper

Characterisation of the vaginal microflora of human immunodeficiency virus (HIV) positive and negative women in a sub-urban population of Kenya

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Lactobacilli predominate normal vaginal microflora and are important in maintenance of vaginal health. The current study set out to identify and compare culture isolates of vaginal microflora of human immunodeficiency virus (HIV) positive (HIV^+) and HIV negative (HIV^-) women at different phases during menstrual cycle from a sub-urban population of Kenya. Seventy four (74) women, 41 HIV^+ and 33 HIV^-, followed up two consecutive menstrual cycles, had high vaginal swabs taken to prepare Gram stains for six visits and anaerobic cultures for four. All 751 isolates identified by t-DNA polymerase chain reaction (PCR) belong to 51 species. Species cultured more frequently in HIV^+ participants were: *Lactobacillus jensenii* (p=0.01), *Lactobacillus iners* (p=0.02), *Gardnerella vaginalis* (p=0.01) and *Peptoniphilus lacrimalis* (p=0.01). Species cultured more frequently in HIV^- women were *Dialister micraerophilus* (p=0.02) and *Streptococcus agalactiae* (p=0.04). *Lactobacillus* predominating both groups were *Lactobacilli crispatus, L. jensenii, L. iners* and *Lactobacilli vaginalis*. Bacterial vaginosis (BV) was equally high in HIV^+ and HIV^- women. *Lactobacillus* and BV-associated species were cultured more frequently in HIV^+ women. Minor species differences were found. Predominant *Lactobacillus* in culture were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. These women had lower concentrations of lactobacilli in vaginal microflora than observed in previous studies of Caucasian women.

Key words: Vaginal microflora, human immunodeficiency virus (HIV), menstrual cycle, t-DNA polymerase chain reaction (PCR), culture, bacterial vaginosis.

INTRODUCTION

Perturbation of the healthy vaginal ecosystem, also by loss of hydrogen peroxide (H_2O_2)-producing

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Abbreviations: BV, Bacterial vaginosis; **STIs,** sexually transmitted infections; **HIV,** human immunodeficiency virus; **ART,** antiretroviral therapy; **pap,** papanicolaou; **ELISA,** enzyme-linked immunosorbent assay; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfate.

referred to as bacterial vaginosis (BV), is characterized lactobacilli and massive overgrowth of *Gardnerella vaginalis* and other Gram positive and negative anaerobic bacteria (Marrazzo, 2004). Of concern are recent observations that BV predisposes to acquisition of sexually transmitted infections (STIs), which raises the risk of mother-to-child transmission of human immunodeficiency virus (HIV). Diminished colonisation resistance during BV renders women vulnerable to various infections such as *Trichomonas vaginalis* and *Neisseria gonorrhoeae* (Martin et al., 1999; Wiesenfeld et al., 2003), *Chlamydia trachomatis* (Wiesenfeld et al., 2003), genital herpes simplex (Kaul et al., 2007), human papillomavirus (Watts et al., 2006) and HIV-1 (Spear et al., 2007; Atashili et al., 2008). Further, it is documented that BV propagates replication and vaginal shedding of the HIV-1 (Sha et al., 2005) and HSV-2 viruses (Cherpes et al., 2005), thus enhancing the spread of STIs. Increased mother-to-child transmission of HIV as a consequence of BV has been documented in a prospective study of 463 HIV-1-infected mothers and infants in Kenya (Farquhar et al., 2010). This study found women with BV to be three times more likely to transmit HIV *in utero*. High prevalence of BV in sub-Saharan Africa (Sewankambo et al., 1997; Taha et al., 1998; Paxton et al., 1998; Martin et al., 1999; van de Wijgert et al., 2000; Demba et al., 2005; Morison et al., 2005; Bukusi et al., 2006; McClelland et al., 2009) has therefore become an issue of global concern. It is estimated, for instance, that in endemic areas, nearly one-third of new HIV cases could be prevented if existing BV were cured (Schwebke, 2005). Tackling the HIV burden through eradicating BV and restoring normal vaginal microflora is currently considered part of the most promising answers to the HIV pandemic (Martin et al., 1999; Shin and Kaul, 2008; Bolton et al., 2008).

The last two decades have seen a significant increase in the volume of research focusing on BV and composition of vaginal microflora (Verhelst et al., 2005; Kalra et al., 2007; Srinivasan et al., 2010; Verstraelen et al., 2010). The prevalence of BV varies widely in different populations. It tends to be high in African women, with rates up to 44% having been documented in a Kenyan cohort and 50% in rural Uganda (Paxton et al., 1998; Bukusi et al., 2006). Studies found African American women to have more than two-fold higher prevalence of BV compared to Caucasian American women. The cause of this disparity remains unknown (Goldenberg et al., 1996; Pastore et al., 1999; Royce et al., 1999; Ness et al., 2003; Allsworth and Peipert, 2007). Studies show African women to be either less likely to harbor H_2O_2-producing lactobacilli which are known to suppress growth of anaerobic bacteria (Antonio et al., 1999) or to have lower concentrations of the same (Anukam et al., 2006; Zhou et al., 2007; McClelland et al., 2009). African women also tend to have significantly higher vaginal pH than do Caucasian women (Ravel et al., 2010). In addition, the prevalence of BV has been shown to be higher in HIV-infected women compared to HIV-uninfected women (Sewankambo et al., 1997; Jamieson et al., 2001; Warren et al., 2001). From the foregoing information, it is apparent that more studies are needed to complete our understanding of BV in affected sub-populations. Further, it is not known how vaginal microflora varies during the phases of the menstrual cycle.

The goal of the present study was to investigate the composition of vaginal microflora of non-pregnant premenopausal HIV^+ and HIV^- women in a sub-urban population of Kenya by PCR-based identification of culture isolates obtained during the follicular and ovulation phases of the normal menstrual cycle.

MATERIALS AND METHODS

Study population

This was a cross-sectional study nested in an on-going Tigoni dysplasia study (TDS) based at Tigoni Hospital, Kiambu West district of Kenya. Recruitment of participants took place at the hospital between August 2006 and March 2008. Of the 215 women screened, 100 of them met the inclusion criteria, and only 74 returned for the scheduled six follow-up visits. Specimens were collected at three points of two consecutive menstrual cycles–follicular (day 5-8), ovulation (day 12-15) and luteal (day 19-22) phases. At each phase, the sampling period was spread out within four days to create flexibility for return visits. The participants were asked to mark all the days of menstrual bleeding as a guide to establishing the regularity of the cycle. Following verbal explanation of the study objectives, written consent to participate was obtained for all participants and hard copies of the study explanation issued to them. The documents were available in both English and the most widely used local language, Kiswahili. The study was approved by the ethical review board of Kenyatta National Hospital/ University of Nairobi (Registration number P122/8/2005).

During the study, all STIs diagnosed were treated according to the Kenya Ministry of Health national guidelines (2007) for both participants and those excluded from the study. All HIV^+ women received treatment free of charge, courtesy of the sponsored partnership of Kenya Ministry of Health, University of Nairobi Institute of Tropical and Infectious Diseases and Pathfinder International. At the time of the study, all HIV^+ persons with a CD_4 count above 200 cells/ mm^3 were exempted from antiretroviral therapy (ART) according to WHO guidelines (1997). Qualifying pre-menopausal women, aged between 18 and 45 years were screened for eligibility using a standard face-to-face questionnaire interview. The HIV^+ women who had CD_4 counts above 250 cells/mm^3 and not undergoing ART were recruited. All the participants had regular menses and a documented normal papanicolaou (pap) smear test result performed within one year.

The exclusion criteria were presence of STIs, ongoing pregnancy, use of contraception except condom, previous hysterectomy, delivery or abortion in the preceding three months and use of systemic antibiotics (except cotrimoxazole prophylaxis for HIV^+ women given under Kenya national guidelines for HIV management). The HIV test was performed in order to group patients as positive and negative.

Following the face-to-face questionnaire interview, subjects provided blood specimens for HIV testing, syphilis and CD_4 count. Vaginal specimens were taken to test for *T. vaginalis, C. trachomatis* and *N. gonorrhoeae*. HIV pre- and post-test counselling was offered by a trained nurse.

