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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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Full Length Research Paper

Induced oxidative stress by *Metarhizium anisopliae* spp. instigates changes in lipid peroxidation and ultra structure in *Periplaneta Americana*

Naren Babu Mutyala* and Padmaja Vadlamani

Department of Botany, Andhra University, Visakhapatnam- 530 003, India.

Accepted 26 August, 2013

Consequent to injection of conidia at LC30, LC50 and LC90 doses of the entomopathogenic fungal isolates of *Metarhizium anisopliae* (Metch.), alterations in lipid peroxidation and ultrastructural changes were observed in cockroaches (*Periplaneta americana*). A decrease in the level of lipid peroxidation was evident in the treated cockroaches compared to those of control. Present investigation reports the effects of fungal infection on the midgut ultrastructure of the adult cockroaches which was manifested in the form of damage to the microvilli, epithelial cell vacuolization, necrosis and disruption of the epithelial cell membrane which occurred with increase in the time interval post treatment with conidia from high virulent isolate (M20) at LC50 dosage. Our study reveals for the first time that fungal infection instigates oxidative stress in the cockroach and that the villi of the midgut are also the target organs for the oxidative damage.

Key words: Entomopathogenic fungi, lethal concentration, cockroach, mid gut villi.

INTRODUCTION

Metarhizium anisopliae (Metschnikoff) Sorokin (Hypocreales: clavicipitaceae), is a ubiquitous insect parasitic fungus (Rehner, 2005) and the causal organism of green muscardine disease. The entomopathogenic fungus, *M. anisopliae* (Metchnikoff) Sorokin has been reported to infect more than 200 species of insects belonging to different orders (Zimmermann, 1993). *M. anisopliae* produces destruxins both in culture and *in vivo* in the infected insects and were reported to be the candidates for mortality in insects. Kershaw et al. (1999) reported that, in the pathogenesis of *M. anisopliae* var *anisopliae*, there is a relationship between the titer of DTX production of isolates *in vitro* and the killing power. DTX A induces adverse ultrastructural changes in the epithelial cells and villi of midgut of the lepidopteran pest *Galleria mellonella* (Dumas et al., 1996). This toxin was found to possess uncompetitive inhibitory effect on the hydrolytic activity of vacuolar-type ATPase in the brush border membrane vesicles of the midgut of *G. mellonella* larvae (Bandani et al., 2001). The detection, characterization and analysis of the role of reactive oxygen species (ROS) is well established in both normal and pathological processes of cellular metabolism.

Lipid peroxidation refers to the oxidative degradation of

*Corresponding author. E-mail: vpadmaja4@gmail.com or narenmutyala@gmail.com. Tel.: +91 9440560258(M) or +91-9640008155(M). Fax- +91 891 2525611.

Abbreviations: ROS, Reactive oxygen species; **SOD,** superoxide dismutase; **CAT,** catalase; **POX,** peroxidase; **APOX,** ascorbate peroxidase; **SDAY,** Sabouraud dextrose agar with yeast extract medium; **TBARS,** thiobarbituric acid-reactive substances; **TCA,** trichloro acetic acid; **MDA,** malondialdehyde; **TEM,** transmission electron microscopic; **SEM,** scanning electron microscopy; **TMB,** tetramethylbenzidine.

lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism and fatty acid radical is produced. The most notable initiators in living cells are ROS, such as Hydroxyl radical ($OH^{\cdot}$) and HO_2, which combines with a hydrogen atom to make water and a fatty acid radical. The peroxidation of lipids is basically damaging because the formation of lipid peroxidation products leads to spread of free radical reactions. Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue.

Studies have revealed the susceptibility of the cellular components from the attack of ROS, but the major multifold effects are manifested in the form of loss of ions and protein cleavage. The generation of ROS like OH^{-} and superoxide radical (O_2^{-}) radicals disintegrates biomembranes by lipid peroxidation which is a general mechanism of stress induced responses in living systems as was reported by Panda and Chaudhury (2003). ROS include oxygen ions, free radicals and peroxides, both inorganic and organic. These molecules are generally very small and highly reactive, because of the presence of unpaired electrons (Aslanturk et al., 2011). ROS are formed as a natural byproduct of the normal metabolism of oxygen. They play an important role in cell signaling and the induction of host defense genes (Dalton et al., 1999). Studies reported that organophosphate pesticides caused lipid peroxidation and the alterations in the antioxidant defence enzymes of insect (Gupta et al., 2010; Wu et al., 2011).

To minimize the potential threats of ROS, the cells are equipped with numerous antioxidant defense systems. Their function is to maintain low steady state levels of ROS and other radicals in the cell, a process involving precise regulation of their location and amount. The antioxidant enzymes, such as superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6) and peroxidase (POX, E.C. 1.11.1.7) form a part of the defence system (Joanisse and Storey, 1996a). Catalases and peroxidases are the most important enzymes that degrade peroxide into water and oxygen. These enzymes and superoxide dismutase are the first lines of cell defence against ROS. Antioxidant defence was measured by activities of SOD, CAT and ascorbate peroxidase (APOX). CAT and POX, more appropriately the specific APOX, act to remove these peroxides. The key step in oxidative stress is the production of ROS which initiate a variety of auto-oxidative chain reactions on membrane unsaturated fatty acids and proteins, producing lipid peroxides and protein carbonyls respectively resulting in a cascade of reactions ultimately leading to destruction of organelles and macromolecules (Jamieson, 1989). Hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$), the ubiquitous products of single electron reductions of dioxygen, are amongst the most reactive compounds known to be produced during oxidative stress (Dietz et al., 1999). Lipid peroxidation is a well-established mecha-nism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues (Esterbauer et al., 1991). Lyakhovich et al. (2006) opined that various antioxidants that were present in the insect may decrease the level of lipid peroxidation.

Cockroaches are insects of the order Blattodea with about 4,500 species, is widely distributed throughout the world. 30 species of cockroaches are associated with human habitations and about four species are well known as pests (Valles et al., 1999). Cockroaches are implicated as vectors of several human disease agents. *Periplaneta americana* (American cockroach) is the well known pest species and ubiquitous throughout the world. Considering cockroach diversity, it is necessary to evaluate virulence of the insect pathogenic fungal isolates of *M. anisopliae* on hand against the locally prevailing populations. There is paucity of information regarding pathogenecity of *M. anisopliae* against *P. americana*. Therefore, the present study is undertaken with the objective of exploring the mode of infection of *M. anisopliae* against *P. americana*.

The aim of the present study was to reveal the induced oxidative stress in terms of levels of lipid peroxidation in *P. americana* when infected with conidia of *M. anisopliae* through injection process with three different lethal concentrations, LC30, LC50 and LC90 at 1, 24th and 48th h post treatment. The dynamics in the levels of lipid peroxidation were studied among the insects that were subjected to infection with three isolates of *M. anisopliae* which differ in their virulence. Microscopic studies were made to observe changes in the ultrastructure of mid gut villi in treated cockroach to unravel the consequences of the oxidative stress induced through fungal infection.

MATERIALS AND METHODS

Fungal culture and maintenance

M. anisopliae isolates, M-20 (*M. anisopliae sensu lato*, ARSEF - 1823, isolated from *Nilaparvatha lugens*), M-48 (*M. anisopliae sensu lato,* ARSEF - 1882, isolated from *Tibraca limbativentres*), and M-19 (*M. anisopliae sensu stricto* (Bischoff et al. 2009), ARSEF - 1080, isolated from *Helicoverpa zea*) were obtained from ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungi), Ithaca culture collections. The microscopic cultures were grown on Sabouraud dextrose agar with yeast extract medium (SDAY - 4% dextrose, 1% peptone, 1% yeast extract, 2% agar, pH 7.0) and slants were incubated at 25±1°C. The sporulated cultures seen with green colored powdery coating on the white mycelial mat were stored at 4°C. Virulence of the isolates was maintained by strain passage. Cockroaches were infected by spraying with spore suspension. After 48 h, the insects were washed with distilled water for removing the ungerminated spores adhered to the insect body. The treated insects after death and mummification were transferred to humid chambers for promoting mycosis. The mycosed and sporulated insects were used to reisolate the fungus. The isolates at the first cycle after reisolation were used for evaluation. Germination of the conidia tested on SDAY prior to experimentation

Table 1. Values of lethal concentrations (conidia/ml) for the three isolates of *Metarhizium* species at 48th h after the treatment through injection mode.

Fungal isolate	LC 30[a]	LC 50[b]	LC 90[c]
M20	7.3×10^{5}	3.5×10^{6}	1.5×10^{8}
M19	3.2×10^{8}	5.0×10^{12}	8.4×10^{22}
M48	1.4×10^{5}	1.4×10^{8}	3.4×10^{15}

[a]Concentration that caused 30% deaths; [b]concentration that caused 50% deaths; [c]concentration that caused 90% deaths.

revealed a range of 90 - 95% values for the four isolates used in the investigation. All the solvents used as medium components of the culture media and artificial diets were from Merck (India) ltd.

Experimental insects

P. americana (American cockroach) adults were initially collected from their natural habitats like godowns. The adult cockroaches were selected and were transferred to wooden framed boxes of 30.5 x 30.5 cm dimensions with iron mesh on one side for aeration. The cages were kept dry and the insects were fed with hardened bread. An absorbent cotton piece soaked in water was kept in a bowl to serve as a source of water and moisture to the roaches. The food was changed for every two days. Rearing conditions were standardized so as to ensure 100% survival in laboratory conditions. The healthy adult roaches were kept for observation for one week before starting the experiment. Insects measuring 3.25 cm (± 0.1) length and 1.5 gms (± 0.1) weight were selected for treatments. The experiment set up was maintained at a temperature of 29± 1°C with photo period conditions of 12 h darkness followed by 12 h light.

Mode of treatment

The lipid peroxidation assays were conducted on *P. americana* using three isolates (M20, M48 and M19) of *M. anisopliae*. Injection method as adopted by Gunnarsson and Lackie (1985) was followed with slight modifications for treating the cockroaches with fungal conidia. Twenty micro liters of conidial suspension at $5x10^{7}$ ($1x10^{6}$ conidia in 20 µl), $5x10^{6}$ ($1x10^{5}$ conidia in 20µl), $5x10^{5}$ ($1x10^{4}$ conidia in 20 µl), $5x10^{4}$ ($1x10^{3}$ conidia in 20 µl), $5x10^{3}$ ($1x10^{2}$ conidia in 20µl) conidia/ml was injected in to the haemocoel of the cockroach. Injection was done using a 1 ml disposable syringe holding a 0.30 x 8.0 mm needle on the ventral side of the roach body piercing through inter segmental region of 5th and 6th segments as was done by Vilcinskas and Matha (1997). The suspension was released gently so as to ensure effective spread of conidial suspension into haemocoel of the insect. Mortality data was recorded at 6 h intervals starting at 6 h post treatment and continued for 5 days. The data obtained from the bioassays was subjected to probit analysis using SPSS 11.0 version software to obtain values of median lethal concentrations that caused 30% (LC30), 50% (LC50) and 90% (LC90) deaths at the 48th h post treatment.

Three lethal concentrations LC30, LC50 and LC90 were selected to treat the cockroaches (Table 1) for conducting lipid peroxidation assay. LC50 was used for scanning electron microscopy (SEM) and transmission electron microscopic (TEM) studies. The changes in the lipid peroxidation levels were recorded at 1, 24, and 48 h post treatment. LC50 and LC90 for M19 isolate and LC90 for M48 isolate were too high to count with haemocytometer and hence were not included in the study. The control cockroaches were treated with 0.02% tween solution.

Lipid peroxidation

The lipid peroxidation level was measured using the thiobarbituric acid-reactive substances (TBARS) assay. Briefly, the treated and control samples were homogenized (1:10 w/v) in 3 ml of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) (w/v). The homogenate was incubated at 95°C for 30 min, and the reaction was stopped in ice. The samples were centrifuged at 10,000 g for 10 min and absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non specific absorbance at 600 nm was subtracted from that at 532 nm. The absorbance coefficient of malondialdehyde (MDA) of 155 /mM`/cm was used for estimating the degree of lipid peroxidation (Heath and Packer, 1968).

Ten (10) insects were used for each treatment and the experiment has been repeated thrice. In each treatment, triplicate readings for lipid peroxidation were taken and the data obtained from the experiments was subjected to analysis of variance (ANOVA) using SPSS version 11.0 statistical software to obtain standard error means (SEM). Since the trend in dynamics of lipidperoxidation was found to be similar in repeated experiments, the results of the final experiment were taken as concluding values.

Transmission electron microscopic (TEM) study

For TEM studies, the anterior thorax region of the live insects after 10,12 16, 20 and 24 h post treatment was cut and gut was transferred to vials and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 41°C. These samples were post-fixed in 0.5% aqueous osmium tetroxide in 0.05 M phosphate buffer for 2 h. After post-fixation, samples were dehydrated in a series of graded alcohol, infiltrated and embedded in spurr's resin. The ultra-thin sections were cut with a glass knife on an ultra microtome (Leica Ultra Cut UCT-GA-D/E-1/100). The sections of 50-70 nm thickness were mounted on grids, stained with saturated aqueous uranyl acetate and counter-stained with 4% lead citrate (Bozzola and Russell, 1999). The sections were observed under a TEM (Hitachi, H-7500).

Scanning electron microscopy (SEM)

Scanning electron microscopy was done by the method proposed by John Bozzolla (1999). The cockroaches were injected with conidia (LC50) from high virulent isolate of *M. anisopliae* (M20). Live insects after 24th and 48th h post treatment were dissected and their fat bodies were processed for SEM. The samples were fixed in 2.5% gluteraldehyde in 0.1 M Phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed in 2% aqueous osmium tetroxide for 4 h in the same buffer. After the fixation, samples were dehydrated in series of graded alcohols and dried to critical point drying with Electron Microscopy Science CPD unit. The dried samples were mounted over the stubs with double-sided carbon tape and applied a thin layer of gold coat over the samples by using an automated sputter coater (JOEL JFC-1600) for 3 min. The samples were scanned under scanning electron microscope (Model: JOEL-JSM 5600) at various magnifications.

RESULTS AND DISCUSSION

The assay of the lipid peroxidation in the cockroaches treated with fungal conidia revealed a decrease compared to those of the untreated samples. The insects treated with M20 isolate at LC30 dose displayed a decrease

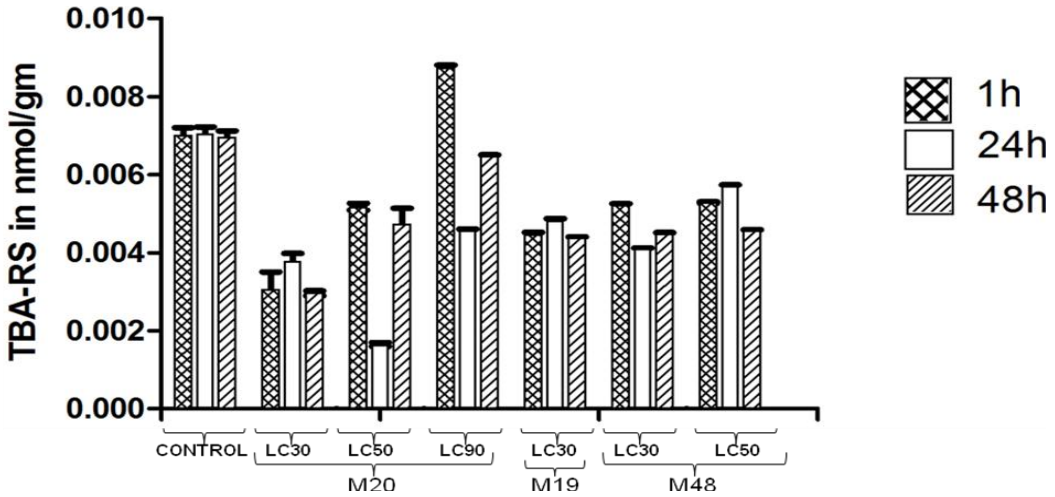

Figure 1. Graph showing lipid peroxidation at 1, 24 and 48 h post treatment in the cockroaches treated with conidia from *M. anisopliae* isolates.

of 54% over the control at 1 h post treatment. At LC50 treatment, an increase in lipid peroxidation was recorded compared to the corresponding values of LC30 at 1st and 48th h post treatment but showed a decrease at the 24th h (Figure 1). On the other hand, at LC90 treatment, the lipid peroxidation levels increased over the control values by 28% at 1 h post treatment but as the time of treatment increased, at the 24th and 48th h, the values decreased. On the other hand, with M19 a high virulent isolate and M48 a low virulent isolate, decrement in lipid peroxidation levels with progression in the post treatment time was displayed.

Among the treated ones with high virulent isolate M20 at 24th h post treatment, the insects infected with LC30 displayed an increase in lipid peroxidation by 11% with the advancement of post treatment time from 1 h and by the 48th h, 11% decrease was revealed. At LC50, there is an initial decrement of lipidperoxidation of about 24% which increased by 50% by the 24th h. For the same isolate, 28% increase in lipid peroxidation in insects infected with LC90 at 1 h post treatment and a gradual decrease by 60% by the 24th h was recorded. With another high virulent isolate M19, at LC30, similar trend as shown by insects treated with LC30 of M20 has been revealed where an increase in lipid peroxidation from 1st to 24th h post treatment and a decrease by 48th h was recorded. With the low virulent isolate M48, at LC30, lipid peroxidation decreased by 13% at the 24th h post treatment and 5% increase from the 24th h to 48th h was recorded (Figure 1).