Screening tests

Study participants were screened for HIV-1 infection using enzyme-linked immunosorbent assay (ELISA) Detect-HIV (BioChem ImmunoSystems, Allentown, PA). Positive samples were confirmed by a second ELISA (Vironostika, BioMérieux, Marnes-la-Coquette, France) (WHO guidelines). Urine pregnancy test was performed using a rapid β–hCG test kit (Plasmatec Laboratory products, Cambridge, UK) and syphilis seroreactivity assessed by the Rapid Plasma Reagin (Becton Dickinson, Baltimore, MD). Confirmatory *Treponema pallidum* haemagglutination assay was not necessary as no seroreactive cases were detected. Two high vaginal swabs were taken for *T. vaginalis* culture (In-Pouch, Biomed Diagnostics, San Jose, CA) and for diagnosis of candidiasis assessed by microscopic examination for presence of budding yeasts or pseudohyphae in a drop of 10% KOH. One endocervical swab was required for combined *C. trachomatis* and *N. gonorrhoeae* polymerase chain reaction (PCR) (Cobas Amplicor, Roche Diagnostics, Basel, Switzerland). The CD_4 count was performed by flow cytometry using specific antibodies (Becton Dickinson), and stained samples analysed using a FacsCaliber instrument and the CELLQuest Software (Becton Dickinson).

Follow-up sample collection

The 74 women (41 HIV^+ and 33 HIV^-) followed up had two high vaginal swabs taken at subsequent visits as follows: with a non-lubricated speculum in place, sterile cotton swabs were consecutively inserted into the vaginal vault. Each swab was rotated against the lateral vaginal wall at the mid-portion of the vault and carefully removed to prevent contamination with the vulva and introitus microflora. The first swab was used to make a Gram stain. The second swab was carried to the laboratory in Amies transport medium (Nuova Aptaca, Canelli, Italy) for anaerobic culture which was performed at follicular (day 5-8) and ovulation (day 12-15) phases. Culture swabs were processed in the microbiology laboratory within 4 h of collection. Gram stain specimens were analysed for the composition of the vaginal microflora by microscopy according to the Nugent criteria (Nugent et al., 1991). An additional category of the gram stains known as grade 0 was included to represent the smears lacking bacteria cells.

Culture and identification of isolates

The swabs were streaked onto Columbia agar (Becton Dickinson) supplemented with 5% sheep blood (obtained freshly defibrinated from the Kabete Veterinary farm, University of Nairobi, Kenya) and incubated anaerobically (GasPak EZ Anaerobe Container System) at 37°C. After 4 days of incubation, all isolates with different colony morphology were selected for species identification. DNA was extracted by simple alkaline lysis as follows: each colony selected for its unique morphology was suspended in 20 µl sodium dodecyl sulfate (SDS) lysis buffer (0.25% sodiumdodecylsulphate in 0.05 N NaOH), heated at 95°C for 15 min and diluted with 180 µl double distilled water. The t-DNA-PCR and capillary electrophoresis were carried out as described previously (Baele et al., 2000; Baele et al., 2002). Species identification of culture isolates was achieved by comparing their respective t-DNA-PCR fingerprints with an existing t-DNA-PCR database using an in-house software program (Baele et al., 2002). The t-DNA-PCR fingerprint database and the software are available upon request to Laboratory for Bacteriology Research, Ghent University, Belgium, where the PCR work was carried out. Isolates that could not be identified in the existing t-DNA-PCR database were sequenced as earlier described (Verhelst et al., 2004).

Statistical analysis

Prevalence rates were compared between groups through Chi-square test or Fischer's Exact Test. Statistical significance was accepted at the significance level $\alpha=0.05$. All analyses were performed with statistical software package PASW v18.0 (Chicago, IL).

RESULTS

Cohort characteristics

Baseline demographic characteristics of 41 HIV^+ and 33 HIV^- women are presented in Table 1. All participants were literate and able to follow the appointment schedule. At enrolment, no significant difference existed in most parameters measured in the two groups. The HIV^+ women had lower CD_4 counts ($p<0.001$) and condom use was more common among them (61%) compared to the HIV^- women (18.2%) ($p=0.0002$). Intake of antibiotic prophylaxis was only in the HIV^+ group at 78% ($p<0.001$). The Kenya national guidelines for treatment and care for HIV persons allows continuous antibiotic intake for prevention of malaria and recurrent bacterial infections. Of the HIV^+ participants, 63.4% were married compared to 39.4% HIV^- ones ($p=0.001$). The HIV^+ women had lower levels of schooling ($p=0.005$).

Vaginal microflora and HIV status

Bacterial vaginosis was diagnosed in 17.5% enrolment visits. Table 2 shows the fluctuation in Gram stain score over two menstrual cycles. Respectively 42.4% of HIV^- and 31.7% of HIV^+ women harboured a normal vaginal microflora on all six visits. As shown in Table 3, the number of vaginal swabs with grades I, II and III was similar throughout the phases of the menstrual cycle in both study groups.

Presence of bacterial species according to HIV status

Species cultured and identified in the HIV^+ and HIV^- women are presented in Table 4 and Figure 1. In total, 1020 isolates were cultured. Of these, 26% of the isolates remained unidentified either because no corresponding t-DNA-PCR fingerprint was found in the existing database or no amplification obtained. In total, 51 species were identified, nine of which belonged to the genus *Lactobacillus,* that is, *L. coleohominis, L. crispatus, L. gasseri, L. iners, L. jensenii, L. mucosae, L. reuteri, L. salivarius* and *L. vaginalis*. In descending order, the most common lactobacilli were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. Respectively, 54.5% of HIV^- and 60.9% of HIV^+ women were colonized by lactobacilli at least one visit, and 9.1 and 29.3% at least three visits. *Lactobacillus crispatus* was cultured on three out of four

Table 1. Descriptive characteristics of the study population at enrolment, expressed as percentages.

Parameter	Category	HIV$^+$ (41)	HIV$^-$ (33)	*p* value
Age (years)	21-28	14.6	24.2	0.4
	29-36	53.7	39.4	
	37-44	31.7	36.4	
CD_4 count (cells/µL)	250-500	56.1	3.0	< 0.001
	501-750	31.7	15.2	
	751-1000	7.3	54.5	
	1001-1500	4.9	27.3	
Vaginal pH	3.0-4.5	73.2	63.6	0.4
	> 4.5	26.8	36.4	
Candida	Positive	36.6	27.3	0.4
	Negative	63.4	72.7	
Gram stain	Grade 0	9.8	6.1	0.7
	Grade I	56.1	72.7	
	Grade II	9.8	6.1	
	Grade III	22.0	12.2	
	No score	2.4	3.0	
Antibiotic prophylaxis	Yes	78.0	0.0	<0.001
	No	22.0	100.00	
Systolic Blood Pressure (mm Hg)	90-110	34.1	30.3	0.8
	111-130	51.2	48.5	
	131-150	12.2	15.2	
	151-170	2.4	3.0	
	> 170	0.0	3.0	
Diastolic Blood Pressure (mm Hg)	60-69	12.2	15.2	0.8
	70-79	43.9	36.4	
	80-89	29.3	27.3	
	90-99	14.6	18.2	
	> 100	0.0	3.0	
Pulse (beats/min)	< 60	0.0	3.0	0.3
	60-69	9.8	24.2	
	70-79	39.0	30.3	
	80-89	36.6	33.3	
	90-99	14.6	6.10	
	> 100	0	3.0	
Height (cm)	130-144	2.4	0.0	0.4
	145-159	46.3	30.3	
	160-174	48.8	66.7	
	> 175	2.4	3.0	
Body weight (kg)	45-54	26.9	18.2	0.3
	55-64	26.9	36.4	
	65-74	31.7	18.2	
	75-84	12.2	15.2	
	85-94	0	9.1	
	> 95	2.4	3.0	

Table 1. Contd.

Parameter	Category	HIV$^+$ (41)	HIV$^-$ (33)	*p* value
Age at first sex (years)	14-15	9.8	6.1	0.7
	16-18	43.9	36.4	
	19-23	31.7	33.3	
	24-28	12.2	18.2	
	> 29	0	3.0	
	N/A	2.4	3.0	
Lifetime partners	0-1	19.5	27.3	0.3
	2-3	48.8	42.4	
	4-7	24.4	30.3	
	8-10	7.3	0.0	
Current partners	0	24.4	30.3	0.6
	1	75.6	69.7	
Marital status	Married	63.4	39.4	0.001
	Single/Separated	22.0	60.6	
	Widow	14.6	0	
Condom use	Yes	61.0	18.2	<0.001
	No	39.0	81.8	
Level of schooling	Primary and below	46.3	12.2	0.005
	Secondary	51.2	78.8	
	Tertiary	2.4	9.1	
Occupation	Housewife	36.6	30.3	0.2
	Farmer	14.6	6.10	
	Business	24.4	15.2	
	Formal employment	24.4	42.4	
	Student	0	6.10	

Table 2. Percentages of the fluctuation of the Gram stain scores of HIV$^-$ and HIV$^+$ women taken at six visits each, spanning two menstrual cycles.