Decrease in the extent of lipid peroxidation, as was revealed by the assay of lipid peroxides, can be attributed to increase in the proportion of antioxidant enzymes like peroxidases and ascorbate peroxidases that the cockroaches secrete to fight the oxidative stress induced by the fungal infection. The antioxidant machinery contributes to decrease in the concentration of lipid peroxides that were the end products of lipid peroxidation. The increased activities of peroxidases and ascorbate peroxidases with increase in the post treatment time (Naren, 2013) could be correlated to decrease in the lipidperoxidation. MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, has been used to determine the degree of lipid peroxidation and as a biological marker of oxidative stress (Rael et al., 2004). Besides, under environmental stress, ultraviolet irradiation, bacterial infections, antibiotics and pesticides exposure, the ROS level may increase remarkably and result in oxidative stress in insects (Lopez-martinez et al., 2008; Buyukguzel and Kalender, 2009; Durak et al., 2009). CAT and POX, more appropriately the specific APOX, act to remove ROS and cellular haemostasis in addition to the non-enzymatic antioxidants such as thiols, ascorbate and glutathione (Joanisse and Storey, 1996a). The decreased lipid peroxidation level is a result of antioxidants that neutralized the lipid peroxides. The increased lipid peroxidation indicates the elevated levels of lipid peroxides as a result of increased oxidative stress which is beyond the reach of insect's antioxidant defense. To neutralize the toxicity of ROS, insects have developed a suite of antioxidant enzymes like other eukaryotes to overcome oxidetive stress. Several antioxidant enzymes may decrease the level of lipid peroxidation in insects (Felton and Summers, 1995). This can be attributed to the scavenging activity of the antioxidant enzymes on the lipid peroxides which, in view of increase in the activity of peroxidases and ascorbate peroxidases, there has been a decrease in the concentration of the lipid peroxides. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate MDA and 4-hydroxyalkenals upon decomposition. Measurement of malondialdehyde and 4-hydroxyalkenals hasbeenusedasanindicatoroflipidperoxidation(Esterbauer

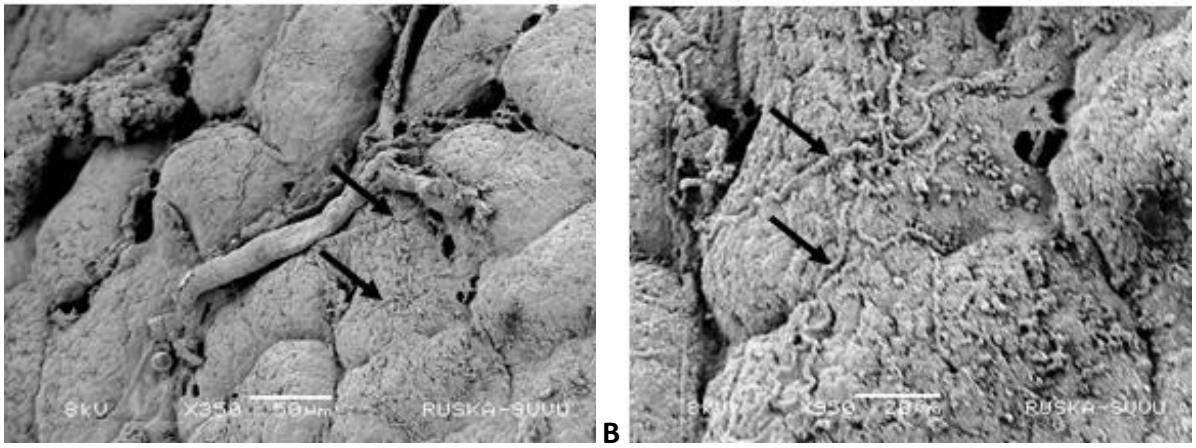

Figure 2. Scanning electron micrograph of the fatbody of treated cockroach. a, 24th h post treatment; b, 48th h post treatment

et al., 1991). Along with lipid peroxides, the APOX activity found in fat body tissues, suggests that ascorbate peroxidase may be important in removing lipid peroxides in insects (Mathews, 1997). With the increase in the fungal dose, the extent of lipid peroxidation increased among the treated insects in all the three fungal isolates. But with the advancement in the post treatment time, for each dose, there are dynamics in the lipid peroxidation levels indicating the response of innate immunity of the insect in the form of antioxidant enzymes viz., POX and APOX (Naren, 2013). Lyakhovich et al. (2006) opined that various antioxidants that were released in the insect as a response to the oxidative stress may decrease the level of lipid peroxidation.

The cockroaches treated with entomopathogenic fungal spores displayed growth of the fungal mycelia in the fat body of the insect at the 24th h post treatment and proliferated all over the insect hemocoel by the 48th h (Figure 2a, b). The TEM observations on the mid gut in the treated cockroaches revealed ultrastructural changes in the form of deformed villi due to lipid peroxidation and structural change. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. Lipid peroxidation in biomembranes is mediated by free radical reactions. It leads to membrane damage and has been proposed to be associated with the pathogenesis to tissue injuries (Tampo, 2000). The extent of damage increased with increase in the post treatment time in the cockroaches treated with fungal conidia. In contrast to the control (Figure 3), at 10th h post treatment, the mid gut of the cockroaches injected with M20 displayed loss of the columnar cell architecture (Figure 4). As the post treatment time incremented at the 12th h post treatment, necrosis of the tissue was observed in the site where villi attach to the gut (Figure 5). At the 16th h post treatment and the extent of damage to the villi increased and the villi were found to be getting separated from the intact columnar epithelial wall (Figure 6). The TEM at the 20th h post treatment displayed an extensively damaged, broken and detached microvillus (Figure 7). The highly reactive properties of ROS make them a potential threat to cellular macromolecules, and if the initiated oxidation processes are not inhibited by the enzymatic and non-enzymatic components of the antioxidant defense system, damage to the DNA, lipid peroxidation and dysfunction of enzymes can result in necrotic or apoptotic cells (Smith et al., 2008). By the 24th h, the villi got totally deformed due to fragmentation of the cells (Figure 8) and the electron dense areas as a consequence of the fat body depositions were observed in transmission electron microscopic observations of midgut wall (Figure 9).

The ultrastructural localization of lipid peroxides was reported by Kayatz et al. (1999) in the glutaraldehyde fixed tissue of rat retina, which was reacted with tetramethylbenzidine (TMB) and then postfixed in osmium tetroxide to visualize the lipid peroxides as electron-dense structures. Joanisse and Storey (1996b) also reported that fatty acids are particularly susceptible to ROS attack and their metabo-lism can in turn lead to ROS formation. To defend the tissue from the harmful effects of these hiked free radical levels, the activity of antioxidant enzymes and the level of antioxidants increases. In the current investi-gation, the deleterious effects of such high levels of free radicals has been ultrastructurally depicted in the villi of the midgut epithelial cell of the adult cockroaches treated with LC 50 of the high virulent isolate (M20). Increase in the post treatment time beyond a certain limit lead to the unbalanced generation of free radicals which suppresses the innate defense mechanism leading to consequent death of the insect.

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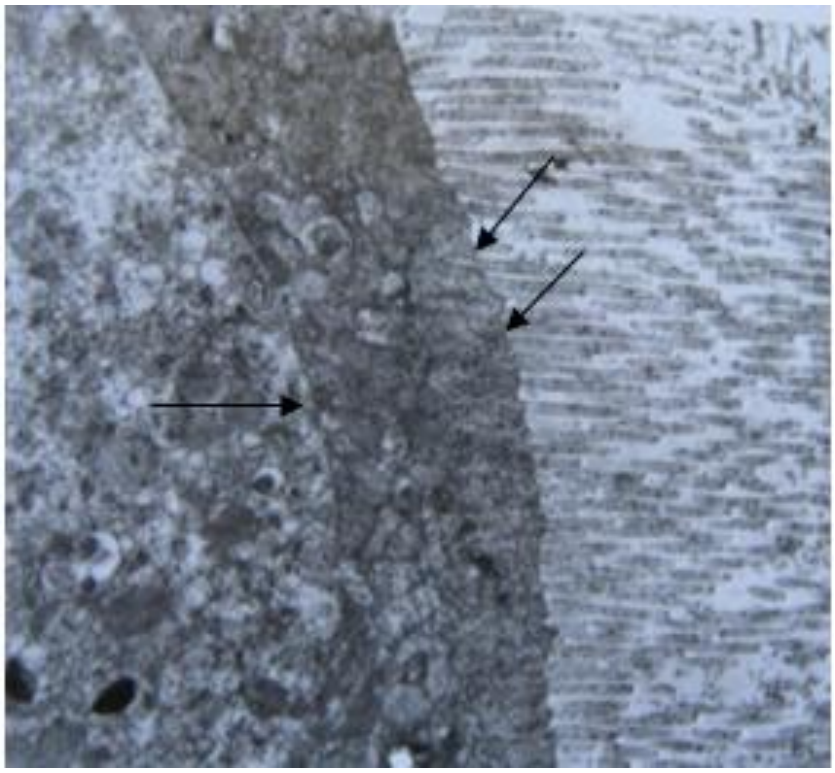

Figure 3. Transmission electron micrograph depicting the ultrastructure of the epithelial architecture of the midgut of *P. americana*. Arrows indicate the intact villi and epithelial cell wall.

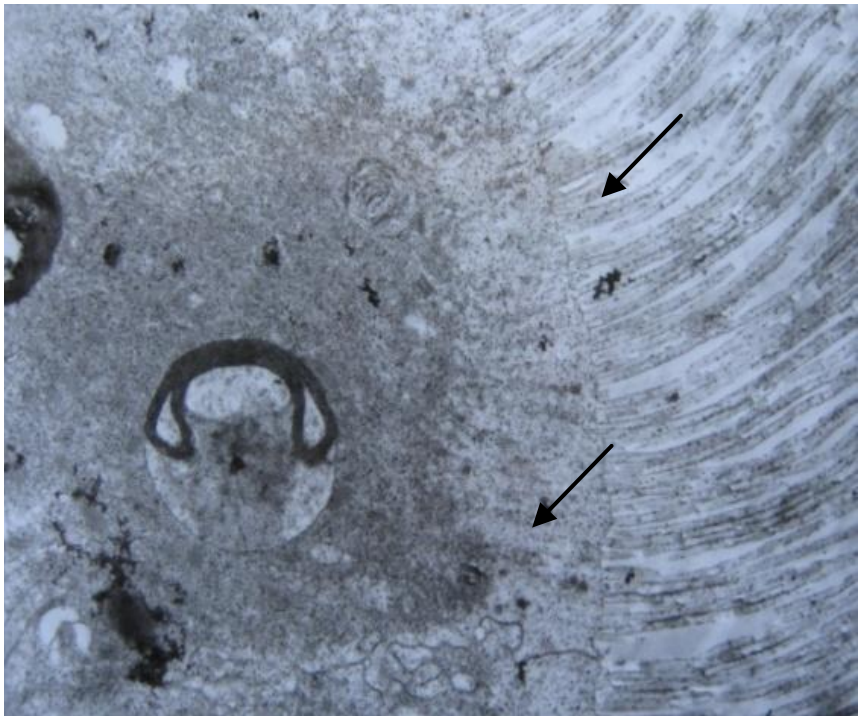

Figure 4. Transmission electron micrograph depicting the ultrastructure of the midgut of *P. americana* injected with LC50 of conidia from M20 isolate at 10^{th} h post treatment. Arrows indicate the deformed structure of epithelia.

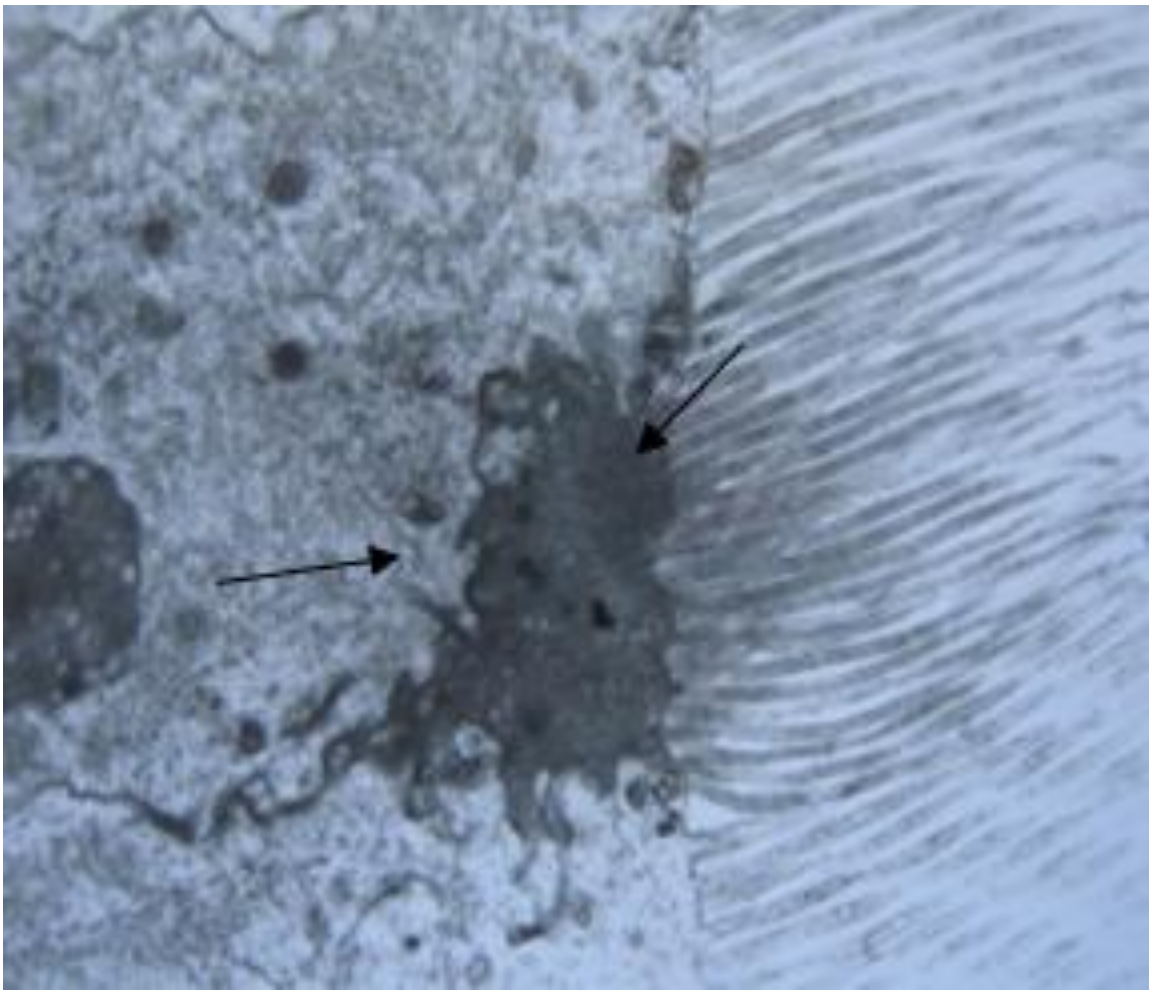

Figure 5. Transmission electron micrograph depicting the ultrastructure of the midgut of *P. americana* injected with LC50 of conidia from M20 isolate at 12th h post treatment. Arrows indicate the necrosis at the base of the villi after the deformation of epithelial structure.

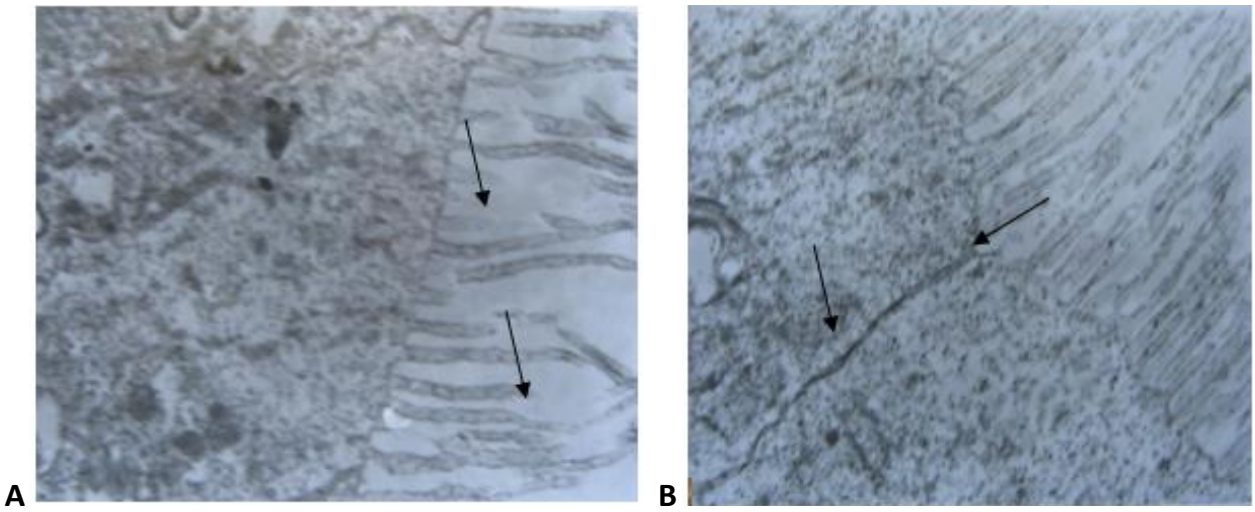

Figure 6. Transmission electron micrographs depicting the ultrastructure of the midgut of *P. americana* injected with LC50 of conidia from M20 isolate at 16th h post treatment. Arrows indicate the (a) progressive to the villi and (b) breakdown of the epithelial structure.

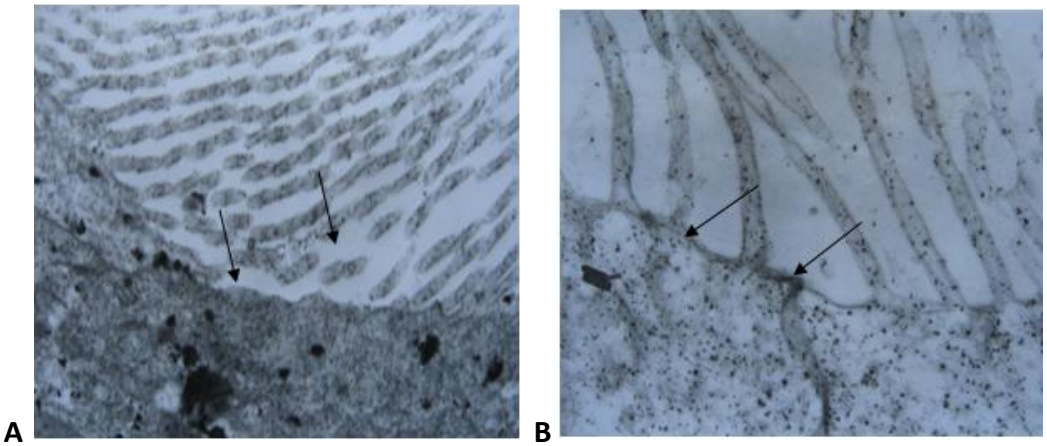

Figure 7. Transmission electron micrographs depicting the ultrastructure of the midgut of *P. americana* injected with LC50 of conidia from M20 isolate at 20th h post treatment. Arrows indicate the (a) detachment of the villi from its base and (b) gaps formed between the villi due to detachment.

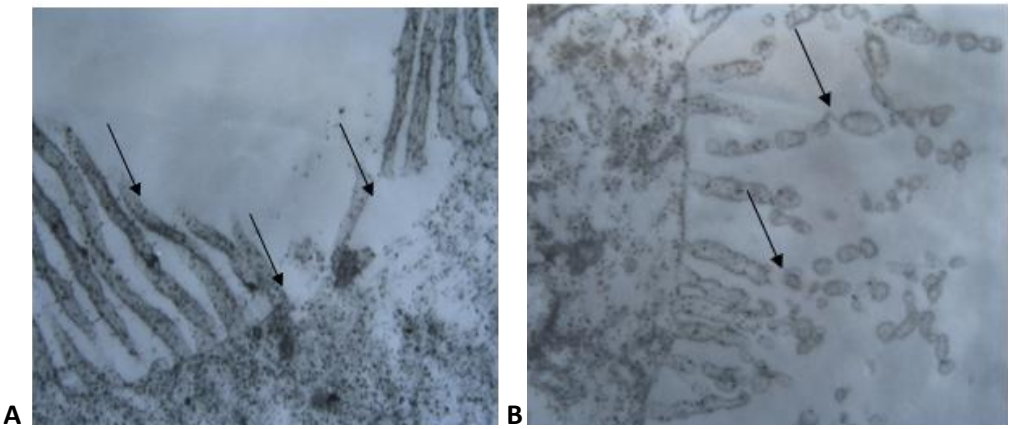

Figure 8. Transmission electron micrographs depicting the ultrastructure of the midgut of *P. americana* injected with LC50 of conidia from M20 isolate at 24th h post treatment. Arrows indicate the presence of (a) lipid peroxides and (b) deformed, fragmented villi.

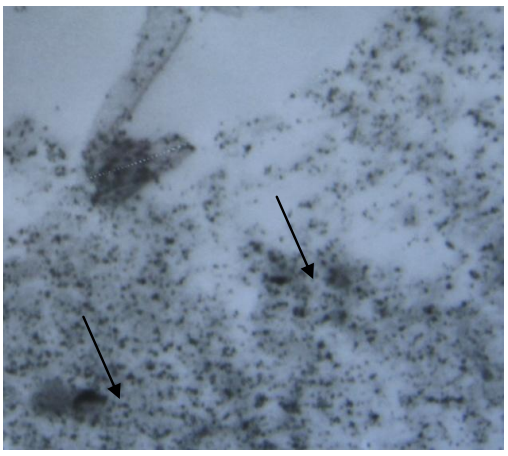

Figure 9. Transmission electron micrograph of mid gut of *P. americana* showing electron dense areas and fatbody in the treated cockroach at 24th h from the time of treatment.

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Full Length Research Paper

Developments in using fatty acids in fungal chemotaxonomy

Mohamed E. Zain[1,2*], Ali H. Bahkali[1] and Monerah R. Al-Othman[1]

[1]Botany and Microbiology Department., College of Science, King Saud University, (KSA), Riyadh, Saudi Arabia.
[2]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt.

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The cellular fatty acid composition of nine species of *Fusarium*; namely, *Fusarium anthophilum*, *F. avenaceum*, *F. cerealis*, *F. graminearum*, *F. graminum*, *F. oxysporum* f. sp. *conglutinans*, *F. pseudograminearum*, *F. roseum* and *F. sacchari* var. *elongatum* growing on malt extract medium were determined. The fatty acid profiles of the investigated fungi showed very little variation and could only differentiate between few species. However, by adding certain chemical compounds including aspartic acid, glutamic acid, methionine, selenium and urea to the growth medium, the variation of the fatty acid profile was greatly increased and differentiated between all the investigated fungi. For example, pentadecanoic acid was not produced by *F. anthophilum* on malt extract broth (MEB) but only produced on MEB supplemented with aspartic acid. On the other hand, linolenic fatty acid was neither produced by *F. anthophilum* nor *F. roseum* grown on MEB, but it was produced by *F. anthophilum* in presence of aspartic acid and by *F. roseum* in the presence of glutamic acid. The fatty acid profiles could be useful for characterization and identification of fungi if determined under different conditions.

Key words: Fungal chemotaxonomy; fatty acids; *Fusarium* spp.; environmental conditions.

INTRODUCTION

The *Fusarium* species are widely distributed in soil and on subterranean and aerial plant parts, plant debris, and other organic substrates. They are common in tropical and temperate regions. They are found in desert, alpine, and arctic areas, where harsh climatic conditions prevail (Nelson et al., 1994). Many *Fusarium* species are abundant in fertile cultivated and rangeland soils but are relatively uncommon in forest soils (Jeschke et al., 1990). *Fusarium* species are often regarded as soil-borne fungi because of their abundance in soil and their frequent association with plant roots, as either parasites or saprophytes.

The taxonomy of *Fusarium* spp. is confusing and various classification systems have been proposed (Nelson, 1991). Species identification by morphological traits is problematic because characteristics like mycelial pigmentation, formation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions. Phenotypic variation is abundant and expertise is required to distinguish between closely related species and to recognize variation within species (Nelson et al., 1983). Chemotaxonomy seems to mean different things to different people depending on their subject area. To a bacteriologist, chemotaxonomy has meant nucleotide, amino acid, carbohydrate or lipid based taxonomy (Fox et al., 1990), and to a mycologist working with yeasts, it often means a carbohydrate or lipid-based taxonomy (Tosch et al., 2006; Velazques et al., 2006).

Fatty acid (FA) profiles are used increasingly as a chemotaxonomic tool for the identification and classification of bacteria (O'Donnell, 1994). There are still few publications on the use of this tool for fungal taxonomy, although it has proved to be useful with fungi

*Corresponding author. E-mail: mohzain@ksu.edu.sa.

that can be grown in carefully standardized axenic culture conditions (Stevens and Jones 1993, 1994; Koppova et al., 2008; Zain, 2009; Dembitsky et al., 2010), as well as for lichens (Sassaki et al., 2001), arbuscular-mycorrhizal fungi (Madan et al., 2002), mushrooms, basidiomycetes (Ben-Ze'ev et al., 2005), and Oomycetes (Larkin and Groves, 2003).

The objectives of this study was: (i) to analyze the cellular fatty acid compositions of some species of the genus *Fusarium*, (ii) to evaluate the usage of FA composition to differentiate each species (iii) to determine the susceptibility of the FA composition to the environmental conditions and (iv) its impact on chemotaxonomy of the fungi.

MATERIALS AND METHODS

Fungal strains

The fungal strains were obtained from different culture collections; *Fusarium oxysporum* Schlechtendahl : Fries f. sp. *conglutinans* (Wollenweber) Snyder & Hansen DSMZ 62045, *F. roseum* Link emend. Snyder & HansenDSMZ 3019, *F. sacchari* var. *elongatum* Nirenberg DSMZ 62272, *F. anthophilum* (A. Braun) Wollenweber DSMZ 63270, *F. graminum* Corda DSMZ 62224, and *F. avenaceum* (Corda: Fr.) Saccardo DSMZ 62161 were obtained from the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) while *Fusarium pseudograminearum* O'Donnell & T. Aoki NRRL 28062, *F. graminearum* Schwabe NRRL 5883, *F. acaciae-mearnsii* Aoki, Kistler, Geiser and O'Donnell NRRL 26752, *F. mesoamericanum* Aoki, Kistler, Geiser and O'Donnell NRRL 25797, and *F. cerealis* (Cooke) Saccardo NRRL 25491 were obtained from Agricultural Research Service Culture Collection (NRRL).

Media

For growing and maintenance of stock cultures, the malt extract agar (MEA) (malt extract, 20 g; peptone, 1 g; glucose, 20; agar, 20 g; and distilled water, 1 L) was used. To determine the effect of chemical compounds, the fungal strains were grown on malt extract broth (MEB) medium separately supplemented with different chemical compound with the concentration of 0.1% (1 gm/L) (aspartic acid, glutamic acid, methionine, and urea) and 0.01% (0.1 gm/L) for selenium selenite and incubated at 25°C for 10 days. Discs, 9 mm in diameter, of agar media containing the fungal materials were picked up from the margin of actively growing colonies, using sterile cork borer and each disc was transferred into 100 ml liquid medium; in 500 ml conical flasks. The flasks were then incubated at 25°C for 10 days.

Determination of the fatty acids

Fungal mycelium was harvested from broth medium by vacuum filtration. The harvested biomass was rinsed with nano-pure water while still in the funnel and then placed on a lipid-free paper towel for several minutes to remove excess moisture. The lipids were extracted from the dried biomass using chloroform/methanol (2:1 v/v) according to the method described by Folch et al. (1957). 1 g (wet weight) samples of fungal tissue were then placed into 4.0 ml of a saponification reagent and homogenized with a tissue grinder. The homogenate was then re-extracted with chloroform/methanol (2:1 v/v).

The extract of each sample was dried under a stream of nitrogen gas, after which the lipid was dissolved in chloroform and methylated. To methylate the liberated fatty acids, 2.0 ml of 6 N HCl in methanol was added to each tube. Subsamples were placed in water bath at 80°C for 10 min and immediately cooled to room temperature. Prior to fatty acid methyl ester (FAMEs) analyses, the samples were evaporated under nitrogen and resuspended in 50 µl of hexane. The fatty acid methyl esters were analysed at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt using a SHIMADZU 5050 type gas chromatograph equipped with mass detector using a 30 x 0.32 mm, 0.53 µm internal diameters, DB1 fused silica capillary column. The carrier gas was helium at a flow rate of 10 ml/min.

The temperature of the injector was 250°C and that of the detector was 280°C. The oven temperature after sample injection (2 µl) was 1 min at 115°C, increasing to 200°C at 7.5°C/min and then raised at a rate of 5°C/min to 240°C held at this temperature for 2 min and then raised at a rate of 3.5°C/min to 260°C and held at this temperature for 2 min. Peaks were identified by reference to authentic standards and verified using mass selective detector.

RESULTS

Nine species of the genus *Fusarium* were grown on malt extract broth medium and media separately supplemented with chemical compounds, incubated at 25°C for 10 days, and the fatty acid profiles were determined (Tables 1 to 9).

The fatty acid profile of *F. anthophilum* revealed that there were some fatty acids produced only in the presence of one or more chemical compound in the growth medium, such as capric fatty acid which is produced in the presence of all the investigated chemical compounds used in our study, while tridecanoic was induced in the presence of the glutamic acid and methionine. On the other hand, pentadecanoic was only induced in the presence of the aspartic acid, while linolenic was induced in the presence of aspartic acid, urea and selenium. Caprylic was not produced in the presence of urea, while linoleic was not produced in the presence of glutamic acid (Table 1).

The fatty acid profile of *F. avenaceum* was affected by the chemical constituents of the growth medium. Eight out of 15 fatty acids were affected by various aspects. Caprylic was not produced in the presence of aspartic acid, glutamic acid, methionine, urea and selenium, while capric was not produced in presence of glutamic acid, methionine, and selenium. Tridecanoic was not produced in the presence of glutamic acid and methionine, whereas palmitoleic was not produced in the presence of urea. Heptadecanoic was not produced in the presence of methionine, while stearic and linolenic were not produced in the presence of aspartic acid, methionine and urea. Seven out of 15 fatty acids produced by *F. avenaceum* including myristic, pentadecanoic, palmitic, oleic, linoleic, arachidic, and behenic were not affected by the addition of certain chemical compounds such as aspartic acid, glutamic acid, methionine, urea, and selenium (Table 2).

Table 1. The fatty acid profile of *Fusarium anthophilum*grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids in presence of					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.40	00.88	00.51	00.08	00.00	00.02
Capric	C10	00.00	00.02	00.08	00.19	00.02	00.02
Lauric	C12	00.20	00.04	00.09	00.46	00.07	00.10
Tridecanoic	C13	00.00	00.00	00.18	00.23	00.00	00.00
Myristic	C14	00.10	00.23	00.20	00.15	00.17	00.10
Pentadecanoic	C15	00.00	00.02	00.00	00.00	00.00	00.00
Palmitoleic	C16:1	00.20	00.32	00.32	01.36	00.25	00.20
Palmitic	C16	04.21	11.24	16.28	13.65	30.93	53.12
Heptadecanoic	C17	00.54	03.50	04.46	11.62	03.38	04.07
Oleic	C18:1	71.60	59.61	56.24	40.18	56.50	13.66
Stearic	C18	22.24	21.29	21.32	27.03	05.73	25.18
Linoleic	C18:2	00.30	00.20	00.00	04.66	02.64	02.40
Linolenic	C18:3	00.00	02.56	00.00	00.00	00.04	00.17
Arachidic	C20	00.05	00.03	00.11	00.31	00.18	00.76
Behenic	C22	00.16	00.06	00.21	00.08	00.09	00.20
Total Saturated		27.90	37.31	43.44	53.80	40.57	83.57
Total Unsaturated		72.10	62.69	56.56	46.20	59.43	16.43

Table 2. The fatty acid profile of *Fusarium avenaceum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids in presence of					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.04	00.00	00.00	00.00	00.00	00.00
Capric	C10	01.33	00.02	00.00	00.00	00.09	00.00
Lauric	C12	01.94	00.07	00.04	00.00	00.20	00.00
Tridecanoic	C13	00.15	00.22	00.00	00.00	00.30	00.10
Myristic	C14	02.83	00.20	00.10	00.41	04.99	00.10
Pentadecanoic	C15	00.51	00.12	00.02	00.10	02.35	00.10
Palmitoleic	C16:1	01.78	00.58	00.37	00.25	01.61	00.00
Palmitic	C16	13.19	22.87	06.20	06.01	15.19	27.99
Heptadecanoic	C17	17.45	03.79	08.50	00.00	03.48	05.25
Oleic	C18:1	15.44	65.82	23.44	86.36	66.60	30.68
Stearic	C18	05.94	00.00	04.27	00.00	00.00	14.63
Linoleic	C18:2	17.64	05.52	39.34	06.30	04.74	19.26
Linolenic	C18:3	20.85	00.00	10.37	00.00	00.00	01.10
Arachidic	C20	00.75	00.46	04.69	00.35	00.42	00.73
Behenic	C22	00.16	00.33	02.66	00.22	00.03	00.06
Total Saturated		44.29	28.08	26.48	07.09	27.05	48.96
Total Unsaturated		55.71	71.92	73.52	92.91	72.95	51.04

The caprylic and capric fatty acids were not produced by *F. cerealis* growing on malt extract medium (control medium) or in presence of the different chemical compounds used in this study while tridecanoic fatty acid was not produced in presence of only urea (Table 3). Most of the fatty acids produced by *F. cerealis* including lauric, myristic, pentadecanoic, palmitoleic, palmitic, heptadecanoic, oleic, stearic, linoleic, linolenic, arachidic, and behenic were not affected by the addition of certain chemical compounds such as aspartic acid, glutamic acid, methionine, urea, and selenium (Table 3). The fatty acid profile of *F. graminearum* showed that the fatty acid

Table 3. The fatty acid profile of *Fusarium cerealis* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.00	00.00	00.00	00.00	00.00	00.00
Capric	C10	00.00	00.00	00.00	00.00	00.00	00.00
Lauric	C12	00.13	00.02	00.02	00.04	00.02	00.02
Tridecanoic	C13	00.02	00.02	00.02	00.03	00.00	00.02
Myristic	C14	00.16	00.10	00.06	00.04	00.02	00.02
Pentadecanoic	C15	00.05	00.02	00.02	00.02	00.02	00.02
Palmitoleic	C16:1	00.10	00.08	00.09	00.06	00.04	00.10
Palmitic	C16	15.50	11.63	04.33	01.53	02.84	13.51
Heptadecanoic	C17	00.14	01.91	01.15	03.76	04.40	03.95
Oleic	C18:1	74.10	69.31	76.20	58.31	19.42	47.14
Stearic	C18	07.84	06.23	02.87	04.27	02.95	09.35
Linoleic	C18:2	00.57	05.42	04.92	23.32	02.73	03.35
Linolenic	C18:3	00.35	04.76	10.10	00.45	65.70	22.19
Arachidic	C20	00.29	00.37	00.08	08.07	00.26	00.10
Behenic	C22	00.75	00.13	00.14	00.10	01.60	00.23
Total Saturated		24.88	20.43	08.69	17.86	12.11	27.22
Total Unsaturated		75.12	79.57	91.31	82.14	87.89	72.78

Table 4. The fatty acid profile of *Fusarium graminearum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.01	00.00	00.01	00.00	00.00	00.01
Capric	C10	00.02	00.00	00.00	00.02	00.04	00.02
Lauric	C12	00.06	00.02	00.67	00.05	00.01	00.04
Tridecanoic	C13	00.00	00.00	00.00	00.00	00.00	00.00
Myristic	C14	00.07	00.06	00.08	00.06	00.36	00.10
Pentadecanoic	C15	00.02	00.00	00.02	00.00	00.34	00.08
Palmitoleic	C16:1	00.12	00.04	00.20	00.10	00.11	00.47
Palmitic	C16	13.30	05.68	09.90	04.37	14.24	56.45
Heptadecanoic	C17	02.05	05.70	00.10	03.60	02.62	00.02
Oleic	C18:1	75.31	32.40	79.20	38.76	64.47	07.72
Stearic	C18	06.28	01.78	09.17	03.37	09.24	00.04
Linoleic	C18:2	01.46	39.80	00.20	40.25	05.22	34.24
Linolenic	C18:3	00.25	14.50	00.28	00.39	00.16	00.04
Arachidic	C20	00.42	00.02	00.15	07.20	01.53	00.51
Behenic	C22	00.63	00.00	00.02	01.83	01.66	00.26
Total Saturated		22.86	13.26	20.12	20.50	30.04	57.53
Total Unsaturated		77.14	86.74	79.88	79.50	69.96	42.47

was not produced by *F. graminearum* growing on malt extract medium (control medium), or any of the treatments used in this study (4). Caprylic, capric, pentadecanoic and behenic were not produced in the presence of aspartic acid which is considered the most influential negative impact on *F. graminearum*, treatment by methionine prevented formation of both caprylic and pentadecanoic. However, only capric fatty acid was not

Table 5. The fatty acid profile of *Fusarium graminum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.05	01.15	00.02	00.06	00.00	00.04
Capric	C10	00.02	00.02	00.02	00.02	00.02	00.05
Lauric	C12	00.04	00.41	00.06	00.03	00.10	00.06
Tridecanoic	C13	00.06	00.02	00.12	00.06	00.16	00.02
Myristic	C14	00.08	00.26	00.08	00.04	00.19	00.20
Pentadecanoic	C15	00.02	00.22	00.17	00.02	00.04	00.08
Palmitoleic	C16:1	00.24	00.79	00.38	00.41	00.08	00.32
Palmitic	C16	13.32	14.54	09.57	15.11	10.49	11.40
Heptadecanoic	C17	00.94	12.11	07.63	35.48	02.85	01.37
Oleic	C18:1	73.81	26.76	48.32	11.17	66.42	30.46
Stearic	C18	07.48	10.83	05.92	07.40	04.98	02.21
Linoleic	C18:2	02.86	07.79	07.65	15.32	06.31	25.68
Linolenic	C18:3	00.76	24.79	19.65	11.99	07.99	27.96
Arachidic	C20	00.27	00.27	00.35	01.07	00.27	00.06
Behenic	C22	00.05	00.04	00.06	01.82	00.10	00.09
Total Saturated		22.33	39.87	24.00	61.11	19.20	15.58
Total Unsaturated		77.67	60.13	76.00	38.89	80.80	84.42