Parameter	HIV $^-$		HIV $^+$	
Condom use (total number)	No (27)	Yes (6)	No (17)	Yes (24)
Invariably normal	33.3	83.3	47.1	20.8
Single intermediate or BV episode	11.1	16.7	11.8	29.2
Two intermediate or BV episodes	14.8	0	5.8	0
Three or more intermediate or BV episodes	29.6	0	17.6	37.5
Invariably intermediate or BV episode	11.1	0	17.6	12.5

Table 3. Distribution of Nugent scored vaginal microflora grades among HIV$^+$ and HIV$^-$ women during different phases of two menstrual cycles. Percentages in parentheses.

Grade	HIV $^+$ (n = 41)			HIV $^-$ (n = 33)		
	Follicular	Ovulation	Luteal	Follicular	Ovulation	Luteal
0	2 (2.4)	4 (4.9)	0 (0.0)	1 (1.5)	1 (1.5)	0 (0.0)
I	42 (51.2)	49 (59.8)	53 (64.6)	41 (62.1)	45 (68.2)	44 (66.7)
II	18 (22.0)	12 (14.6)	15 (18.3)	11 (16.7)	8 (12.1)	9 (13.6)
III	17 (20.7)	16 (19.5)	14 (17.1)	11 (16.7)	12 (18.2)	11 (16.7)
Missing	3 (3.7)	1 (1.2)	0 (0.0)	2 (3.0)	0 (0.0)	2 (3.0)
Total	82	82	82	66	66	66

Table 4. Percentage of 250 visits during which 44 HIV$^+$ and 33 HIV$^-$ women with a positive culture.

Species	HIV$^+$women[a]	HIV$^-$ women[b]	P value
Acinetobacter haemolyticus	1.4	0	0.2
Acinetobacter lwoffii	0.7	0	0.3
Anaerococcus prevotii	2.1	4.6	0.2
Anaerococcus tetradius	0.7	0	0.3
Anaerococcus vaginalis	0.7	0.9	0.4
Atopobium vaginae	0	0.9	0.2
Bacteroides coagulans	0.7	0	0.3
Bacteroides ureolyticus	0.7	0.9	0.4
Clostridia bacterium	0.7	0	0.3
Dialister micraerophilus	0	3.7	0.02
Enterococcus faecalis	7.7	14.8	0.07
Escherichia coli	9.2	10.2	0.7
Finegoldia magna	16.2	22.2	0.2
Gardnerella vaginalis	12	3.7	0.01
Klebsiella pneumoniae	0.7	0	0.3
Lactobacillus coleohominis	0	1.9	0.09
Lactobacillus crispatus	11.3	14.8	0.4
Lactobacillus gasseri	1.4	1.9	0.7
Lactobacillus iners	15.5	6.5	0.02
Lactobacillus jensenii	13.4	4.6	0.01
Lactobacillus mucosae	0.7	0	0.3
Lactobacillus reuteri	0	1.9	0.09
Lactobacillus salivarius	2.8	0	0.07
Lactobacillus vaginalis	7.7	4.6	0.3
Mobiluncus curtisii	0.7	0	0.3
Peptoniphilus asaccharolyticus	27.5	29.6	0.7
Peptoniphilus lacrimalis	7	0.9	0.01
Peptoniphilus sp.	0.7	2.8	0.1
Peptostreptococcus anaerobius	13.4	14.8	0.7
Peptostreptococcus hydrogenalis	0.7	0	0.3
Peptostreptococcus indolicus	0	0.9	0.2
Porphyromonas somerae	0.7	1.9	0.3
Porphyromonas sp.	0	0.9	0.2
Prevotella bivia	12.7	13	0.9
Prevotella buccalis	3.5	0.9	0.1
Prevotella corporis	0.7	2.8	0.1
Prevotella disiens	2.1	0	0.1
Prevotella timonensis	0.7	1.9	0.3
Propionibacterium acnes	0.7	0	0.3
Pseudomonas mendocina	0	0.9	0.2
Staphylococcus aureus	0.7	1.9	0.3
Staphylococcs epidermidis	17.6	19.4	0.7
Staphylococcus hominis	1.4	0	0.2
Streptococcus agalactiae	4.9	12	0.04
Streptococcus anginosus	9.9	9.3	0.8
Streptococcus mitis	2.1	3.7	0.4
Streptococcus salivarius	0.7	0	0.3
Streptococcus sp.	0.7	0	0.3
Ureaplasma parvum	0.7	0	0.3
Veillonella atypica	5.6	11.1	0.1
Veillonella parvula	0.7	0	0.3

[a]142 visits; [b]108 visits.

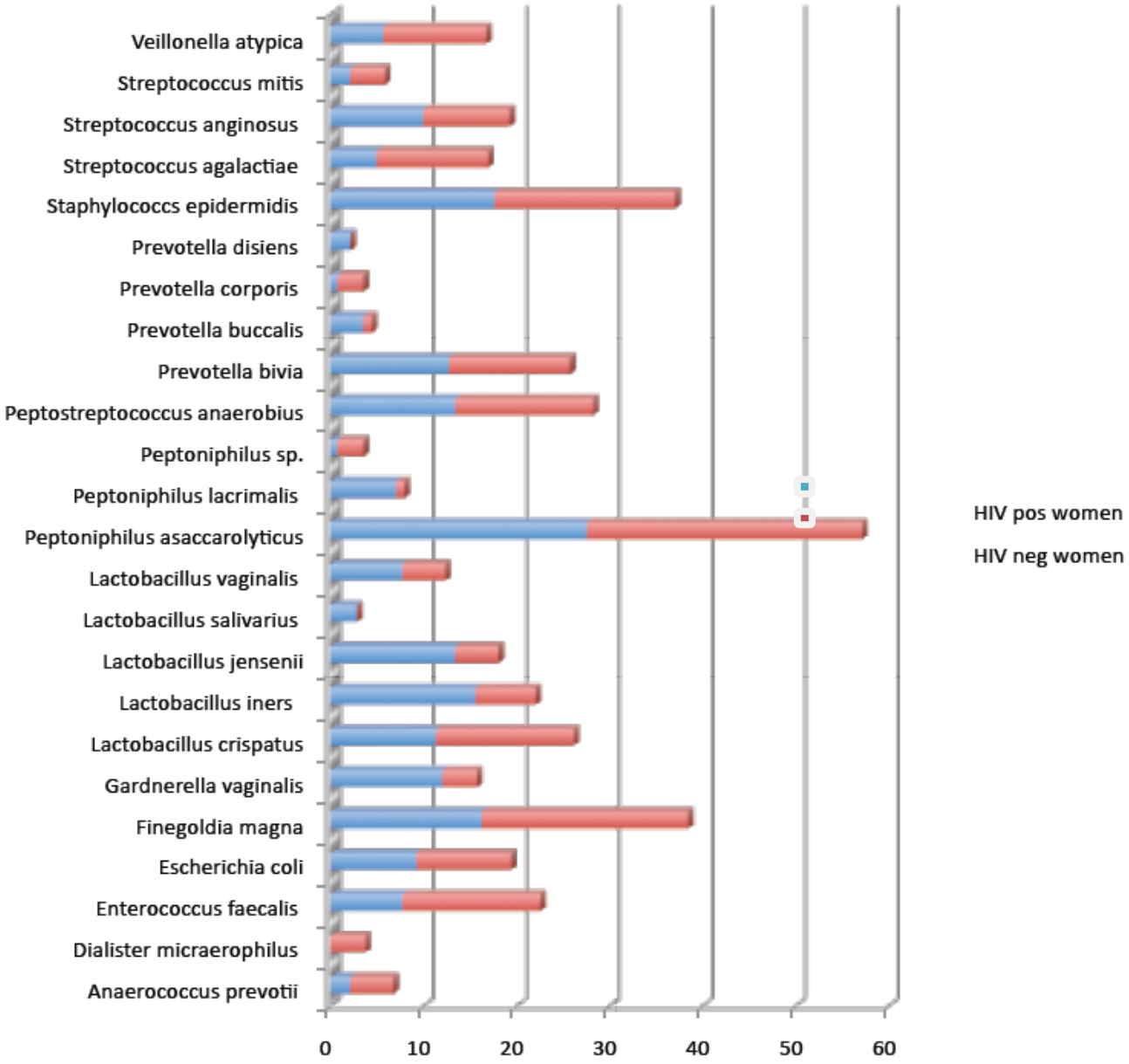

Figure 1. Bacterial species present in more than 2% of the 74 women studied.

visits for two women and it occurred twice in five women and once in 11 women. Lactobacillus iners was cultured on three visits for two women, twice in two women and only once in 13 women. Lactobacillus jensenii was recovered on three out of four visits in five women, on two visits in two women and one visit in four women. Two species occurred more frequently in the HIV+ women compared to HIV- women: L. iners (15.5% vs 6.5%; p=0.02) and L. jensenii (13.4% vs 4.6%; p=0.01). G. vaginalis and P. lacrimalis occurred more frequently in HIV^+ women (respectively 12% vs 3.7% and 7% vs 0.9%; p=0.01). *D. micraerophilus* was found more frequently in the HIV^- group (3.7% vs 0%; p=0.02) as was *S. agalactiae* (12% vs 4.9%; p=0.04).