Table 6. The fatty acid profile of *Fusarium oxysporum*f. sp. *conglutinans* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.03	00.02	00.00	01.90	00.00	00.00
Capric	C10	00.02	00.03	00.00	00.06	00.00	00.00
Lauric	C12	01.87	00.05	02.55	01.12	00.20	00.10
Tridecanoic	C13	00.20	00.04	00.00	00.47	00.08	00.10
Myristic	C14	01.62	00.30	00.20	01.87	03.12	00.10
Pentadecanoic	C15	00.32	00.20	00.02	00.10	00.08	00.10
Palmitoleic	C16:1	02.94	00.90	09.58	00.20	03.63	00.10
Palmitic	C16	28.90	30.70	40.98	29.60	20.07	29.70
Heptadecanoic	C17	06.40	00.50	04.32	00.20	02.08	00.10
Oleic	C18:1	19.20	02.04	06.66	49.81	32.40	37.20
Stearic	C18	09.43	08.72	05.60	00.00	02.30	32.07
Linoleic	C18:2	16.61	50.50	11.37	06.33	22.80	00.20
Linolenic	C18:3	11.52	05.40	18.60	08.20	12.60	00.10
Arachidic	C20	00.82	00.40	00.10	00.10	00.56	00.10
Behenic	C22	00.12	00.20	00.02	00.04	00.08	00.03
Total Saturated		49.73	41.16	53.79	35.46	28.57	62.40
Total Unsaturated		50.27	58.84	46.21	64.54	71.43	37.60

produced in presence of glutamic acid. However, the caprylic and pentadecanoic were not produced in the presence of methionine. Caprylic was not produced in the presence of urea; only four fatty acids acids produced by *F. graminearum* were affected by the addition of certain chemical compounds (Table 4). On the other hand, fatty

Table 7. The fatty acid profile of *Fusarium pseudograminearum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.04	00.00	00.04	00.30	00.02	00.71
Capric	C10	00.58	00.02	00.20	00.10	00.04	00.02
Lauric	C12	00.51	00.50	00.43	01.66	06.64	00.33
Tridecanoic	C13	00.06	00.00	00.05	00.02	00.00	00.00
Myristic	C14	00.89	00.20	01.04	00.30	00.35	00.95
Pentadecanoic	C15	00.05	00.04	00.00	00.46	00.40	00.23
Palmitoleic	C16:1	00.76	00.51	02.11	01.08	00.66	00.49
Palmitic	C16	26.78	23.90	46.55	40.97	26.72	26.04
Heptadecanoic	C17	06.29	00.02	00.00	02.86	05.48	00.80
Oleic	C18:1	37.23	72.26	00.96	29.80	42.41	42.49
Stearic	C18	14.03	00.00	00.53	13.27	10.39	09.06
Linoleic	C18:2	08.21	00.07	47.40	06.67	01.91	11.17
Linolenic	C18:3	03.62	00.00	00.00	00.83	04.39	04.51
Arachidic	C20	00.67	01.23	00.08	01.18	00.28	00.53
Behenic	C22	00.28	01.25	00.61	00.50	00.31	02.67
Total Saturated		50.18	27.16	49.53	61.62	50.63	41.34
Total Unsaturated		49.82	72.84	50.47	38.38	49.37	58.66

Table 8. The fatty acid profile of *Fusarium roseum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.02	00.07	00.02	00.02	00.02	00.04
Capric	C10	00.08	00.05	00.04	00.38	00.04	00.08
Lauric	C12	01.50	00.14	01.06	01.10	06.21	00.10
Tridecanoic	C13	01.74	00.02	00.23	00.30	00.04	00.08
Myristic	C14	00.63	00.20	01.30	00.91	01.76	00.24
Pentadecanoic	C15	00.78	00.04	00.04	00.35	12.92	00.05
Palmitoleic	C16:1	01.40	00.39	01.13	00.20	01.03	00.30
Palmitic	C16	14.13	15.52	35.60	21.20	33.92	31.67
Heptadecanoic	C17	18.82	06.21	02.69	43.03	00.10	02.10
Oleic	C18:1	41.60	58.21	18.21	04.86	03.27	40.19
Stearic	C18	04.61	00.39	04.95	09.86	11.60	18.02
Linoleic	C18:2	14.35	18.32	15.36	17.15	08.57	04.33
Linolenic	C18:3	00.00	00.00	19.14	00.00	20.42	02.40
Arachidic	C20	00.25	00.34	00.18	00.02	00.06	00.30
Behenic	C22	00.09	00.10	00.05	00.62	00.04	00.10
Total Saturated		42.65	23.08	46.16	77.79	66.71	52.78
Total Unsaturated		57.35	76.92	53.84	22.21	33.29	47.22

acid profile of *F. graminum* was not affected by the chemical constituents of the growth medium. However, only caprylic was affected; not produced in presence of urea (Table 5).

The fatty acid profile of *F. oxysporum* f. sp. *conglutinans* was affected by the addition of certain compounds to growth medium (Table 6). Caprylic and capric were not produced when urea or selenium were added to the growth medium. However, both fatty acids in addition to tridecanoic were not produced in the presence of glutamic acid. The stearic was not produced in the presence of methionine. Most of the fatty acids produced by *F. oxysporum* f. sp. *conglutinans* including lauric, myristic, pentadecanoic, palmitoleic, palmitic, heptadecanoic, oleic, linoleic, linolenic, arachidic, and behenic were not affected by the addition of aspartic acid,

Table 9. The fatty acid profile of *Fusarium sacchari* var. *elongatum* grown on MEB medium amended with different chemical compounds.

Fatty acid		Percentage of fatty acids					
		Control	Aspartic acid	Glutamic acid	Methionine	Urea	Selenium
Caprylic	C8	00.05	00.01	00.02	00.05	00.00	00.01
Capric	C10	00.16	00.02	00.02	00.04	00.01	00.06
Lauric	C12	02.39	00.05	00.49	00.32	00.03	06.51
Tridecanoic	C13	00.10	00.00	00.00	00.00	00.00	00.00
Myristic	C14	02.08	00.85	0.52	00.43	00.35	01.80
Pentadecanoic	C15	00.10	00.11	00.00	00.16	00.40	00.10
Palmitoleic	C16:1	00.63	01.16	00.64	00.71	00.16	00.82
Palmitic	C16	20.31	66.68	56.34	38.83	68.05	14.99
Heptadecanoic	C17	24.38	01.72	00.20	02.86	00.05	12.64
Oleic	C18:1	18.99	24.38	04.36	30.92	05.26	11.86
Stearic	C18	16.67	00.10	03.62	11.93	04.78	13.46
Linoleic	C18:2	02.64	00.43	33.30	12.41	20.64	23.08
Linolenic	C18:3	11.00	03.70	00.00	00.79	00.05	12.28
Arachidic	C20	00.30	00.69	00.08	00.29	00.18	00.10
Behenic	C22	00.20	00.10	00.41	00.26	00.04	02.29
Total Saturated		66.74	70.33	61.70	55.17	73.89	51.96
Total Unsaturated		33.26	29.67	38.30	44.83	26.11	48.04

glutamic acid, methionine, urea, and selenium. On the other hand, the production of caprylic and capric was strongly affected and coined to the chemical constituents of growth medium. However, tridecanoic and stearic were slightly susceptible to the constituents of the growth medium (Table 6).

The fatty acid profile of *F. pseudograminearum* was affected by the chemical constituents of the growth medium (Table 7). Caprylic, tridecanoic, stearic, in addition to linolenic were not produced in the presence of aspartic acid. Tridecanoic was not produced in the presence of urea, and selenium as well. Linolenic was not produced in presence of aspartic acid and glutamic acid. However, pentadecanoic, heptadecanoic in addition to linolenic fatty acids were not produced in the presence of glutamic acid (Table 7). The fatty acid profile of *Fusarium roseum* was slightly affected by the chemical constituents of the growth medium (Table 8). However, linolenic was produced only in the presence of glutamic acid, urea, and selenium (Table 8).

Most of the fatty acids produced by *Fusariumsacchari* var. *elongatum* including capric, lauric, myristic, palmitoleic, palmitic, heptadecanoic, oleic, stearic, linoleic, arachidic, and behenic were not affected by the addition of certain chemical compounds such as aspartic acid, glutamic acid, methionine, urea, and selenium. On the other hand, the production of tridecanoic was strongly affected and coined to the chemical constituents of growth medium. However, caprylic, pentadecanoic and linolenic were slightly susceptible to the constituents of the growth medium (Table 9).

DISCUSSION

The results of the current study reveal that the fatty acid profiles of the investigated *Fusarium* species were varied. Most of the *Fusarium* species, seven out of nine, contained the same fatty acids but differed in the relative amounts of each, however, *F. anthophilium* and *F. roseum* differed in both the type and amount of fatty acids produced .The cellular fatty acid composition is now routinely used for the identification and differentiation of microorganisms (Lechevalier and Lechevalier, 1988; Tuner et al., 1992; Graham et al., 1995; Stahl and Klug, 1996; Bentivenga and Morton, 1996; Tighe et al., 2000; Whittaker et al., 2005, 2007; Devi et al., 2006; Koppova et al*.,* 2008; Zain, 2009; Dembitsky et al., 2010).

However, addition of certain chemical compounds to the growth medium affected both the kinds and amounts of the fatty acid composition. It is strongly believed that when employing fatty acid profiles to differentiate or compare fungi, it is important to minimize sources of variation in fatty acid composition from culture conditions (Stahl and Klug, 1996; Zain et al., 2009). The results of the present study confirmed this postulation since the fatty acid profiles changed when certain chemical compounds were added to the growth medium. However, the addition of chemical compounds to the growth medium revealed significant disparities in both the amounts and type of fatty acid composition for all the investigated *Fusarium* species.

The results of this study strongly demonstrate that the use of fatty acid composition in characterization and iden-

tification of fungi could be significantly improved by using more than two different type of growth medium.

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Full Length Research Paper

Escherichia coli from Nigeria exhibit a high prevalence of antibiotic resistance where reliance on antibiotics in poultry production is a potential contributing factor

Chijioke A. Nsofor[1], Isaac O. Olatoye[2,4], Elizabeth A. Amosun[3], Christian U. Iroegbu[1], Margaret A. Davis[4], Lisa H. Orfe[5] and Douglas R. Call[4,5]*

[1]Department of Microbiology, University of Nigeria, Nsukka, Nigeria.
[2]Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria.
[3]Department of Veterinary Microbiology and Parasitology, University of Ibadan, Nigeria.
[4]Paul G. Allen School for Global Animal Health, Washington State University, Pullman, WA, USA.
[5]Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA.

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To assess the prevalence of antibiotic resistance in Nigeria, single *Escherichia coli* isolates were collected from a geographically diverse panel of fecal samples collected from human clinical and non-clinical donors (n=77), livestock (cattle, swine, and goats) and chickens (n=71 total). There was no difference in the proportion of isolates resistant to ≥1 antibiotics from human clinical and non-clinical samples, but overall, this was significantly higher for human (85.7%) compared to animal (53.5%) isolates (*P*<0.0001). The average number of resistance phenotypes per isolate was significantly higher for human (5.0), goat (4.0), and poultry (3.4) compared with cattle (2.4) and swine (2.0) (*P*<0.05). There were 25 different resistance phenotypes with more diversity from animal compared with human isolates. A survey of management practices at 30 poultry farms in the vicinity of Ibadan found that all respondents self-milled feed and most (87.7%) routinely added antibiotics to feed. Tetracyclines were the dominant antibiotics of choice followed by tylosin and gentamicin and some use of chloramphenicol, ciprofloxacin, and enrofloxacin. If this pattern of antibiotic resistance and use is repeated across the different sectors of food-animal production and in multiple developing countries, then trade and travel are likely to disseminate resistance traits to other countries potentially negating local policies that are designed to limit selection for antibiotic resistant bacteria.

Key words: Antibiotic resistance, *Escherichia coli*, growth promotion, poultry, chloramphenicol.

INTRODUCTION

Antibiotic resistance is a significant worldwide public health concern (French, 2010; Kumarasamy et al., 2010) both because of resistance found in specific pathogens (Delsol et al., 2004; Hurd et al., 2010), and because resistance harbored by commensal organisms may serve as a reservoir of traits that can be disseminated to pathogens (Pallecchi et al., 2007; Shoemaker et al., 2001; Li and Wang, 2010). Antibiotic resistant bacteria that normally pose no immediate disease risk can also become opportunistic pathogens that complicate post-operative recovery (Patel and Kirby, 2008) or otherwise become problematic for immunocompromised individuals (Bonomo, 2001). While a number of ecological factors probably contribute to the dissemination and maintenance

*Corresponding author. E-mail: drcall@vetmed.wsu.edu. Tel: 509-335-6313. Fax: 509-335-8529.

of antibiotic resistance traits, it is clear that antibiotic use is a primary factor that selects for the evolution and amplification of antibiotic resistant bacteria in humans and animals.

The rising prevalence of antibiotic resistance is a particularly important problem in deve-loping countries where there is limited control of the quality, distribution and use of antibiotics in human medicine, veterinary medicine, and food-animal agricul-ture (Okeke et al., 1999). The relative contribution of these three sectors to the evolution and amplification of antibiotic resistance is not clearly understood (McDermott et al., 2002; Oliver et al., 2011) although it probably varies with different countries. Indeed, many of the highly complex issues in developing countries that influence antibiotic use practices in human medicine (Okeke et al., 1999) are likely to apply to both veterinary and food-animal applications.

At a broader level, the evolution and amplification of antibiotic resistance traits in developing countries should be a significant focus of attention for industrialized countries as well. For example, while policy makers in the USA debate the merits of limiting antibiotic use in food animals (DHHS, 2010b), the potential benefits of such limits could be overshadowed by amplification in developing countries and dissemination of antibiotic resistant bacteria and resistance traits through travel and trade. More efforts are needed to understand how antibiotic use practices at an international scale ultimately influence the prevalence and dissemination of antibiotic traits within individual countries.

Bacterial antibiotic resistance has been highlighted in Nigeria through a series of published studies since the 1990's (Okeke et al., 1999; Fashae et al., 2010; Akinyemi et al., 2011; Ogbolu et al., 2011) and thus Nigeria provides a model for considering how antibiotic use in different sectors of a developing country can impact the prevalence of antibiotic resistant bacteria. Our first objective was to conduct a comparative analysis of antibiotic resistance for bacteria from humans and domestic animals under the null hypothesis that the prevalence of antibiotic resistant bacteria would be equal for human and animal populations in Nigeria. We focused on *E. coli* because this species includes a broad range of both pathogenic and commensal serovars that can harbor antibiotic resistance traits of interest to human and veterinary medicine. Our second objective was to survey antibiotic use practices in poultry production. Widespread consumption of poultry by Nigerian consumers makes this sector of food production a potentially important source of antibiotic resistant bacteria in the human diet.

MATERIALS AND METHODS

Sample collection

The study population included humans (who were either ill or presumptively healthy) and a variety of presumptively healthy domestic livestock viz. cattle, goats, pigs and chicken obtained from five geopolitical zones of Nigeria viz. south-east, south-west, south-south, north-central and north-north. In the south-south and south-east, clinical specimens were collected at the University of Port Harcourt Teaching Hospital, Port Harcourt, Rivers State, and the Abia State University Teaching Hospital, Aba, Abia State, respectively. The Lagos State University Teaching Hospital, Ikeja, Lagos, was the site of specimen collection for the south-west, while the National Hospital, Abuja and Military Reference Hospital, Kaduna State, were the sources of specimens from the north-central and north-north, respectively. All fecal samples from these hospitals were clinical specimens from patients presenting with gastroenteritis. Fecal samples were also collected from apparently healthy undergraduate students at Madonna University Elele, Rivers State. For this convenience sample, individuals were limited to those reporting no exposure to antibiotics for six months prior to sampling and each person received an explanation of the study objectives and consent form for inclusion in the study.

All sampling procedures were in accordance with guidelines that are promulgated by the National Health Research Ethics Committee in Nigeria (www.nhrec.net). None of the animals included in this study (at the time of specimen collection) exhibited signs and symptoms of abnormal health. The cattle and goat specimens came from the Obinze livestock market Owerri, Imo State, while the Madonna University Poultry Elele, Rivers State, was the source of poultry specimens. The specimens from swine came from a farm located at the Ogbor-Hill area of Aba, Abia State. There was no documented evidence of antibiotics use in the farms from which the specimens were collected, although the manager of the poultry farm indicated occasional antibiotic use at this facility. In all, a single non-duplicate *E. coli* isolate was selected from each human and animal fecal sample to maximize biological independence between observations.

Isolation of *E. coli*

Fresh fecal droppings were collected from animals and care was taken to avoid collecting more than one fecal sample per individual animal. Feces were packed in a sterile plastic container and were transported to the laboratory on ice for processing and cultivation. 1 g of each animal's feces was diluted in 9 ml of 0.85% sterile saline solution. The contents were mixed thoroughly and serially diluted (10-fold) before plating on Eosin Methylene Blue agar (EMB) (Oxoid, England) to isolate Gram-negative enteric bacilli (Holt-Harris and Teague, 1916; Levine, 1918). Human fecal specimens were streaked directly on EMB agar with a sterile inoculating loop. No antibiotic was included in the EMB agar plates used for cultivation. The inoculated plates were incubated overnight at 37°C. A single isolate resembling *E. coli* (green metallic sheen on EMB) was selected from an individual fecal sample for further characterization. Identification of *E. coli* was confirmed using conventional microbiological tests; indole positive, methyl-red positive and citrate negative (Cheesbrough, 2000). Isolates were transported to Washington State University Field Diseases Investigation Unit (WSU-FDIU, Pullman, WA) where identity was further confirmed by characteristic growth on 4-methylumbelliferyl-b-D-glucuronide (MUG) supplemented with violet red bile (VRB-MUG) (Venkateswaran et al., 1996) and by an indole test.

Antibiotic susceptibility testing

The antibiotic susceptibility pattern of the isolates was determined using the disk diffusion method on Mueller-Hinton agar at the WSU-FDIU. Inhibition zone sizes were interpreted using standard recommendations of the Clinical Laboratory Standard Institute (CLSI, 2008). Susceptibility was tested against ampicillin (AM; 10 ug),

Table 1. Percentage of antibiotic resistant *E. coli* from humans and animals in Nigeria.

Antibiotic	Human			Animal				
	Clinical (n=65)	Non-clinical (n=12)	Human combined (n=77)	Cattle (n=27)	Goats (n=13)	Poultry (n=14)	Swine (n=17)	Animal combined (n=71)
Am[a]	78.5	83.3	79.2	29.6	61.5	64.3	35.3	43.7
Amc	32.3	41.7	33.8	14.8	30.8	28.6	5.9	18.3
An	0.0	0.0	0.0	3.7	0.0	0.0	0.0	1.4
C	23.1	8.3	20.8	18.5	23.1	21.4	5.9	16.9
Caz	1.5	0.0	1.3	0.0	0.0	7.1	0.0	1.4
G	83.1	91.7	84.4	37.0	69.2	64.3	35.3	47.9
Gm	13.8	16.7	14.3	11.1	0.0	0.0	5.9	5.6
K	12.3	16.7	13.0	11.1	0.0	0.0	5.9	5.6
Nal	24.6	25.0	24.7	18.5	0.0	14.3	5.9	11.3
S	67.7	66.7	67.5	22.2	61.5	35.7	29.4	33.8
Sxt	83.1	83.3	83.1	37.0	69.2	57.1	35.3	46.5
T	81.5	83.3	81.8	37.0	84.6	50.0	35.3	47.9

[a]Am = ampicillin; Amc = amoxicillin/clavulanic acid; An = amikacin; C = chloramphenicol; Caz = ceftazidime; G = sulfisoxazole; Gm = Gentamicin; K = kanamycin; Nal = nalidixic acid; S = streptomycin; Sxt = trimethoprim/sulfamethoxazole; and T = tetracycline.

amikacin (AN; 10 ug), amoxicillin/clavulanic acid (AmC; 20/10 ug), chloramphenicol (C; 30 ug), ceftazidime (CAZ; 30 ug), sulfisoxazole (G; 0.25 mg), gentamycin (GM; 10 ug), kanamycin (K; 30 ug), nalidixic acid (NA; 30 ug), streptomycin (S; 10 ug), sulfamethaxazole/trimethoprim (SxT; 23.75 ug/1.25 ug), and tetracycline (TE; 30 ug). All antibiotic discs were purchased from Becton Dickinson (Franklin Lakes, NJ). *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25932), and *Enterococcus fecalis* (ATCC 29212) were used as reference strains for culture identification and antibiotic susceptibility testing.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using XbaI (New England Biolabs) based on the PulseNet protocol with minor modifications (www.cdc.gov/pulsenet/protocols.htm). Briefly, DNA fragments were resolved by electrophoresis in 1% SeaKem Gold agarose gels with a CHEF DRII machine (Bio-Rad), using 0.5X Tris-borate-EDTA as the buffer. Gels were run for 18 h at 14°C, using a linearly ramped switching time from 2.2 s to 63.8 s and a voltage of 6.0 V/cm^2. After electrophoresis, the gels were stained in 400 ml of deionized water containing 40 ul of 10 mg/ml of ethidium bromide for 20 min on a rocker and destained three times for 20 min each with distilled water. Bands were visualized by a UV transilluminator (Fisher Scientific) and photographed using an Alpha imager (Alpha Innotech Corporation, San Leandro, CA, USA). Digitalized gel images were analyzed using BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by using the Unweighted Pair Group Method using arithmetic Averages (UPGMA) based on Dice coefficients to quantify the similarities.

Survey of poultry producers

During an eight week survey between April and June 2009, semi-structured questionnaires were administered to 30 poultry farmers randomly selected from six local governing areas of the Ibadan metropolis. Data was obtained for management practices including commonly used antibiotics, indications for use, sources and knowledge of withdrawal period and antibiotic resistant food-borne pathogens. Invariably, it was not possible to provide precise case definitions or diagnostic confirmation, but self-reported diseases could be characterized into ten categories.

RESULTS

Antibiotic resistance

In total, we collected a single isolate of *E. coli* from each of 77 human and 71 animal fecal samples (Table 1). There was less overall variance in the proportion of resistant isolates for human clinical and non-clinical isolates (average 4.1% difference) compared with the proportion of resistant isolates between different animal hosts (average 23.7% difference) (Table 1). The prevalence of resistance ranged from nearly zero for amikacin to a high of 84.4% for human isolates resistant to sulfisoxazole and a high of 47.9% for animal isolates resistant to sulfisoxazole or tetracycline. We detected resistance to ceftazidime (a third-generation cephalosporin) in one poultry isolate (7.1%).

Sample sizes precluded analysis across individual antibiotics, but it is notable that there was no statistical difference in the proportion of isolates that were resistant to ≥ 1 antibiotic for human clinical (84.6%) and non-clinical (91.7%) isolates (Fisher's exact test, P = 1.0). Human isolates were more likely to be resistant to ≥ 1 antibiotic compared with animal isolates (85.7 and 53.5%, respectively, Fisher's exact test, P < 0.0001). The average number of resistance phenotypes per isolate was significantly higher for human (5.0), goat (4.0) and poultry (3.4) compared with cattle (2.4) and swine (2.0) (one-way ANOVA, P<0.0001; Tukey-Kramer multiple comparison test, P<0.05).

Table 2. Distribution of multidrug resistance phenotypes amongst human and animal isolates collected in Nigeria.

Antibiotic resistance phenotype	Human		Animal	
	Number	Percent (%)	Number	Percent (%)
None	11	14.3	33	46.5
Am[a]	0	0.0	1	1.4
AmCGmKSxtSTAmcNalG	1	1.3	1	1.4
AmCGmKSxtSTNalG	1	1.3	0	0.0
AmCGmSxtSTNalG	2	2.6	0	0.0
AmCSxtSG	0	0.0	2	2.8
AmCSxtSTAmcG	0	0.0	3	4.2
AmCSxtSTAmcNalG	2	2.6	1	1.4
AmCSxtSTG	3	3.9	0	0.0
AmCSxtSTNalG	4	5.2	2	2.8
AmGmKAnSxtSTAmcNalG	0	0.0	1	1.4
AmGmKSxtSTAmcNalG	6	7.8	1	1.4
AmGmKSxtSTNalG	1	1.3	0	0.0
AmGmKSxtTAmcNalG	0	0.0	1	1.4
AmKSxtSAmcNalGCaz	1	1.3	0	0.0
AmSxtSNalG	1	1.3	0	0.0
AmSxtSTAmcG	15	19.5	1	1.4
AmSxtSTG	15	19.5	11	15.5
AmSxtTAmcG	1	1.3	3	4.2
AmSxtTAmcNalGCaz	0	0.0	1	1.4
AmSxtTG	8	10.4	1	1.4
AmTG	0	0.0	1	1.4
CSxtTG	3	3.9	3	4.2
G	1	1.3	0	0.0
SxtSG	0	0.0	1	1.4
T	1	1.3	3	4.2
Total	77	100	71	100

[a]Am = ampicillin; Amc = amoxicillin/clavulanic acid; An = amikacin; C = chloramphenicol; Caz = ceftazidime; G = sulfisoxazole; Gm = Gentamicin; K = kanamycin; Nal = nalidixic acid; S = streptomycin; Sxt = trimethoprim/sulfamethoxazole; and T = tetracycline.

We detected 26 different resistance phenotypes with 22 having >1 trait (Table 2). Approximately 50% of human isolates were classified into one of three resistance phenotypes that included a core resistance to ampicillin, trimethoprim/sulfamethoxazole, tetracycline and sulfixo-xazole. Resistance phenotypes for animals were more diverse with the largest proportion (15.5%) sharing the same core resistance traits as described above with the addition of resistance to streptomycin (Table 2). The PFGE analysis showed that animal isolates were interspersed without any clear host association (data not shown). Interestingly, two clusters of human isolates (n=18 and n=15 isolates) were mostly distinct from animal isolates and included both human clinical and non-clinical *E. coli*. Both clusters were comprised of isolates from geographically separated parts of the country indicating that there was no spatial separation in the distribution of antibiotic resistant *E. coli* from humans. Using an arbitrarily selected 85% similarity threshold for the PFGE data grouped 52.4% of isolates into clusters having ≥3 isolates. A slight majority of human isolates (53.3%) were found in human dominated clusters (>50%) while only 38.0% of animals isolates were found in animal dominated clusters.

Poultry practices survey

In total we interviewed 30 poultry producers to assess their animal husbandry and antibiotic use practices. The majority of producers had primary (16.7%) or secondary (36.7%) education with an additional 20% having earned a secondary education diploma or certificate. The remaining farmers had some university experience (13.3%) while 2 (6.7%) were veterinarians. Two other producers did not disclose their education experience. The majority of producers (64%) had >10 years of poultry farming experience, with an average of 14.6 years.

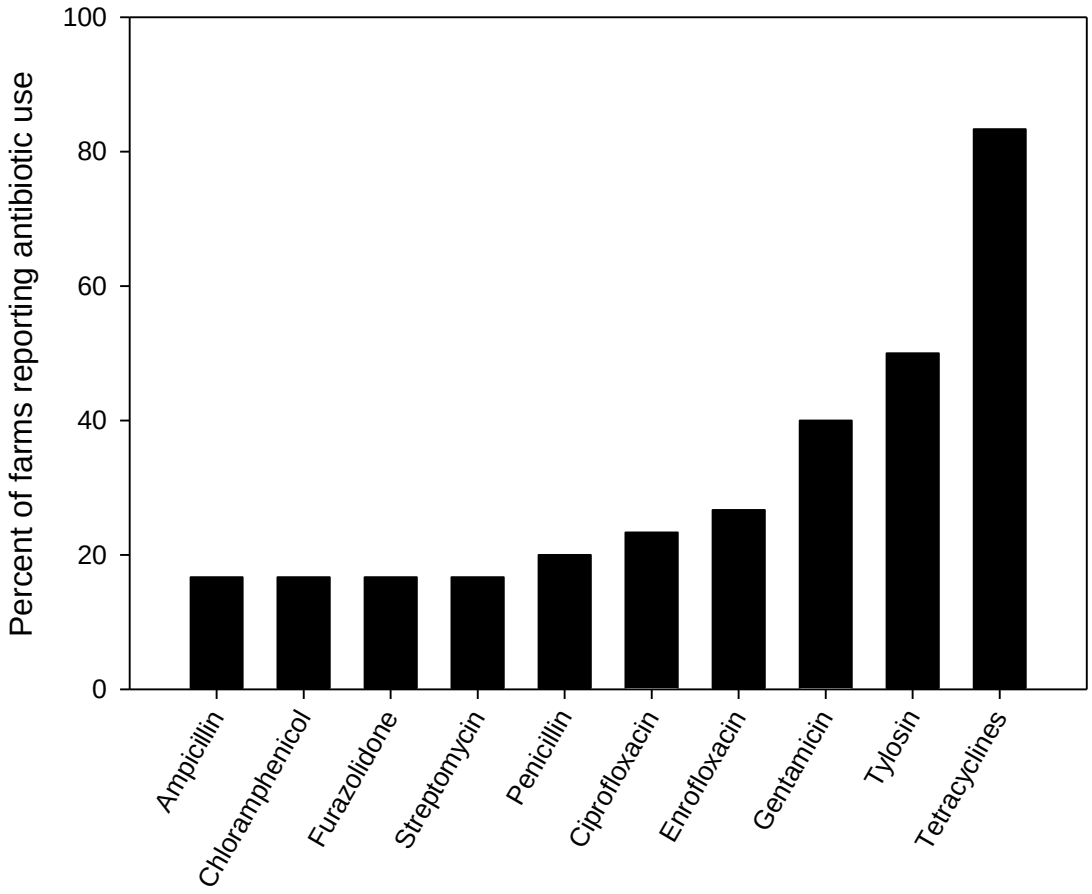

Figure 1. Percent of Nigerian poultry producers (n=30 total) reporting use of different antibiotics.

Sixty percent (n = 18) of farmers practiced a “deep litter” system while 23.3% (n = 7) practiced both deep litter and a “battery cage” system to house their flocks. The median flock population was 4,500 (range=250 to 65,000) birds with 83.3% of farmers considered “large-scale” producers. All the respondents engaged in self-milling of feed for their flocks and all farms reported use of at least two antibiotics. The majority of producers used at least three antibiotics (n=15) while seven other producers reported using between four and six different antibiotics. The majority of producers (87.7%) routinely added antibiotics to the feed for disease prevention and to improve production. Tetracyclines (oxytetracycline and chlortetracycline) were the most commonly used antibiotic (Figure 1). Approximately, 50% of producers also used gentamicin while approximately 20% of producers employed fluoroquinolones (ciprofloxacin or enrofloxacin) and chloramphenicol. A majority of respondents (86.7%) did not engage the services of a veterinarian for disease diagnosis or for drug prescriptions and use. Records of diseases and treatments were not available in most of the surveyed farms. A majority of producers (63.3%) were not aware of or did not adhere to antibiotic withdrawal periods before selling poultry products for human consumption. Producers typically purchased antibiotics from retail sources.

To better understand the motivation for antibiotic use, we also attempted to identify the most common infectious disease challenges that are faced by poultry producers and the history of antibiotic use in the flocks. The majority (93.3%) of the farmers diagnosed disease conditions based on clinical signs and post mortem findings while 6.7% engaged laboratory confirmation and antibiotic sensitivity tests for bacterial isolates. There was a diverse array of infectious disease challenges including chronic respiratory disease (etiology unknown) and more specific bacterial and viral diseases (Figure 2).

DISCUSSION

One of the strengths of the current study is that we restricted our analysis to only one *E. coli* isolate per fecal sample thereby maximizing biological independence between isolates and thereby limiting bias from any single individual. The independence between isolates was consistent with the generally high diversity of PFGE

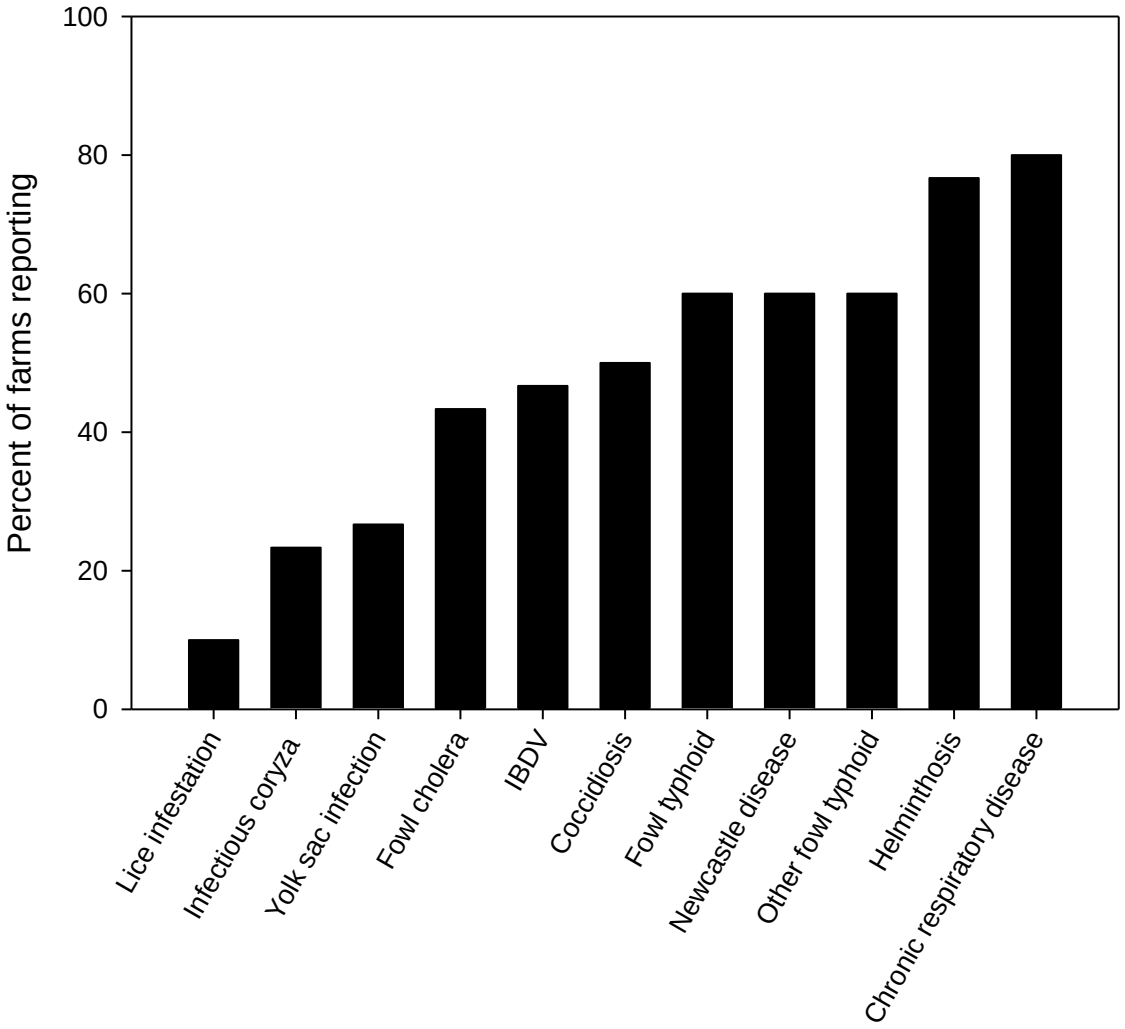

Figure 2. Percent of Nigerian poultry producers (n=30 total) reporting occurrence of different infectious disease or parasite problems. Disease classifications include chronic respiratory disease (cough; suspect *Mycoplasma*); helminthosis (parasitic worm infestation); Newcastle disease (suspect avian paramyxovirus); coccidiosis (bloody diarrhea caused by bacterium or protozoa); infectious bursal disease virus (IBDV); fowl cholera (pasteurellosis); yolk sac infection (salmonellosis or colibacillosis); infectious coryza (suspect *Hemophilus paragallinarum*); other fowl typhoid (suspect non-bloody diarrhea salmonellosis); lice infestation.

profiles detected in this study. Human isolates were mostly distinct from animal isolates by PFGE, and the human isolates were collected from multiple geographic locations suggesting that there are probably distinct sources of *E. coli* that colonize humans while animal isolates are more diverse in origin. It is also important to note that human clinical isolates can represent a potential source of bias because isolates collected at hospitals may have been enriched in numbers due to antibiotic treatment prior to collection. If this was a significant bias in our study we would expect the proportion of resistant strains to be significantly lower for non-clinical isolates. Nevertheless, while our non-clinical isolate collection was limited in number, the proportion of antibiotic resistant *E. coli* was not different from clinical samples.

Overall, it is clear that *E. coli* isolates from humans were disproportionately more resistant to antibiotics (85.7%) compared with *E. coli* isolates from animals (53.5%). Furthermore, the majority of antibiotic resistant isolates expressed multidrug resistance phenotypes (97 and 89.5% for human and animal isolates, respectively). While it is impossible, from our data, to estimate selection coefficients for different antibiotic resistance traits, the difference in proportion of resistance from human and animal isolates is consistent with a higher degree of selection in the human population. It is also notable that the *E. coli* collected from livestock and poultry came from adult- or market-age animals and is generally higher than reported for similar studies in developed countries (Khachatryan et al., 2004; Sato et al., 2005; Cho et al., 2007).