DISCUSSION

The objective of this study was to compare vaginal microflora of HIV^+ and HIV^- Kenyan women of reproductive age by means of culture and Nugent scoring of Gram stains. There were existing differences in the species present in the two groups of women. This confirms findings of Spear et al. (2008) who identified a trend towards increased diversity in HIV^+ women, suggesting that HIV infection may be associated with

changes in the diversity of genital microflora.

Presence of BV has been associated with increased susceptibility to acquisition of STIs (Martin et al., 1999; Sewankambo et al., 1997; Kaul et al., 2007). Since BV increases the risk of HIV acquisition, it would be expected that higher rates of BV be confirmed among HIV^+ women. However, in this study, no significant difference was found in the rates of BV in HIV^+ and HIV^- women. This agrees with findings of Demba et al. (2005) and Watts et al. (2006), but is in contradiction to observation of other researchers (Jamieson et al., 2001; Sewankambo et al., 1997; Warren et al., 2001) who found prevalence of BV to be higher among HIV-infected women compared to HIV-uninfected women.

Possible explanation for the lack of correlation between the prevalence of BV and HIV status observed in the current study may be due to differences in antibiotic intake between HIV^+ and HIV^- women. The imbalance of vaginal microflora in this HIV^+ group may further be explained by the fact that 78% of them were on co-trimoxazole (trimethoprim-sulfamethoxazole) prophylaxis. Although this antibiotic has been shown to reduce mortality of HIV patients and to have a beneficial effect by decreasing the rate of CD_4 cell depletion (Mermin et al., 2004), use of antibiotics decimates H_2O_2-producing lactobacilli in the vagina (Vallor et al., 2001; Hummelen et al., 2010). Co-trimoxazole is the prophylaxis antibiotic approved by the Kenya Ministry of Health (2007) and is readily available to HIV^+ persons in health centers. It is used similarly in other sub-Saharan African countries (Hamer and Gill, 2008). Since the HIV^+ women continue to have more lactobacilli despite continuous intake of co-trimoxazole, it is suggested that resistance to this antibiotic may have been attained. A previous study found that women with BV have decreased colonization rates for H_2O_2-producing strains of lactobacilli and increased colonization rates for non-H_2O_2-producing strains (Onderdonk et al., 2003; Ferris et al., 2007). This observation is supported by the present findings that *L. iners, G. vaginalis* and *P. lacrimalis* occurred more frequently in HIV^+ women regularly using antibiotics.

Some studies found infection with HIV-1 to be associated with abnormal vaginal microflora. Sewankambo et al. (1997) who studied 4718 women in a community survey in rural Uganda, concluded that loss of lactobacilli or presence of BV may increase susceptibility to acquisition of HIV-1. Jamieson et al. (2001) and Warren et al. (2001) while studying different aspects of a comparable high-risk cohort of 1288 women in the USA, found HIV infection to be positively correlated with presence of BV. However, they could not determine whether HIV-infected women have a higher incidence of BV or more persistent infections. Participants of the said study were not excluded on account of low CD_4 counts or other STIs like in the current one. Jamieson et al. (2001) found that immunocompromised women (CD_4 cell count < 200 cells/µL) were more likely to have prevalent and persistent BV than HIV-infected women with higher CD_4 cell counts (>500 cells/µL), but not more likely to have incident infections. This could be explained by the fact that the immunocompromised women were probably on HAART and the accompanying cotrimoxazole prophylaxis (Hamer and Gill, 2008; Watts et al., 2006). In the current cohort, none of the 74 women were immunocompromised or on ART, as illustrated by the fact that, of the 41 HIV^+ women, 56.1% had CD_4 cell count in the range 250-500 cells/µL and the rest in the range 501-1500 cells/µL.

Further to the differences in immune status and therapeutic intervention between the two study groups, differences in sexual dynamics may also play a role. At the time of the study, most of the HIV-infected women had one (75.6%) or no (24.4%) sexual partners while 63.4% were married. This may partly explain why no differences were detected in the prevalence of BV between the HIV- infected and uninfected subjects. Confounders not measured but varying between populations may also contribute to the difference in findings. In the current study more HIV^+ women were married, which may imply that marriage increased the risk of exposure to HIV infection in this population. This finding is consistent with data obtained by the Kenya Aids Indicator Survey (KAIS, 2007). Compared to the HIV^- group, the HIV^+ women had lower levels of schooling, which may contribute to the observed lower rate of formal employment of HIV^+ women (24.4% compared to 42.4% HIV^- women). A previous study in Kenya (Steele et al., 2004) found genital hygiene practices to be associated with resource access factors such as higher education and income. Further, a more recent study clearly shows low socio-economic status to be associated with prevalence of BV in African American women (Allsworth and Peipert, 2007). The presence of BV is believed to predispose women to acquisition of HIV-1 (Sewankambo et al., 1997), whereas the psychosocial stress created by being HIV infected could predispose to acquisition of BV (Nansel et al., 2005), thus creating a complex inter-relationship between the two infections.

In agreement with other culture-based studies of African populations (Martin et al., 1999; McClelland et al., 2009), the concentrations of vaginal *Lactobacillus* species were comparatively low, and prevalence of abnormal vaginal microflora and BV-associated species high in the current study group. High rates of BV were previously observed in cohorts of African women (Sewankambo et al., 1997; Taha et al., 1998) and in Kenya (Bukusi et al., 2006; McClelland et al., 2009). It appears, therefore, that African women have lower concentration of lactobacilli in their vaginal microflora, which may explain the high rates of BV in this population. Reasons for this are unclear, although vaginal hygiene practices and sexual behaviour may play a role. No significant effect of the menstrual cycle on the prevalence of BV was observed. Earlier studies found quantities of lactobacilli to decrease during menstruation (Keane et al., 1997) and vaginal microflora to be more unstable during the first week (Morison et al., 2005). The current study

found almost equal numbers of grade I to III samples evenly spread out in the three sampling points at follicular (day 5-8), ovulation (day 12-15) and luteal (day 19-22) phases. This difference in observations may be explained by the varying time frame of sample collection in the current study. Keane et al. (1997) had a daily collection of the vaginal swabs while Morison et al. (2005) collected a sample on alternate days. As recent report by Brotman et al. (2010) showed that episodes of BV may be missed if specimens are collected at weekly or monthly intervals since fluctuations occur more frequently, and in some episodes remission may occur without intervention. However the current study had the strength of having accounted for most known behavioural confounders commonly associated with increased dynamism of the vaginal microflora (such as age, douching, contraceptive use, vaginal lubricants, irregular condom use, STIs and multiple partners) (Schwebke et al., 1999). This makes the observation of almost equal numbers of grade I to III samples evenly spread out in the three sampling points of the menstrual cycle more reliable.

Demba et al. (2005), also using culture methods, found *Prevotella* and *Bacteroides* species to occur more frequently in Nigerian HIV^+ women. The current study found these species to be equally distributed among both HIV^+ and HIV^- groups. Other bacteria isolated in the vaginas of the participants that are not associated with normal vaginal microflora include the streptococci. *S. agalactiae* was most common in the HIV^-group. Most of the other species identified occurred at more or less the same frequency in the two groups, and in other cases where a species was altogether absent in one group, statistical significance in the differences was not attained.