Thus, the high level of resistance documented for livestock and poultry in the current study is not biased by the tendency for younger animals to harbor disproportionately more resistant *E. coli*. On average, resistance among animal isolates was ordered with swine < cattle < poultry < goat. This finding combined with the diversity of resistance phenotypes across different animal groups (Table 1) is consistent with distinct ecologies and husbandry practices between different food animal sectors, although unequal sampling of animal populations across geographic space is a confounding factor in this study. Regardless, our samples were collected from facilities that purportedly used little or no antibiotics. If correct, our findings highlight the fact that once antibiotic resistant traits are circulating in a population, a variety of factors may favor their long-term persistence even in the absence of antibiotic selection pressure (Khachatryan et al., 2006; Singer et al., 2006).

The Nigerian livestock industry provides about 94% of the animal protein that is consumed in Nigeria and this sector of the economy contributes 4-5% of the national gross domestic product (FGN, 2009). The poultry sector accounts for about 25% of local meat production in Nigeria and poultry represents a source of quality protein and employment for a sizeable proportion of the populace. About 20% of the poultry population in Nigeria is reared within intensive commercial systems, most of which are located in the southwestern Nigeria with Ibadan being the main entry point for imported poultry products. This is also the location of the major poultry breeders and retailers that distribute poultry around the country and beyond (Owoade et al., 2004). Consequently, poultry represents an important component of the Nigerian diet and a potentially important source of selection for antibiotic resistant bacteria.

Our surveys found that the majority of the poultry farmers routinely used antibiotics as feed additives to prevent and treat infections and to boost production without veterinarian oversight. These practices, which appear to be common among developing countries (Mitema et al., 2001; Kabir et al., 2004), contribute to both selection for antibiotic resistance and probably introduce antibiotic residues into local food products (Riviere and Spoo, 1995). The most widely used antibiotic reported by poultry farmers was a tetracycline compound, which is consistent with other studies in developing countries (Al-Ghamdi et al., 2000; Nonga et al., 2010). Respondents to our survey indicated that they used several antibiotics that could be problematic from a public health perspective. For instance, the U.S. Food and Drug Administration has prohibited use of chloramphenicol in food animals (DHHS, 2010a) in part because residues in meat products can cause aplastic anemia and other blood disorders in humans (Fraunfelder et al., 1993; Young and Alter, 1994). Consequently, this practice represents a genuine public health risk beyond selecting for antibiotic resistant bacteria and it will certainly contribute to trade complications. A number of producers also used either ciprofloxacin or enrofloxacin, both of which are fluoroquinolones. In the U.S, fluoroquinolones have been banned from use in poultry primarily because of the potential to select for ciprofloxacin resistant *Campylobacter jejuni* (Delsol et al., 2004; DHHS, 2005; Hurd et al., 2010). In general, fluoroquinolone use should be limited whenever possible because of the high probability of *de novo* resistance arising from simple chromosomal point mutations. There is also growing evidence of proliferation of quinolone resistance in Nigeria including detection of horizontally transmitted resistance traits (Aypak et al., 2009; Fortini et al., 2011).

It is important to note that the Nigerian poultry industry is constantly faced with challenges of high input costs, low egg production, diseases and pests, low and poor performing breeds, poor weight gain/feed conversion, feeding and management problems and lack of capital (Isiaka, 1998; van Eekeren et al., 2004). Fowl typhoid is one example of a significant problem for poultry producers in developing countries; in Nigeria an estimated 18.4% of flocks are affected (Mbuko et al., 2009). Prevention and treatment of salmonellosis and other bacterial infections are major challenges of profitable poultry production and this is a primary motivation for the heavy reliance on antibiotics (Kabir et al., 2004). In developing countries including Nigeria, antibiotics are easily acquired and used without veterinary oversight (Dina and Arowolo, 1991; Kabir et al., 2004). While potentially important to sustain production, these practices most likely contribute to development and expansion of antibiotic resistance.

In 2001, the WHO launched the first global strategy to develop antibiotic use and resistance surveillance programs and networks across several regions of the world (Simonsen et al., 2004). There is no coordinated effort, however, to monitor or control antibiotic resistance in Nigeria and there is no coordinated intervention effort to provide resources and education to livestock and poultry producers to help them limit their need for antibiotics. Clearly, there are multiple opportunities for meaningful interventions to improve production and animal welfare in Nigeria while reducing the dependence on antibiotics for successful food production. This effort is probably needed within most developing countries before efforts to control antibiotic resistance within any single country are going to prove effective.

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Full Length Research Paper

Environmental influence on cultivable microbial community in the sediment of Sundarban mangrove forest, India

Subhajit Das[1]*, Minati De[2], Tapan Kumar Jana[1] and Tarun Kuma De[1]

[1]Department of Marine Science, Calcutta University, 35 B.C.Road, Kolkata -700019, WB, India.
[2]Maniktala Siksha Bhavan, Calcutta, India.

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In the Sundarban Mangrove forest, microbial activities are dominantly involved both in the mineralization and decomposition processes that regulate nutrient profile of sediment of different depth. It was found that besides changing the water and sediment quality, monsoonal cycle plays a crucial role to regulate microbial population distribution in the mangrove sediment. Statistical analysis revealed that organic carbon was the most significant factor that regulates the total microbial population. The cellulose degrading bacteria, [mean value of CFU 14.320×10^6 (gm dry weight of sediment)$^{-1}$] was dominant throughout the year. The sulphate reducing bacteria showed an increasing trend along depth with a minimum value at the surface that is 6.113×10^6 (g dry weight of sediment)$^{-1}$ and 12.312×10^6 (g dry weight of sediment)$^{-1}$ at a depth of 60 cm. Both rooted and deep mangrove forest regions showed distinct stratification of microbial population and nutrient distribution whereas the un-rooted regions did not show any such stratification which may be attributed to frequent wave and tidal action that make it a higher energy zone. Intensification of monsoonal cycle could heavily affect microbe dominated sediment biogeochemistry and subsequent change in the regional ecology of Sundarban mangrove forest.

Key words: Sundarban Mangrove, microbial population, monsoonal cycle, nutrient concentration.

INTRODUCTION

Mangroves are highly productive marine ecosystem where bacteria actively participate in biomineralization and biotransformation of minerals (Gonzalez-Acosta et al., 2006). The distribution of microbial activities in estuarine systems is clearly complex and variable. Much research remains to be done in order to define the distributions of microbial activities and the major factors involved in controlling these distributions in estuaries. Leaves and wood provided by mangrove plants to the sediment are degraded primarily by large variety of microbes which actively participated in the heterotrophic food chain (Thatoi et al., 2012; Alongi et al., 1989, 1993; Alongi,1994). Major products of general recycling of organic matter are detritus which is rich in enzymes and proteins and contains large microbial population (Holguin et al., 2001). Bacteria are the major participants in the carbon, sulphur, nitrogen and phosphorous cycles in mangrove forest (Toledo et al.,1995,Vazquez et al., 2000; Rojas et al., 2001). Bacterial activity is responsible for most of the carbon recycling in mangrove sediment under both oxic and anoxic condition (Das and Dangar, 2008). Many species of phosphate solubilizing rhizosphere

*Corresponding author. E-mail: subhajit_310@yahoo.com. Tel: +919038752251.

bacteria associated with black mangrove roots were found. The mechanism for phosphate solubilization probably involves the production of several organic acids (Vazquez et al., 2000).

Saprophytic fungi are fundamental to many aspects of decomposition and energy flow in mangrove forests (Nedwell, 1982; Radhakrishnan et al., 2011). Most investigation of anaerobic metabolism in natural ecosystem have dealt with sulfate rich marine sediments where sulfate reduction is the dominating process or eutrophic lake sediments where sulfate and nitrate is depleted in the hypolimnionn and in the superficial sediment layers leaving terminal carbon mineralization principally to methane producing bacteria (Sahoo and Dhal, 2009; Senior et al., 1982; Lovley and Klug, 1982). Sulfate reduction, methane production, and denitrification are the important processes for the terminal electron removal during decomposition of organic matter in anoxic environment. The methanogens are characterized by their ability to produce methane from hydrogen and carbon di oxide, formate, acetate, methanol, etc (Chen et al., 2010; Mohanraju and Natarajan, 1992). Methanotrophs are a subset of a physiological group of bacteria known as methylotrophs. They are unique in their ability to utilize methane as a source of carbon and energy (Liebner et al., 2008; Chen-rul et al., 2003). Nitrogen fixing bacteria are the other group of bacteria that are involved in formation of ammonia or organic nitrogen from atmospheric nitrogen. They may be free living or symbiotic in nature. It has been studied that N_2 fixation by heterotrophic bacteria are generally regulated by specific environmental factors like oxygen, combined Nitrogen and the availability of carbon source for energy requirement (Teri and Mary, 2005). Aerobic, autotrophic nitrifiers oxidize ammonia to nitrite and nitrate, with molecular oxygen as electron acceptor (Nicol et al., 2008). Nitrite and nitrate are reduced to dinitrogen gas by heterotrophic denitrifying bacteria that use NO_x instead of oxygen as electron acceptor (Hayatsu et al., 2008; Riley et al., 1995).

The purpose of the present study was to examine seasonal and spatial variations in microbial population (bacteria and fungi) in mangrove soil and to find out the correlation between different microbes with nutrients.

Study area

Sundarban Mangrove forest is located geographically in between 21° 31´ N and 22°30´ N and longitude 88° 10´ E and 89° 51´ E along the North East coast of Bay of Bengal, India. This mangrove forest is a part of the estuarine system of the River Ganges, NE coast of Bay of Bengal (Figure 1), which covers 9630 km^2, out of which 4264 km^2 of inter-tidal area, covered with thick mangroves, is subdivided as forest sub-ecosystem and 1781 km^2 of water area as aquatic sub-ecosystem. The tide in this estuarine complex is semidiurnal in nature with spring tide range between 4.27 and 4.75 m and neap tide range between 1.83 and 2.83 m. It is a unique bioclimatic zone in land ocean boundaries of Bay of Bengal and the largest delta on the globe. Several numbers of discrete islands constitute Sundarbans. One of these Islands, Lothian Island covering an area of 38 km^2 has been notified as a sanctuary and is situated at the confluence of Saptamukhi River and Bay of Bengal. In the southern part of the island, the ground level is high while in the northern areas, the land is low and gets inundated during highest high tide. Mangroves, *Avicennia alba*, *Avicennia marina* and *Avicennia officinalis* are the dominant species, *Excoecaria agallocha* and *Heritiera fomes* are thinly distributed and *Ceriops decandra* is found scattered all over the island. The deltaic soil of Sundarban Biosphere Reserve comprises mainly with saline alluvial soil consisting of clay, silt, fine sand and coarse sand particles. It is described as very deep, poorly drained, fine soils occurring on level to nearly level lower delta with loamy surface, severe flooding and very strong salinity (extensive extent) associated with very deep, very poorly drained, fine loamy soil. Sediment samples were collected from different sites namely rooted, un-rooted and deep forest regions at Lothian Island to critically examine the spatial variations of depth integrated microbial diversity in these mangrove ecosystem.

MATERIALS AND METHODS

Sediment cores were collected aseptically using a hand -held stainless steel core sampler (3.2 cm diameter, 100 cm long) from the different tidal zone of Sundarban Mangrove Forest and from different depth (0-10, 10-20, 20-30, 30-40, 40-50, 50-60 cm) during pre-monsoon, monsoon and post monsoon. Samples were collected into sterilized container and were transferred to laboratory in iced condition for both chemical and microbiological assay. The three tidal zones were sediment from dense forest region, sediment from the region with pneumatophores near mid littoral zone (rooted), and the sediment from the lower littoral zone where no pneumatophores were found (un-rooted).

Quantification of bacteria and fungi

Sediment samples were stored at 4°C immediately after collection and transported to the laboratory, for analysis with adequate care. 10 g of sample from different depth of different regions were homogenized with sterilized phosphate buffer solution. Serial dilutions up to 10^{-4} were made and inoculation was done with 0.1 ml. Quantification of bacteria and fungi from mangrove sediments was carried out by spread plate method for different type of bacteria such as phosphorous solubilising bacteria (PSB), cellulose degrading bacteria (CDB), nitrifying bacteria, free living nitrogen fixing bacteria and fungi and they were incubated at different condition (Ramanathan et al., 2008). Sulfate reducing bacteria was cultured in Starkey's medium in anaerobic condition (Fathul et al., 2008).

Sediment quality measurement

Sulphate, nitrite-nitrogen, nitrate-nitrogen, phosphate, silicate

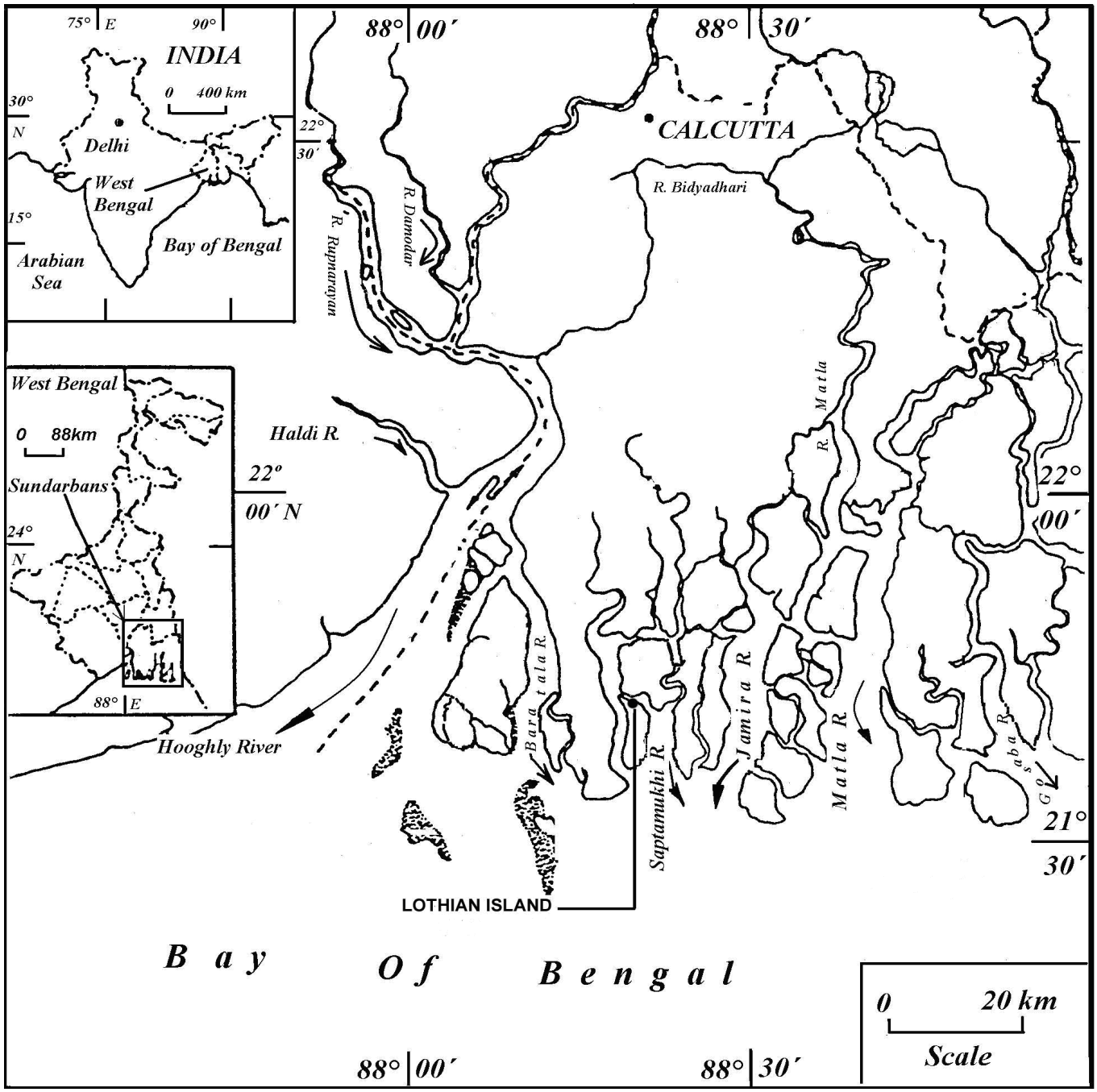

Figure 1. Map showing the location of study area

concentration of the sediment sample was measured at 10 cm interval (from 0 to 60 cm depth). 30 g of soil subsample was collected from the different depth and was immediately extracted in 75 mL of 2 mol L^{-1} potassium chloride (KCl). The mixture was shaken until well mixed and allowed to stand overnight (Riley et al., 1995). After 24 h, 4 mL of the supernatant was collected for the estimation of different nutrients using standard spectrophotometric methods (Grasshoff, 1983). The pH value was measured in a 1:5 (w/w) soil water suspension using electric digital pH meter (Tiwari et al., 1989) and soil organic carbon was measured by standard methods (Walkley and Black, 1934).

RESULTS AND DISCUSSION

Mangrove sediment at Indian Sundarban showed seasonal variation with respect to both major nutrient concentrations and microbial population. Beside monsoonal addition of nutrients to the system mangrove, litters also played a significant role in regulating the nutrient status that in turn controls the microbial population (Das et al., 2012). Among several physical factors

tidal inundation, wave action, presence of mangrove roots and bioturbation are the important factors considered for determining microbial abundance in the mangrove sediment from surface to a depth up to 60 cm (Laing et al., 2009; Bharathkumar et al., 2008). During pre-monsoon, nutrient concentration in soil sample of deep forest region showed very weak stratification from surface to 30 cm of depth with almost uniform distribution. Intense bioturbation up to 30 cm depth by several benthic organisms could cause uniform mixing of soil nutrients. No significant variation of silicate concentration was found throughout the entire depth.

Again silicate concentration was found to be more in unrooted and rooted region than that of deep forest region, because rooted and un-rooted region is at the nearest part of the river and the ultimate source of silicate is riverine system. Gradual decrease in organic carbon and phosphate-phosphorous concentration was observed from 30 to 60 m. During transportation of organic matter from surface to bottom, it is decomposed by microbes. As a result, organic content of soil decreased with increasing depth (Kristensen et al., 2008). Organic carbon in the deep forest sediment was found to be more than that of rooted and un-rooted region. It could be attributed to mangrove litter fall with an annual rate of 1603 g m^{-2} $year^{-1}$ (Ghosh et al., 1990). Both rooted and un-rooted regions regularly experience significant tidal flushing which carried away significant amount of mangrove litter. In contrary, the deep forest seldom gets inundated by tidal water resulting to organic carbon rich sediment. In all the sediments, organic carbon was found maximum during monsoon followed by post monsoon and pre-monsoon (Figure 2a).

The microbial population was also found maximum in the deep forest sediment relative to the other two sites (Figure 2a). Nitrate-Nitrogen concentration was increased from surface to 40 cm of depth but decreased from 60 to 40 cm. Vertical movement of materials, nutrient cycling and reuse driven by various burrowing organisms could have an effect on this nitrate-nitrogen distribution along the depth profile up to 40 cm. Less abundance of bioturbation below 40 cm could enhance the anoxic condition which in turn initiate denitrification causing sudden depletion of nitrate-nitrogen. The nitrite-nitrogen concentration showed no significant variation throughout depth but slight increased below 50 cm of depth which may be an indication of denitrification. Population of SRB was found to be increased with increasing depth. The Eh value of surface soil and soil from 60 cm of depth was found to be -78 mV and -163 mV respectively. Thus, more anoxic condition preferred the more population of SRB in the bottom soil than that of surface soil (Brune et al., 2000*;* Sass et al., 1997). Fungal population showed decreasing trend with increasing depth. Freeliving nitrogen fixing bacterial population that showed from surface to 30 cm depth increased again from 30 cm to 50 cm of depth. After the death of plant, the woods are carried carried away by tidal action or consumed by herbivorous animal but the root that remains attached to the bottom soil below the 50 cm depth seldom may act as the source of corbon to fungus and cellulose degrading bacteria (Figure 2a).

During monsoon, nitrate-nitrogen organic carbon content of soil showed decreasig pattern along with decrease in population of nitrifying bacteria with increase in depth. Silicate concentration showed little variation with increasing depth. Population of PSB was found to decreased with increasing depth and at same time, phosphate-phosphorous concentration was also decreased with increase in depth. Population of CDB decreased with increas in depth as the organic carbon content of the soil was also decreased with increase in depth (Zemin et al., 2010). Population of SRB showed increase in trend as Eh value found in the surface and below 60 cm of depth were -83 mV and -169 mV (Figure 2b).

During postmonsoon, nitrate-nitrogen organic carbon content of soil showed decreasing pattern along with decrease in population of nitrifying bacteria with increase in depth. Silicate concentration showed little variation with increasing depth. Population of PSB was found to be decreasing with increasing depth and at a same time phosphate-phosphorous concentration was also decreased with increasing depth. Free living nitrogen fixing bacteria showed decrease in population upto 30 cm of depth but below 30 cm to the next 60 cm of depth their population was increased. Population of CDB decrased with increase in depth as the organic carbon content of the soil was also decreased with increase in depth. Sulfate concentration did not show distinct stratification though population of SRB showed increase in trend as Eh value was found on surface and below 60 cm of depth were -87 mV and -198 mv (Figure 2c).

In the region of pneumatophores or rooted region during pre-monsoon, organic carbon content of soil showed decreasing pattern along with decrease in population of CDB from surface to 30 cm of depth and from 30 cm to the next 60 cm of depth, reverse image was found. Same profile was found for population of PSB and Phosphate-Phosphorous concentration. Silicate concentration showed little variation with increasing depth. Free living nitrogen fixing bacteria showed no gradiation along the deph. Sulfate concentration did not show distint stratification though population of SRB showed increase in trend as Eh value was found to surface and below 60 cm of depth were -89 mV and -198 mV (Figure 3a). Other nutrient and microbial population showed little gradiation along the depth. This may be due to perforation of soil because of presence of pneumatophores (Figure 3a).

During monsoon season, sediment of rooted region showed more or less uniform distribution of nutrients along depth profile. During that season, effective tidal force becomes more active to mix up the nutrients vertically. Sulfate concentration increased suddenly at

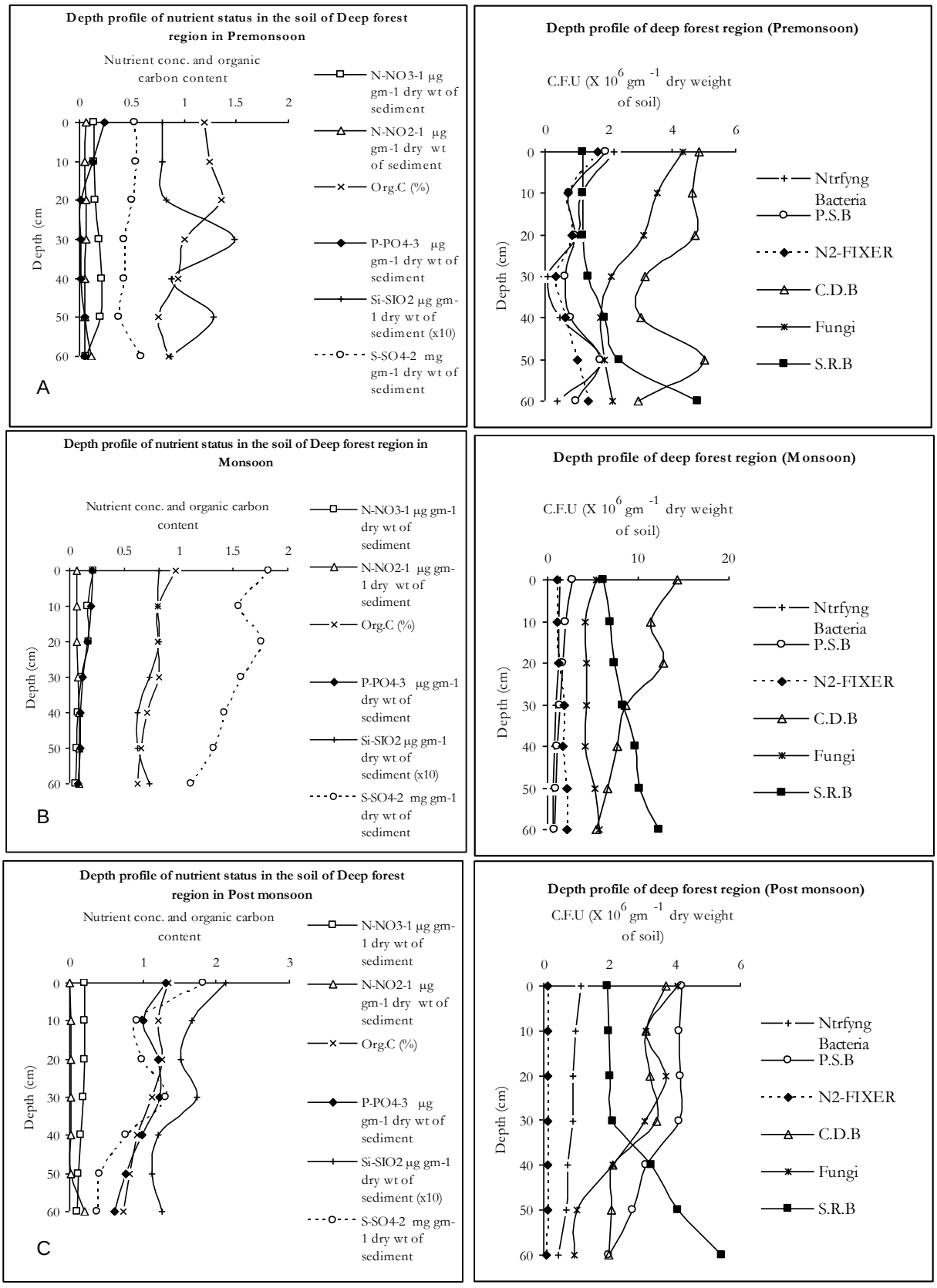

Figure 2. Graphical representation of deep forest region that shows profile of nutrient concentration and CFU of microbes of different category along depth profile during during pre monsoon **(a)**, monsoon (**b**) and post monsoon (c) respectively.

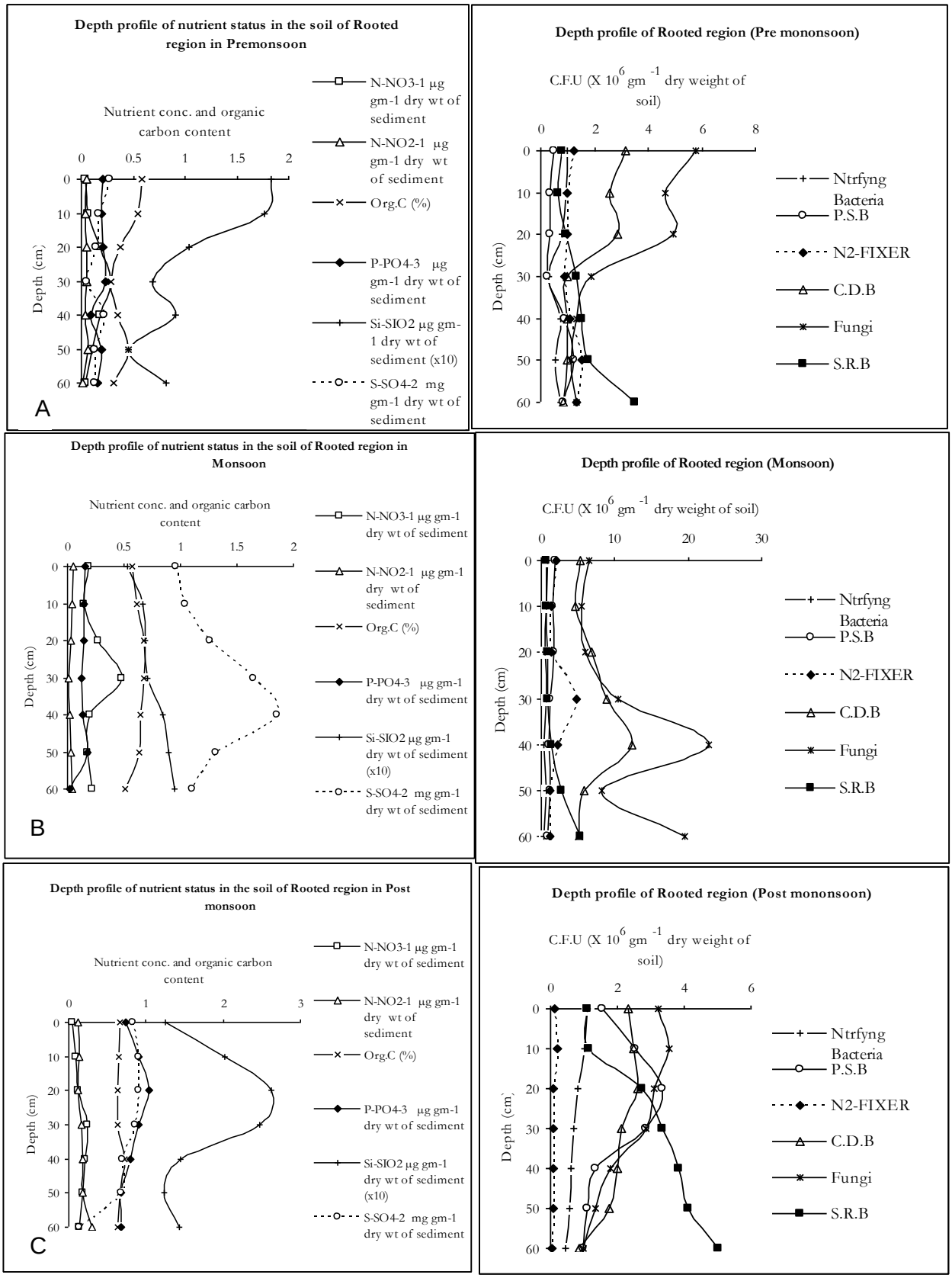

Figure 3. Graphical representation of rooted region that shows profile of nutrient concentration and CFU of microbes of different category along depth profile during during pre monsoon **(a)**, monsoon (**b**) and post monsoon (c) respectively.

about 40 cm and it may be due to sudden intrution of inorganic sulfate from remote environment. Again C.F.U of SRB showed increase in their population from 30 to 50 cm of depth. Same type of trend was also found for population of fungus and CDB. It may be due to accumulation of such group of bacteria through the pores present in that zone (Figure 3b).

During postmonsoon, uniformity of nutrient concentration was found with increase in depth. Random change in silicate concentration was found along depth profile. Same time, fungal population, population of cellulose decomposing bacteria and population of nitrifying basteria showed decreasing pattern with increasing depth. Free living nitrogen fixing bacteria showed more or less same population with increasing depth. Population of sulfate reducing bacteria was found to be increased with increase in depth (Figure 3c).

During pre-monsoon sulfate concentration was found to decrease with increasing depth followed by gradual increase of CFU of sulfate reducing bacteria. This may be due to more anoxic condition which is evident from sediment decreasing Eh value with increasing depth (Feng et al., 2003). Eh value was found maximum and minimum at the surface and 60 cm depth of un-rooted region with a value of -64.7 mV and -116.9 mV, respectively. Nitrite concentration did not vary with depth profile. Phosphate, nitrate, organic carbon content was found to show no proper gradation. Population of free living nitrogen fixing bacteria was found to show increase with depth. Other group of microbes showed no distinct gradation with increase in depth (Figure 4a).

During monsoon, organic carbon content and nitrate concentration showed a decreasing trend with increasing depth (Figure 4b). Monsoon played an important role in regulating the microbial population in Sundarban mangrove sediment. During post monsoon, no distinct gradation was found with respect to nutrient conce-tration and organic carbon content of soil along the depth profile. Microbial population showed same profile like nutrient concentration and organic carbon content of soil along the depth profile but only fungal population was found to decrease with increasing depth (Figure 4c).

Annual mean microbial population for rooted region (14.57±12.42 C.F.U $\times 10^6$) was found relatively higher than un-rooted region (16.29±6.29 C.F.U $\times 10^6$). The annual range of total microbial population fluctuated to maximum at the un-rooted exposed region (22.24±9.46 C.F.U $\times 10^6$) and minimum in the deep forest region (15.21±6.14 C.F.U $\times 10^6$). This indicates the occurrence of relatively stable condition over the deep forest region than that of the other two sites (Figure 5).

In the deep forest region, the most dominating group of microbe was found to be cellulose decomposing bacteria because that region contained more organic carbon content than that of the other two regions. Fungal population was less with respect to the other two regions. It may be attributed to less water content in deep forest region than that of other two regions. Population of SRB also showed more in proportion due to anoxic condition. In rooted region, fungal population was found to be the most dominant. CDB and SRB showed more or less same relative abundance. Population of PSB and free living nitrogen fixing bacteria was more in proportion than population of nitrifying bacteria. In un-rooted region, fungal population was found to be the most dominating group of microbe. The second highest was SRB. Un-rooted region was found to retain less organic carbon content for which population of CDB was less than population of fungus and SRB. Un-rooted region is inundated by water in most of the time and it increases water content of soil. More water content caused increase in fungal population. Anoxic condition also created ideal condition for much population of SRB (Figure 6).

Organic carbon content of the soil was found to be most significant on the growth rate of cellulose decomposing bacteria (Pearson correlation of Org.C (%) and C.D.B. (C.F.U $\times 10^6$) = 0.500 P-Value = 0.000). The population of cellulose decomposing bacteria was found to be more in monsoon period than that of pre-monsoon and post monsoon. Again, the zone with more population of phosphate solubilising bacteria showed more concentration of available phosphate. Presence of phosphatase enzyme within such type of bacteria might be responsible for those findings (Cheng and Zhiping, 2007). It might be for availability of more organic carbon source. Rooted region showed a little stratification of nutrients along with microbial population with increasing depth. Rooted region makes the soil perforated for which during high tide nutrients and microbes present in sea water get mixed with soil vertically.

Again the little stratification in that region may be due to absorption of nutrients readily by the roots present in that region. Un-rooted region showed insignificant stratification of nutrient concentration and also for microbial population in those three seasons. That region experiences daily tidal action with great tidal wave and that high energy facilitates to mix the soil vertically (Cyr, 1998). Sulfate reducing bacteria was found to be correlated with sulfate concentration of soil sample (Pearson correlation of Sulfate-Sulfur (mg g^{-1} dry wt of sediment) and S.R.B (C.F.U $\times 10^6$) = 0.595 P-Value = 0.000). Phosphate solubilizing bacteria was also found to be correlated with phosphate concentration of the Sundarban mangrove soil (Pearson correlation of phosphate-phosphorous (µg g^{-1} dry wt of sediment) and P.S.B (C.F.U $\times 10^6$) = 0.766 P-Value = 0.000). No such correlation was found for nitrogen fixing bacteria with nitrate and nitrite concentration.

Conclusion

From the present study, the following conclusions have

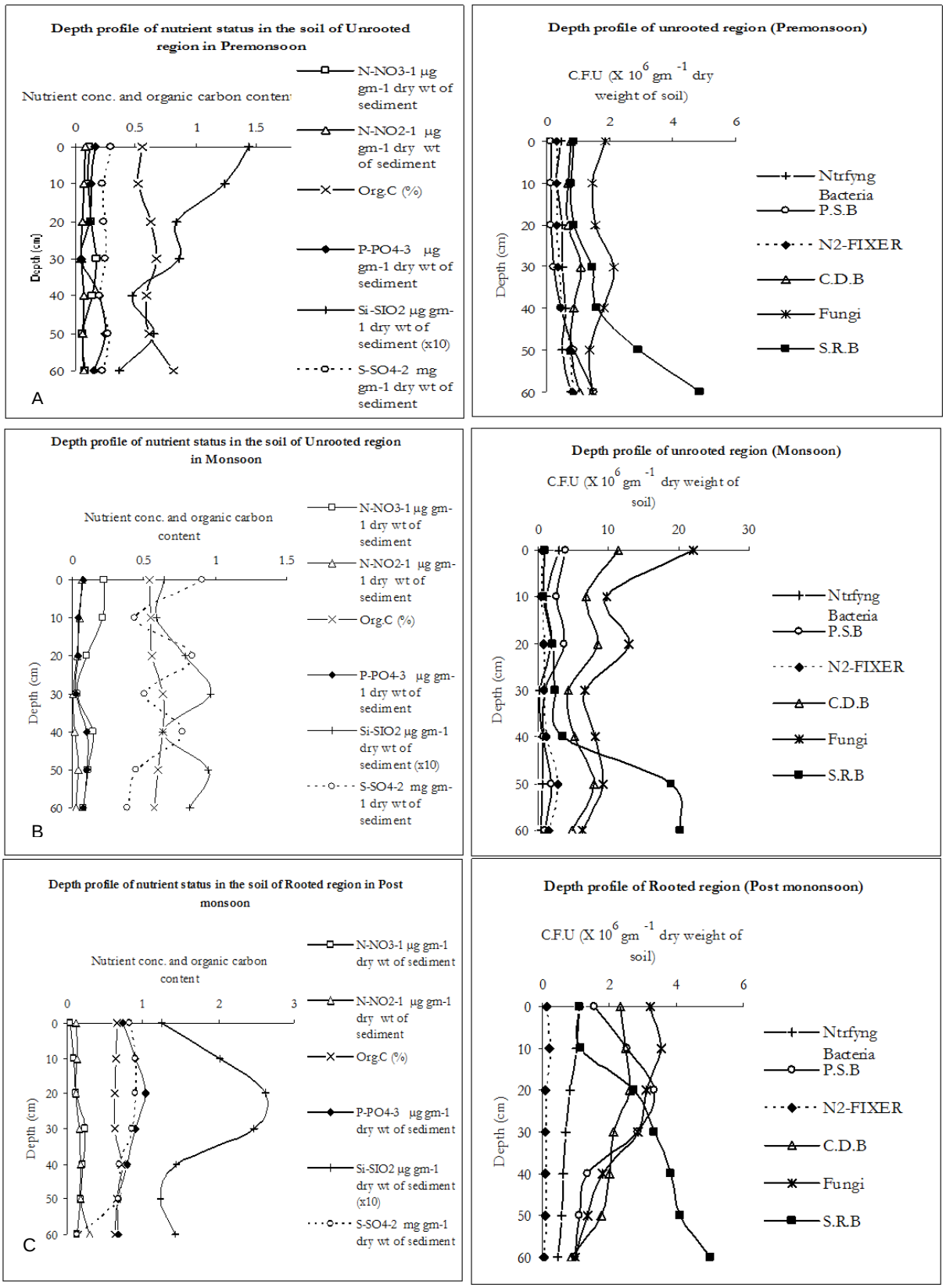

Figure 4. Graphical representation of deep forest region that shows profile of nutrient concentration and CFU of microbes of different category along depth profile during pre monsoon (a), monsoon (b) and post monsoon respectively (c).