The finding that the most abundant *Lactobacillus* species in the grade I category were *L. iners* (17.1%), *L. crispatus* (16.4%), *L. jensenii* (12.5%) and *L. vaginalis* (9.2%), in that order, are largely in agreement with previous studies of African women. In Nigeria, using denaturing gradient gel electrophoresis, one group (Anukam et al., 2006) found *L. iners* and *L. gasseri* to be the predominant species while in Uganda others (Jin et al., 2007) found *L. reuteri*, *L. crispatus*, *L. vaginalis* and *L. jensenii* to be most abundant using culture and PCR identification as in the current study. A more recent study conducted in Kenya (Matu et al., 2009) found *L. jensenii* to be the most abundant species using culture and phenotypic identification methods. In Belgian women, *L. iners* is more dominant in altered vaginal microflora (De Backer et al., 2007). One group (Anukam et al., 2005; Anukam et al., 2006) did not however detect lactobacilli in BV cases whereas in the current study, of the nine *Lactobacillus* species detected, eight of them were present in the non-grade I samples. The causes of racial difference in concentrations of lactobacilli remain unknown. A highly intriguing observation came from a study of Nigerian women (Anukam et al., 2006) that found only 3% of the healthy vaginal microflora to be dominated by *L. crispatus*, while *L. iners* was present in 64%. Overall African women tend to have lower concentrations of lactobacilli (McClelland et al., 2009). This observation may be a possible explanation for the high prevalence of BV among African women (Allsworth and Peipert, 2007; Bukusi et al., 2006) since it is known that most *L. iners* strains are very weak H_2O_2 producers. In contrast, *L. crispatus* is known to produce large amounts of H_2O_2 and as such provides better colonization resistance (Antonio et al., 1999; Vallor et al., 2001; Verstraelen et al., 2009; Verstraelen et al., 2010).

Low concentrations of lactobacilli in Kenyan women have been observed by other researchers (McClelland et al., 2009). Further, among 185 and 241 Nigerian women respectively, two studies found 12 and 10 *Lactobacillus* spp. to be present (Anukam et al., 2005; Anukam et al., 2006). An earlier study comparing Korean and Ugandan women found that vaginal microflora may vary in women of different geographical communities (Jin et al., 2007). In a US study African American women with BV were found to be predominantly colonized by *Mobiluncus* spp., based on gram staining (Royce et al., 1999). Researchers in Nigeria (Anukam et al., 2006) and The Gambia (Demba et al., 2005) found *Mycoplasma hominis* to be commonly associated with BV. In the present study *Mobiluncus* spp. was cultured in only one subject in the non-grade I category despite the observation of *Mobiluncus*–like organisms in the vaginal Gram-stained smears. *M. hominis* was not cultured at all. Although this could point to culture bias, the Demba group (Demba et al., 2005)also failed to isolate *Mobiluncus* in the Nigerian population of 277 women using a 72 h incubation protocol. Like their group, the current study used Columbia blood agar and longer incubations of 96 h.

Some species identified in greater proportions in either the HIV^+ or HIV^- women did not attain statistical significance due to small sample size. Studies of this nature conducted on a larger sample size will help to draw conclusions on other types of microflora predominant in HIV^+ and HIV^- women, thus enabling more specific approaches to probiotic therapy. Probiotic therapy may need to be geographically determined in order to take care of differences in the species and populations of *Lactobacillus*. Treating BV in HIV^+ women would subsequently reduce the risk of mother-to-child-transmission.

Conclusions

The current data provide evidence that differences exist in the species that colonize the vaginas of HIV^+ and HIV^- women in the course of the normal menstrual cycle. BV was equally high in HIV^+ and HIV^- women. *Lactobacillus* and BV-associated species were cultured more frequently in HIV^+ women. Predominant *Lactobacillus* in culture were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. These Kenyan women had lower concentrations of lactobacilli in vaginal microflora than observed

in previous studies of Caucasian women.

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Full Length Research Paper

Characterization of blue green algae isolated from Egyptian rice field with potential anti-hepatitis C active components

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Several species of cyanobacteria has been recognized for its therapeutic value that can be used for treatment of malnutrition, cancer and viral infection. Many natural occurring cyanobacteria are known to produce toxins, for example, species of the genera *Microcystis, Nodularia, Nostoc, Anabaena, Aphanizomenon, Cylindrospermopsis, and Planktothrix* (*Oscillatoria*). Cyanotoxins are classified according to their mode of action in vertebrates as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritants. Microcystin is a hepatotoxin which commonly found in *Microcystis* and it was found to be produced by other genera, including *Anabaena, Nostoc, Nodularia*, and *Planktothrix*. In the present study cyanobacteria strain isolated from Egyptian soil was purified, characterized and identified as *Nostoc* sp. and named *Nostoc EGY*. PCR-based techniques targeting the toxin biosynthesis genes were used verifying absence of toxic genes in the newly purified cyanobacteria. Cell lysate was prepared from the purified strain; the efficacy of this lysate to prevent hepatitis C virus (HCV) replication in vitro was proved qualitatively and quantitatively. Lysate prepared from isolated cyanobacteria after 10 and 25 days of cultivation was able to prevent replication of *in vitro* cultivated HCV.

Key words: Hepatitis C, green algae, cyanobacteria, polymerase chain reaction.

INTRODUCTION

Cyanobacteria as one of the oldest fossils on earth, more than 3.5 billion years, they are aquatic and photosynthetic unicellular microorganisms (Botos and Wlodawer, 2003; Laura G. Barrientos, 2008). Cyanobacteria are growing in a wide range of habitats and were named for the blue-green pigment, phycocyanin. Usually, they grow in large colonies include unicellular, colonial, and filamentous forms and can be considered as one of the largest and most important groups of bacteria on earth. Cyanobacteria that lack toxins are widely used as food supplement as well as in complementary and alternative medicine (Gao, 1998; Liu Chenglong, 2009). Several

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Species of cyanobacteria has been recognized for its therapeutic value that can be used for treatment of malnutrition, cancer and viral infection due to presence of medicinally active component that varied according to cyanobacteria species (Helle et al., 2006). For example CV-N is an 11 kDa protein of known three-dimensional structure produced mainly by *Nostoc* strains of cyanobacteria, where CV-N blocks HIV infection by binding to the surface envelope glycoprotein, gp120. Further study of the mechanism of CV-N/gp120 interaction revealed that CV-N bound to high-mannose oligosaccharides on gp120, specifically, oligomannose-8 (Man-8) and oligomannose-9 (Man-9). Moreover CV-N was proved to be active against certain other viruses, such as the Ebola virus and HCV, whose envelope proteins possess similar oligosaccharide structures. Therefore in HCV infection, CV-N as mannose binding protein has the ability to bind to hepatitis C (HCV) viral particle where HCV envelop as mannose rich protein and prevent its entry to the host cells, consequently prevent viral replication. Also the same action by CV-N is applicable with other viruses with envelope similar to that of HCV viral particles (Buffa et al., 2009; Liu et al., 2009; O'Keefe et al., 2003; Tsai et al., 2004).

Many natural occurring cyanobacteria are known to produce toxins, for example, species of the genera *Microcystis, Nodularia, Nostoc, Anabaena, Aphanizomenon, Cylindrospermopsis, and Planktothrix* (*Oscillatoria*) (Haider et al., 2003; O.M. Skulberge et al., 1993; Rao et al., 2002) Cyanotoxins are classified according to their mode of action in vertebrates as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritants (Carmichael et al., 2001). Microcystin is a hepatotoxin which commonly found in *Microcystis* and it was found to be produced by other genera, including *Anabaena, Nostoc, Nodularia*, and *Planktothrix*. It is a cyclic heptapeptide produced non-ribosomally by a multifunctional enzyme complex consisting of peptide synthetase (PS)-polyketide synthase (PKS) modules and tailoring enzymes, they act by inhibiting protein phosphatase 1 and 2 (PP1 and PP2A) (Goldberg et al., 1995; MacKintosh et al., 1990).

PCR-based techniques were developed for targeting the toxin biosynthesis genes and they have become popular methods for detecting and quantifying toxic species due to their specificity, sensitivity, and speed (Dittmann and Wiegand, 2006; Furukawa et al., 2006; Ouellette et al., 2006). Characterization of the genes responsible for hepatotoxin biosynthesis has made it possible to design general PCR primers that target the aminotransferase (AMT) domains of the *mycE* and *ndaF* genes (Jungblut et al., 2006) allowing detection of microcystin and nodularin-producing cyanobacteria.

MATERIALS AND METHODS

Isolation and cultivation of blue green algae

Blue green algae had been isolated from soil of Rice field in river Nile Delta. The isolation of a single algal unit into medium for growth is required to establish a clonal, unialgal culture by streak plating on BG12 or BG13 media. This method serves to isolate an algal unit less than 10 µm diameter (Ferris and Hirsch, 1991). Plates were incubated at 30°C for 4 to 8 days under suitable growth conditions (light cycle). Using stereomicroscope the desired colonies that are free of other microorganism were selected for further isolation. The purity of the selected colonies was determined by examining samples from each selected colony using a fine wire needle and examined with a high power objective of the compound microscope. The pure colonies were transferred into liquid or agar media (BG12 or BG13) (Ferris and Hirsch, 1991).

The purified colonies of blue green algae were cultivated in a BG12 sterile modified medium (Elango, 2008); the algae were grown in a batch culture at 30°C illuminated by cool white lamp. The cells were allowed to grow for 10 days to density equal to 32 mg/ml and were harvested by cooling centrifugation; the pellets were stored at -20°C.

Light and scanning electron microscopy

The fresh unialgal culture from the isolated cyanobacteria was examined with an Olympus BX 40 Phase contrast light microscope. A part from the fresh culture was dried; the specimen was sputter-coated with gold and examined with a Joel scanning electron microscope (SEM, JSM-6360; Joel, Japan) at 10 kV. The morphological identification of cyanobacteria was done according to Komárek and Anagnostidis (Komárek, 1998; 2005).

Identification and amplification of 16S rDNA and ITS-23S rDNA genes

DNA was isolated from the samples as described by Tillett and Neilan (2000) and quantified using UV spectrophotometer (NanoDrop Technologies, Thermo Scientific, USA). All PCR was performed using 0.2 U of Dream Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, USA) in a 20 ml reaction. The PCR mixture contained 1x Taq polymerase buffer, 0.5 pmol of forward and reverse primers (Table 1), 0.2 mM dNTPs, and 100 ng template DNA. For the molecular identification of the cyanobacteria, 16S rDNA oligonucleotide primers, CYA106F-CYA781R (Nubel et al., 1997) and ITS-23S rDNA (Tillett et al., 2000) P322-P340, (Iteman et al., 2000) were used and synthesized by (Bioneer, Korea). PCR reactions were performed as previously described for each primer pair (Al-Tebrineh et al., 2011; Iteman et al., 2000; Jungblut et al., 2006; Nubel et al., 1997).

PCR amplification of HEP genes

The HEP-F and HEP-R oligonucleotide primers targeting the aminotransferase (AMT) domain of the hepatotoxin synthetase *mcyE* and *ndaF* genes (Bioneer, Korea) were used for determining whether that toxicity gene is present or absent in the isolated bacteria (Amer et al., 2009; Jungblut et al., 2006). The AMT primers HEP-F and HEP-R (Table 1) were used to amplify the 472 bp PCR product. DNA isolated from EGY isolate and positive control DNA isolated from toxin producing *Nostoc* sp were used as template for PCR amplification using this primers. The initial temperature of 94°C for 2 min, followed by 35 cycles at 92°C for 50 s, 52°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min.

Preparation of blue green algae (BGA) lysate

Cultivated cells of EGY strain were collected by centrifugation at

Table 1. List of primers used for PCR and sequencing in this study.

Primer ID	Primer sequence 5'-3'	Target	Reference
CYA106F	CGGACGGGTGAGTAACGCGTGA	16S rDNA	(Nubel et al., 1997)
CYA781R	GACTACTGGGGTATCTAATCCC(A/T)TT	16S rDNA	(Nubel et al., 1997)
P322F	TGTACACACCGCCCGTC	End of 16S rDNA	(Iteman et al., 2000)
P340R	CTCTGTGTGCCTAGGTATCC	23S rDNA	(Iteman et al., 2000)
HEPF	TTTGGGGTTAACTTTTTTGGGCATAGTC	mcyE/ndaF	(Jungblut et al., 2006)
HEPR	AATTCTTGAGGCTGTAAATCGGGTTT	mcyE/ndaF	(Jungblut et al., 2006)

5000 rpm for 15 min at 4°C followed by lypholization. Lyophilized cells were broken down into powder using liquid nitrogen (10 times volume of the dried cells) the resulted powder were dissolved in normal saline with EDTA free protease inhibitor cocktail (Roche, USA) at 20 mg/ml, sterilized by filtration to be ready for testing. In order to find out relation between the cell density/suitable cultivation time and anti-HCV activity, BGA lysate was also prepared from cultivated EGY cells and collected at time intervals from 5 up to 60 days. Cell density and dry cell weight were determined and anti HCV activity was determined.

Anti HCV activity

During the last few years, a number of cell culture systems showed to have the ability to harbor and support reliable and efficient progression of this virus. Among several human hepatocyte cell lines analyzed, the hepatocellular carcinoma HepG2 cell line was found to be most susceptible to the HCV infection (El-Awady et al., 2006). Also, human peripheral blood mononuclear cells (PBMC) showed the ability to harbor the replication of HCV *in vitro* and also HCV viral particles was reported to be harbored inside the PBMC of the HCV infected patients (el-Awady et al., 2005).

On the other hand, monitoring of the HCV viremia pre- and post-antiviral therapy through the detection of viral (+) and/or (-) RNA strands by the use of qualitative reverse transcription-polymerase chain reaction (RT-PCR) has become the most frequently-used, reliable and sensitive technique. Recently, it has been reported that the detection of the (-) strand HCV-RNA using the RT-PCR is a very important tool for understanding the life cycle of the HCV and provides a reliable marker for the diagnosis of HCV and monitoring the viral response to antiviral therapy (El-Awady et al., 1999).

Neutral red uptake assay to measure cytotoxicity on human cells

Peripheral blood lymphocytes (PBMC) were separated from whole blood of healthy volunteer using gradient separation by Ficoll-Paque™ Plus (MP Biomedicals, France), $10X10^4$ lymphocyte cells were seeded per well in 96 well plates and incubated in culture media (CM, RPMI1640 (Loza) media supplemented with 200 µM L-glutamine (Lonza), 25 µM HEPES buffer; N- [2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (lonza) and 10% Fetal bovine serum, FBS, Lonza) containing different concentrations of the BGA lysate for 24, 48, 72 and 96 h. The fraction of viable lymphocyte cells was measured by the Neutral Red assay (Guillermo Repetto, 2008). The neutral red assay is based on the initial protocol described by Borenfreund and Puerner (Borenfreund and Puerner, 1984) and determines the accumulation of the neutral red dye in the lysosomes of viable cells (Fotakis and Timbrell, 2006). Following exposure of isolated PBMC to different concentrations of BGA lysate, cells were incubated for 3 h with neutral red dye (40 µg/ml) dissolved in culture media RPMI. Cells were then washed with Phosphate Buffered Saline (PBS) and the addition of 1 ml of elution medium (50% ethanol /1% glacial acetic acid /49% water) followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to 96-well plates and absorbance at 490 nm was recorded using microtiter plate reader spectrophotometer (Biotek, USA). IC50 was estimated by plot x-y and fit the data with a straight line (linear regression) using IC50 was determined using Prism statistics software program.

Detection of the effect of the BGA lysate on HCV cultivated in human cancer cell line

Human cancer cell line, HepG2 cells, were washed twice in RPMI1640 (Loza) media supplemented with 200 µM L-glutamine (Lonza) and 25 µM HEPES buffer; N- [2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (lonza) and were suspended at 2×10^5cells ml^{-1}in RPMI CM. The cells were left to adhere on the polystyrene 6-well plates for 24 h in 37°C, 5% CO_2, 95% humidity incubator. After 24 h the cells were washed twice from debris and dead cells by using RPMI supplemented media. Cells were infected by incubation for 24 h with 4% HCV infected serum, followed by addition of culture media and depending on th value of the determined IC50, different concentrations of BGA (1, 10, 160, 1, 1.6 µg/ml) were added. Positive (HCV infected cells) and negative control (uninfected cells) cultures were included. Cultures were incubated for 96 h in 37°C, 5% CO_2, 95% humidity. The tested BGA concentration is considered to be active when it is capable of inhibiting the viral replication inside the HCV-infected HepG2 cells, as evidenced by the disappearance of the viral RNA-amplified products detected by the RT-PCR (compared with the positive control).

RNA extraction and RT-PCR of HCV RNA

Total RNA was extracted from HCV-infected HepG2 cells using the method described by El-Awady et al. (El-Awady et al., 1999). Briefly, culture cells were collected in 200 µl of Solution D (4 M guanidinium isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β- mercaptoethanol, 100 µl sodium acetate). The cell lysate was mixed with an equal volume of RNA phenol, chloroform and isoamyl alcohol mixture (pH 4, Fisher Scientific, USA), mixing vigorously by vortex, followed by centrifugation at 14 K rpm for 10 min at 4°C. The aqueous layer was collected and mixed with an equal volume of isopropanol then incubated overnight at -20°C. RNA was collected by centrifugation at 14 K rpm for 30 min at 4°C followed by washing twice with 70% ethanol then suspended in RNase free water. Concentration and purity (260/280 nm ratio) was determined using UV spectrophotometer. The complimentary DNA (cDNA) and the first PCR reaction of the

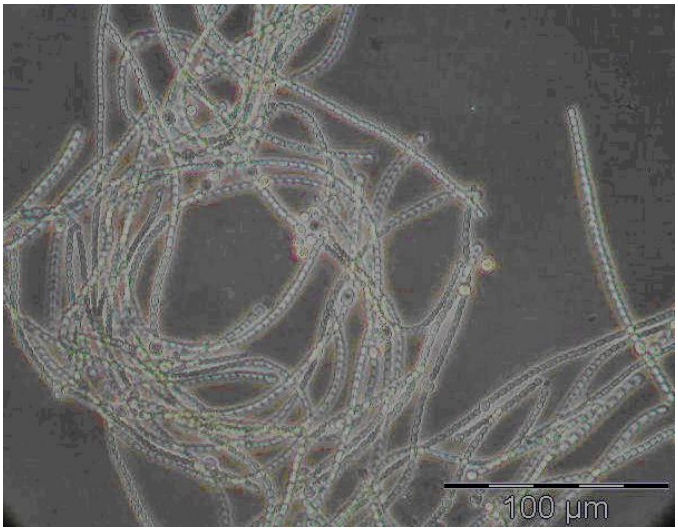

Figure 1. Ligth microscope filed showing the obtained pure culture EGY of isolate with magnification of 100X.

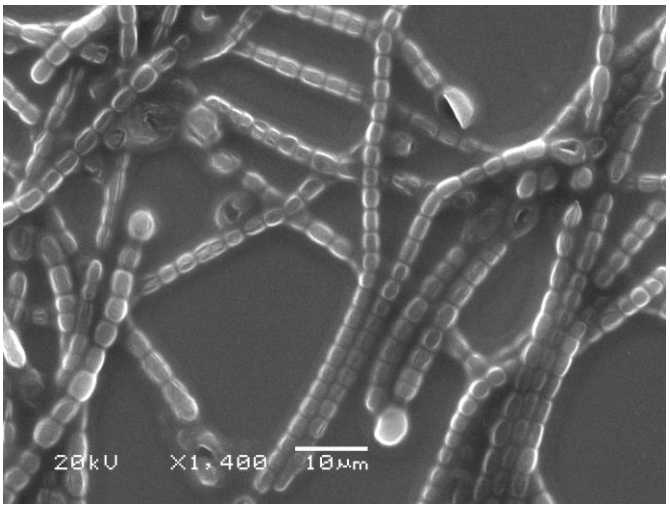

Figure 2. Scanning electron microscope for EGY by magnification 400 X.

nested PCR detection system for the HCV RNA was performed in a 50 µL volume single-step reaction using the Ready-To-Go RT-PCR beads (GE Healthcare, USA) followed the detailed protocol described by El Awady et al, 2002 (El Awady et al., 2002). The Final nested PCR product was detected using 2% agarose gel electrophoresis.

Quanititative *in-vitro* anti-HCV screening

Same BGA concentrations were examined for its anti HCV activity quantitatively using same culture system used for qualitative test and analytikjena HCV viral RNA extraction kit and Robogen HCV quantitative kit (Bometera, Germany)

RESULTS

Isolation and microscopic examination

The unialgal culture was obtained after primary and secondary screening on BG12 media plates and broth. The pure isolate EGY was examined for its morphological characterization under the light microscope (Figure 1) going to scanning electron microscope (SEM) (Figure 2). The figures showed that the isolated cyanobacterium was filamentous with the presence of hetercysts and akinetes that may belongs to group Nostocales.

Molecular identification of EGY by amplification of 16S rDNA gene

The DNA was extracted from EGY by the method described by Tillett and Neilan 2000. The cyanobacterial universal primers CYA106F-CYA781R were used for the identification of the pure isolate EGY. The obtained 16S rDNA PCR product was amplified and partially sequenced and analyzed by Bioneer Company, Korea. The resulting phylogenetic reconstructions were shown in Figure 3 on the basis of neighbor-joining analysis. The data showed in Figure 3 ensure the results of the microscopic examination that the EGY belongs to family Nostocales. It showed highest similarity to 91% to *Nostoc* sp. PCC 9426. The obtained sequence was added to the GenBank data base under the accession number JQ220408.1 and designated as *Nostoc* sp. EGY. As the similarity obtained from the partial sequence of the 16S rDNA is slightly low the 16S-ITS-23S rDNA which was amplified to confirm the 16S rDNA results and to confirm the genus of the isolated bacteria. As shown in Figure 4, the sequence of the PCR product of 16S-23S ITS showed 98% similarity with *Nostoc* sp. NR1 (2012) 16S-23S ribosomal RNA intergenic spacer from the GenBank. The obtained sequence was added to the data base under accession number JQ220407.1 and designated as *Nostoc* sp. EGY 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence.

Amplification of HEP genes

The identification of the hepatotoxin cyanobacteria is performed by amplification of AMT domain of hepatotoxin synthetase *mcyE* gene (Amer et al., 2009; Jungblut et al., 2006). The amplification of HEP genes of *Nostoc* sp. EGY versus the positive control (microcystine producing strain) was visualized on 2% agarose gel. The data showed the absence of 472 bp band for *Nostoc* sp. EGY and presence of the same band is the positive control. Therefore, *Nostoc* sp. EGY can be used for farther investigations.

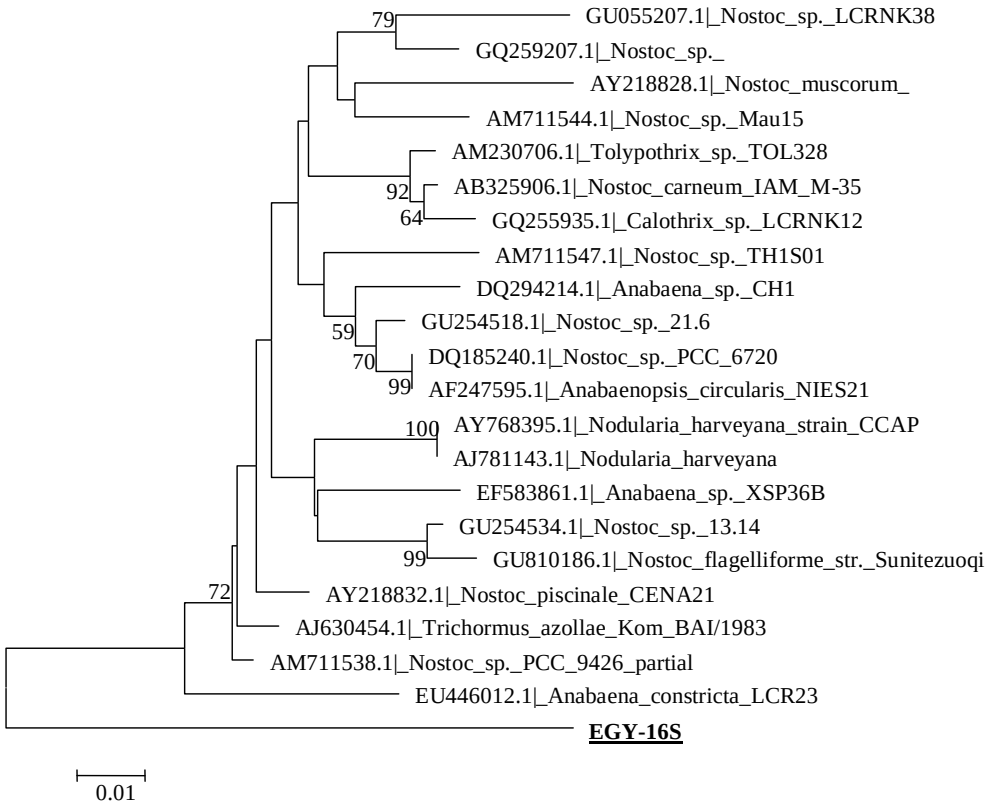

Figure 3. Neighbor-joining tree based on 16S rDNA sequences (CYA primers) of the EGY. Evolutionary distances were calculated using the Kimura 2 model using MEGA5 software. The numerals show the results of the bootstrap analysis values from 1000 replicates (only bootstrap values above 50% were shown).

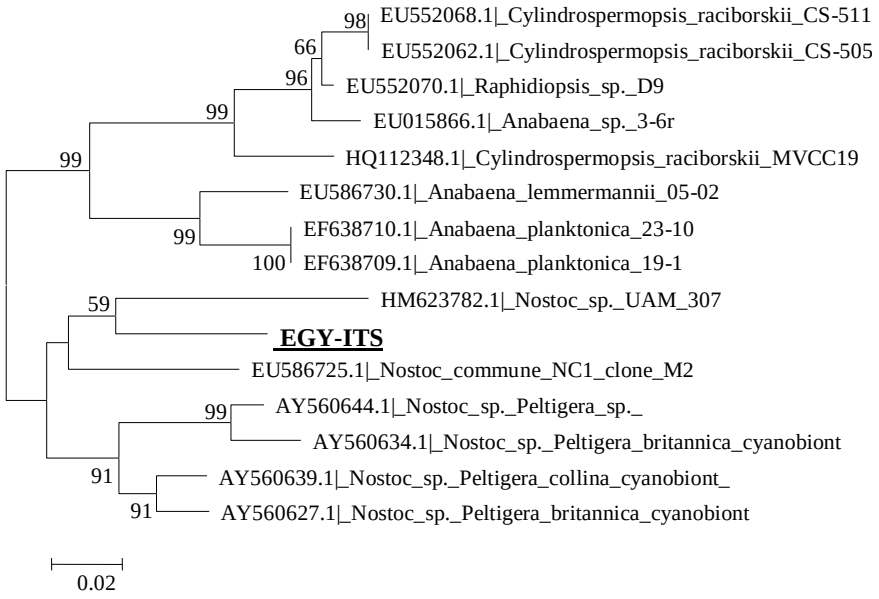

Figure 4. Neighbor-joining tree based on ITS sequences (P322-P340 primers) of Iso-1 and Iso-2 isolates isolated from Nile River. Evolutionary distances were calculated using the Kimura 2 model using MEGA5 software. The numerals show the results of the bootstrap analysis values from 1000 replicates. The sequence in bold was determined in this work.

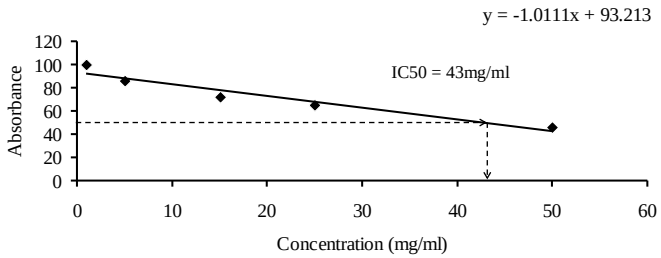

Figure 5. Cytotoxicity of BGA lysate and IC50 value.

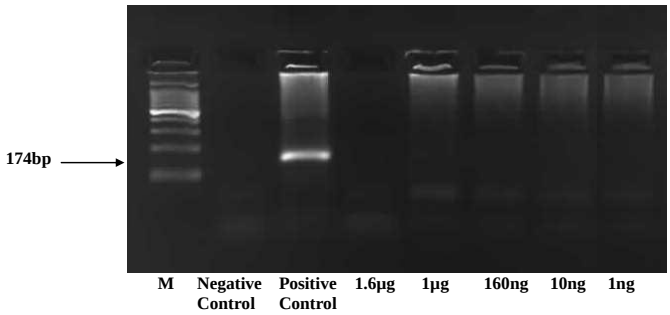

Figure 6. Anti HCV activity of different concentrations of BGA.

Table 2. Cyanobacteria growth curve and anti-HCV activity.

Cultivation interval (days)	Anti HCV activity
5	-ve
10	+ve
15	-ve
20	-ve
25	+ve
30	-ve
35	-ve
40	-ve
45	-ve
50	-ve
55	-ve
60	-ve

Anti HCV activity

As indicated in Figure 5 the IC50 of BGA lysate is 43 mg/ml. Safe concentration ≤ 1/100 the IC50 (0.4 mg/ml) was examined for the anti HCV activity. Safe concentration was able to prevent HCV replication (data not showed). Much Lower concentrations were tested for anti-HCV activity (1, 10, 160, 1, 1.6 µg/ml) all of selected concentrations were able to prevent HCV replication in the *in vitro* HCV culture system (Figure 6). Where, viral load was almost equal to zero at concentrations 10 160, 1, 1.6 µg/ml while 1 ng and 0.5 ng/ml showed very low viral load as compared to the positive control but the percent inhibition of viral replication was almost 100% at all used BGA concentrations (Table 3).

Table 3. Quantitative RT-PCR of anti HCV activity of the lowest BGA concentrations.

BGA concentration (ng/ml)	HCV viral load (IU/ml)	Percent inhibition
Positive control	$1.28X10^9$	
0.5	322	99.99999975
1	172	99.99999987

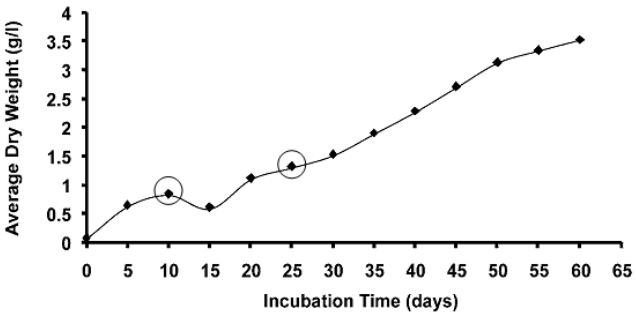

Figure 7. Cultivation of the organism in its selected suitable media for high cell density.

As presented in Table 2 and Figure 7 anti HCV activity was influenced by cultivation time where anti-HCV activity was detected with lysate of 10 and 25 days of culture while the lysate of the rest of cultivation time intervals have no anti HCV activity. This data indicated that cultivation time is very crucial for BGA activity against HCV while cell dynasty is not.

DISCUSSION

According to the taxonomic identification of Komárek and Anagnostidis (Komárek, 2005) the phenotype of the isolated cyanobacteria was characterized as a genus belongs to group of Nostocales as the filaments showed the presence of heterocysts and akinetes. The analysis of 16S rDNA and 23S-ITS rDNA together with the microscopic examination revealed that the isolated cyanobacterium was designated as *Nostoc* sp. EGY. The association of N2-fixing cyanobacteria is commonly known in the rice filed due to the lack of nitrogen. *Nostoc* as one of the N2-fixing cyanobacteria has been isolated and characterized by many authors from the roots of the rice plants (Nilsson et al., 2002). The absence of amplicon from the PCR amplification of aminotransferase (AMT) domain of the hepatotoxin synthetase *mcyE* and

ndaF genes by using HEP primers revealed that the isolated strain was not a hepatoxic strain. Whereas, these primers were used before for the characterization of the genes responsible for hepatotoxin biosynthesis (Amer et al., 2009; Jungblut et al., 2006).

Recently, Hashtroudi et al (Hashtroudi et al., 2013) was able to isolate four major carotenoids from *Nostoc* sp that can be used for nutrition or production of supportive medicine. Also, edible *Nostoc* sp was proven to have component that can act as anti herpes simplex virus (Kanekiyo et al., 2007). Other *Nostoc* sp was showed to have hypocholesterolemic efficacy (Rasmussen et al., 2009). Several reports have indicated the ability of small protein, cyanivirin N, of cyanobacteria *Nostoc* sp act as potent antivirus to HIV, Ebola, Influanza, and HCV (Barrientos et al., 2003; Boyd et al., 1997; Buffa et al., 2009; Dey et al., 2000; Helle et al., 2006; O'Keefe et al., 2003; Tsai et al., 2004). Also, cyanovirin-N has been recently evaluated for treatment of HIV infection (Buffa et al., 2009; Zappe et al., 2008). Accordingly, the new identified isolate presented in this study *Nostoc* sp. EGY may have the same anti viral component. However, more detailed study is required for the identification of detailed effect of the new isolated strain lysate on HCV replication. The absence of hepatotoxin synthetase *mcyE* gene indicate acceptable safety of the isolated *Nostoc* sp. EGY which increase the possibility of using this isolated strain for designing an effective anti HCV medication.

Conflict of interests

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

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