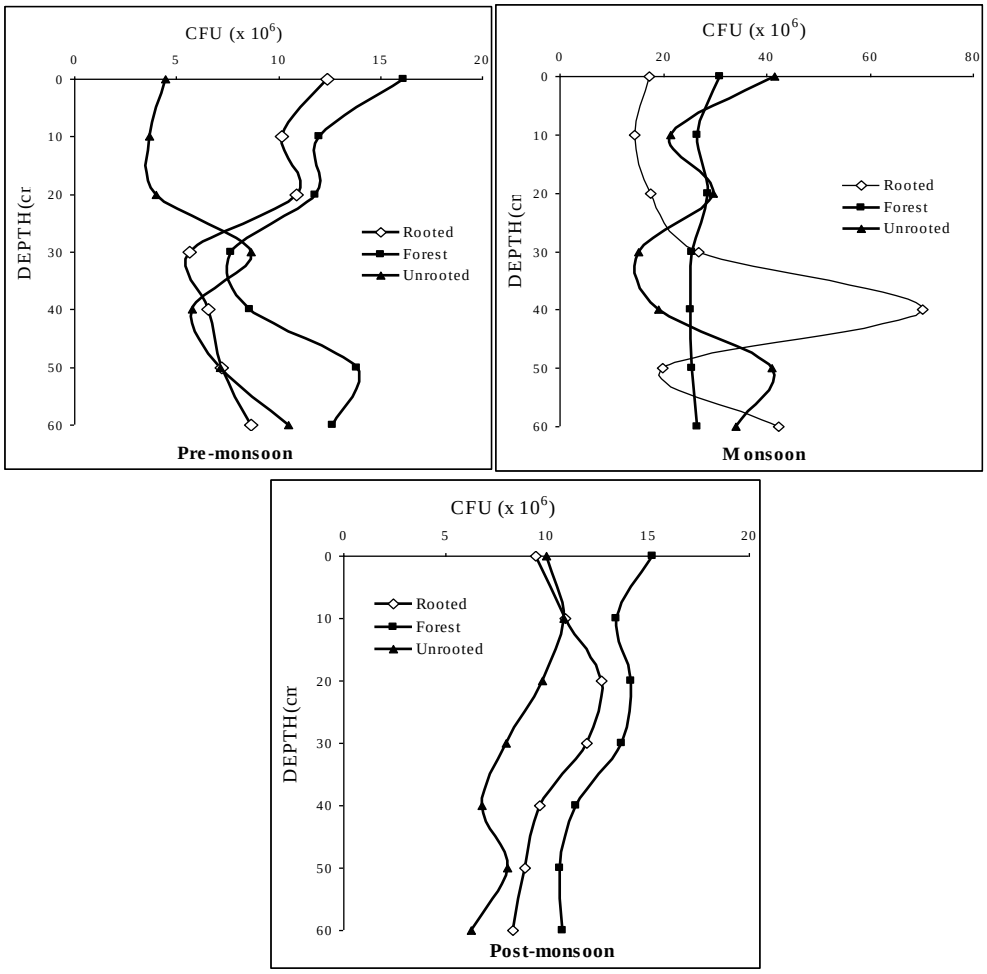

Figure 5. Depth profile of total CFU in 3 different seasons.

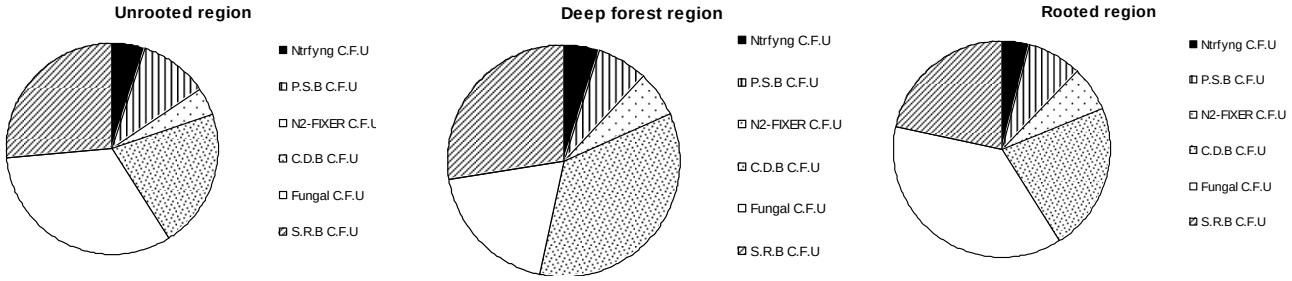

Figure 6. Relative abundance of microbial population at different site.

been drawn as a result of our research on depth integrated microbial diversity pattern of Sundarban Mangrove forest, along the shore of North East coast of Bay Of Bengal, India:

1) For in deep forest region, a decrease in nutrient concentration was observed with increase in depth below 30 cm. Active bioturbation could result in vertical mixing of nutrients up to a depth of 30 cm below which effect of bioturbation become insignificant.

2) The overall concentration of organic C was found more in deep forest region than that of rooted and un-rooted

region. It might be attributed to undisturbed supply of mangrove litter which converts this zone to most suitable for microbial population.

3) Organic carbon content of the soil was found to be most significant on the population of cellulose decomposing bacteria (Pearson correlation of Organic C (%) and C.D.B C.F.U ($\times 10^6$) = 0.500 P-Value = 0.000).

4) The zone with higher population of phosphate solubilising bacteria showed more concentration of available phosphate could be attributed to significant activity of phosphatase enzyme (Cheng and Zhiping, 2007). Phosphate solubilizing bacteria was also found to be correlated with phosphate concentration of the Sundarban mangrove soil (Pearson correlation of Phosphate-Phosphorous µg g^{-1} dry wt of sediment and P.S.B C.F.U (×106) = 0.766 P-Value = 0.000). No such correlation was found for nitrogen fixing bacteria with nitrate and nitrite concentration.

Organic carbon from the leaves, wood from forest and other organic dead or waste products from other living creatures are easily degraded by cellulose degrading bacteria in the mangrove sediment because they are the most dominating group of microbes prior to fungi. Other group of microbes also showed significant population which is a good sign for such mangrove forest with respect to mineralization of organic debris and as a result mangrove plants can easily get nutrient in simplest form. It can also be predicted that deep forest region is ecologically more stable than rooted region and un-rooted region. Sea level rise due to global warming may hamper the stable ecological zone of Sundarban Mangrove Forest which may ultimately reflect to net flux of several biologically active trace gases between soil and atmosphere.

Introduction of huge amount of nutrients during monsoon have a positive feedback on the bacterial population of mangrove sediment. Beside the changes in several physicochemical parameters, transport of huge amount of aquatic microbes could lead to the significant increase in the microbial population in the sediment of this mangrove ecosystem. This may contribute to the aquatic biogeochemistry of this tropical wetland.

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Structural characterization and biological activity of exopolysaccharide from *Lysinibacillus fusiformis*

S. Mahendran[1]*, P. Vijayabaskar[2], S.Saravanan[1], K.T.K. Anandapandian[2] and T. Shankar[2]

[1]Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai –608 502, Tamilnadu, India.
[2]Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi- 626 124, Tamil Nadu, India.

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The exopolysaccharide (EPS) of *Lysinibacillus fusiformis* were evaluated for their antioxidant properties. The total antioxidant capacity of EPS extracts was found to be maximum in malt medium 80.13 ± 0.26%. The hydrogen peroxide radical scavenging activity was found to be maximum in malt medium 78.3 ± 0.26%. The DPPH radical scavenging activity was found to be maximum in malt medium 48.39 ± 2.15%. 3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) inhibition assay was found to be maximum in malt medium 50.75 ± 3.85%. This study suggested that the EPS from *L. fusiformis* could potentially be used for antioxidant activity. Agarose gel electrophoresis was used to find the structure of mobility using acetate buffer (pH 3.6) and citrate buffer (pH 9.0). The physiochemical characterization of EPS was studied and the structure was confirmed by the Fourier transform infrared spectroscopy (FTIR), gas chromatography–mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) analysis.

Key words: Exopolysaccharide (EPS), *Lysinibacillus fusiformis*, Fourier transform infrared spectroscopy (**FTIR**), high performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (**GC-MS**).

INTRODUCTION

The biosynthesis of exopolysaccharide (EPS) is believed to serve many functions concerning promotion of the initial attachment of cells to solid surfaces formation maintenance of microcolony and mature biofilm structure and enhanced biofilm resistance to environmental stress and disinfectants. In some cases EPS matrix also enables the bacteria to capture nutrients. The production of EPS by attached microorganisms is a very complicated process, which is affected by many unique parameters. It is also considered that the mechanisms of biofilm development process are vastly different from species to species (Dunne, 2002). Thus, it is essential to develop and utilize effective and natural antioxidants, so that can be protecting the human body from free radicals and retard the progress of many chronic diseases (Nandita and Rajini, 2004). Many natural resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods. Some polysaccharides have been demonstrated to play an important role as free-radical scavengers in vitro and as antioxidants for the prevention of oxidative damage in living organisms (Zhang et al., 2004). The microbial biofilm formed on a

*Corresponding author. E-mail: mahi.ran682@gmail.com. Tel: +919884570709. Fax: 91 4144 243555.

biotic surface is an important area of research because of the wide range of possible affects and the disinfectant resistance of the cells. The colonization of solid surfaces by microorganisms is a very complicated process that depends mostly on extracellular molecule production. The biosynthesis of EPS reflected not only the attachment and aggregation process but also provided an optimal environment for the exchange of genetic material between the cells. The comparative and comprehensive analysis of all documented data concerning EPS production can enable the development and effective control strategies for biofilms (Czaczyk and Myszka, 2007).

The polysaccharides play important roles in many biological processes, and they can function as the virulence determinants in the pathogens. Occurrence in nature the biological activities of polysaccharides have attracted more and more attention in biochemistry and medicine (Peng et al., 2008). EPS producing strains are of commercial value for both their technological and putative probiotic properties. Microbial polysaccharides of economic interest are usually produced at the industrial level by fermentation. Problems associated with the reproducibility of fermentation during the industrial scale-up may result in inconsistent productivity, yield and quality, all of which can translate into financial losses. This can be solved by optimising the inoculation methodology and the fermentation conditions. A high EPS production could result in the formation of a highly viscous fermentation broth, making the recovery of cells and further downstream processing difficult (Champagne et al., 2007).

The exopolysaccharides (EPS) are high molecular weight polymers which are long chain composed of sugar residues and secreted by microorganisms into the surrounding environment. Bacterial EPS consist of a complex mixture of macro molecular poly electrolytes including polysaccharides, proteins and nucleic acids. Each comprises of variable molecular mass and struc-tural properties (Vijayabaskar et al., 2011). *L. fusiformis* produce polysaccharides respond to environmental factors directly and for some, the nutritional conditions determine the degree of exopolysaccharide formation. To evaluate the potential antioxidant activities and characterization of AGE, FTIR, HPLC and GC-MS for exopolysaccharides (EPS) produced *L. fusiformis.*

MATERIALS AND METHODS

Isolation and identification of the bacteria

Exopolysaccharide producing bacteria were isolated from soil samples collected from Ayya Nadar Janaki Ammal College campus near the garden. Isolates were obtained by serial dilution plating on nutrient agar medium. A total of 100 colonies were isolated and the exopolysaccharide producing bacteria were screened for their ability to produce exopolysaccharide, based on colony morphology (mucous and ropy). A mucous colony was isolated and identified by biochemical characterization and 16S rRNA sequencing.

Identification of bacteria by 16S rRNA sequencing analysis

Genomic DNA isolation from isolates

The isolated bacterial strain was grown in 25 ml LB broth overnight at 35°C. The culture was spin at 5000 rpm for 5 min. The pellet was resuspended in 400 µl of sucrose TE buffer (Tris EDTA). Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1 h at 35°C. To this tube, 100 µl of 0.5 M EDTA (pH 8.0), 60 µl of SDS and 3 µl of proteinase-K (20 mg/ml) were added and incubated at 55°C. After incubation, they were centrifuged at 7000 rpm for 3 min and then the supernatant were extracted twice with phenol: chloroform (1:10 and again with chloroform: isoamylalcohol (24:1). It was precipitated with ethanol. The DNA pellet was resuspended in sterile buffer.

Amplification of 16S rRNA gene sequence

Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5'ACGGCTACCTTGTTACGACTT 3'. Polymerase chain reaction was performed in a typical reaction mixture was 2 µl of template DNA and 1.5 µl of forward primer, 1.5 µl of reverse primer, 10 µl of 2X PCR master mixes and 5 µl of nuclease free water for 20 µl reaction. The reaction was performed with an initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 1 min, extension at 72°C for 1 min 30 s followed by final extension at 72°C for 5 min and hold at 4°C. The amplification of 16S rRNA gene was confirmed by running the amplification product in 1% agarose gel electrophoresis.

Sequencing of 16S rRNA sequence

Partial sequencing of the rRNA gene (about 1210 bp) for the isolated bacteria was carried out in Royal life science Pvt Ltd., (Hyderabad).

Culture process and exopolysaccharide production from bacteria

Bacteria culture was maintained on nutrient agar plates. It was sub cultured and slants were inoculated and maintained at 28ºC for 24 h. Experiments were done using 250 ml flask each containing 100 ml of basal and malt medium inoculated with the bacterial culture. The basal medium contains Glucose 10 g, yeast extract 3 g, malt extracts 3 g, peptone 5 g, $MgSo_4.7H_2O$ 1 g, KH_2PO_4 0.3 g and 10 mg of vitamin B1 incorporated at 28ºC with initial pH 7 in 1000 ml distilled water. Malt medium, malt extracts 40 g, peptone 5 g, distilled water 1000 ml and pH 7. The flask was incubated at 28°C on an orbital shaker incubator at 110 rpm for 72 h.

Bacterial exopolysaccharide (EPS) quantification

After 72 h of incubation both basal and malt medium samples were centrifuged at 5000 rpm for 20 min. The EPS was then precipitated from the supernatant by addition of equal volume of methanol. The mixture were agitated with addition of methanol to prevent local high concentration of the precipitate and left over night at 4ºC and centrifuged at 7000 rpm for 20 min. After centrifugation the precipitate was collected in a Petri plate and dried at 60ºC.

Analysis of antioxidant activity on bacterial exopolysaccharide (EPS) extract

Determination of total antioxidant capacity

Total antioxidant activity of exopolysaccharides extract was determined according to the method (Mitsuda et al., 1996). 45 ml of sul-

furic acid (0.6 M), 0.9942 g of sodium sulfate (28 mM) and 1.235 g of ammonium molybtate (4 mM) were mixed together in 250 ml with distilled water and labeled as total antioxidant capacity. 0.1 ml of the EPS extract (50, 100, 250, 500 and 1000 µg) was dissolved in 1 ml of total antioxidant capacity and absorbance was read at 695 nm after 15 min. Ascorbic acid was used as standard.

Determination of reducing power

Reducing power of the exopolysaccharides extract was determined by the following method (Yamaguchi et al., 1998). 0.75 ml of various concentrations EPS (200, 400, 600, 800 and 1000 µg) was mixed with 0.75 ml phosphate buffer (pH 6.6) and 0.75 ml of 1% of potassium ferriccyanide was added. The mixture was incubated at 50ºC for 20 min. 0.75 ml of 10% trichloro acetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. 1.5 ml of distilled water and 0.1 ml of 0.1% ferric chloride was added. After an incubation of 10 min the absorbance was read at 700 nm.

Hydrogen peroxide scavenging assay

The free radical scavenging activity of exopolysaccharides extract was determined by Hydrogen peroxide assay (Gulcin et al., 2004). Hydrogen peroxide (10 mM) solution was prepared in phosphate buffered saline (0.1 M, pH 7.4). 1 ml of exopolysaccharides extract (50, 100, 250, 500 and 1000 µg) was rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer after 10 min of incubation at 37ºC against a blank (without hydrogen peroxide). The percentage scavenging of Hydrogen peroxide was calculated using the formula,

Percentage of scavenging = $(A_\circ - A1) / A_\circ \times 100$

$A_\circ$ - Absorbance of control; A1 - Absorbance of sample

Picrylhydrazyl free radical (DPPH) radical scavenging assay

The free radical scavenging activity of exopolysaccharides extract was measured by the 1-1-Diphemy1-2picryl-hydrazyl (DPPH) following the method (Blois, 1958). Used as a reagent, DPPH evidently offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of exopolysaccharides extract at different concentrations (50, 100, 250, 500 and 1000 µg). After 10 min, absorbance was read at 517 nm. The percentage scavenging activity values were calculated as below.

Percentage of scavenging = $(A_\circ - A1) / A_\circ \times 100$

$A_\circ$ - Absorbance of control; A1 - Absorbance of sample

3-Ethylbenzothiazoline-6-sulfonic acid) (ABTS) inhibition assay

The free radical scavenging activity of exopolysaccharides extract was determined by ABTS (2,2 azino bis (3-etheylbenzothiazoline-6-sulphonicacid) diammonium salt) radical cation decolourization assay (Re et al., 1999). ABTS was generated by mixing 5 ml of 7 mM ABTS with 88 µl of 140 mM potassium persulfate under darkness at room temperature for 16 hours. The solution was diluted with 50% ethanol and the absorbance at 734 nm was measured. The ABTS radical cation scavenging activity was assessed by mixing 5 ml ABTS solution (absorbance of 0.7 ± 0.05) with 0.1 ml exopolysaccharides extract (50, 100, 250, 500 and 1000 µg). The final absorbance was measured at 743 nm with spectrophotometer. The percentage scavenging of was calculated by the following formula

Percentage of scavenging = $(A_\circ - A1) / A_\circ \times 100$

$A_\circ$ - Absorbance of control; A1 - Absorbance of sample

Infra-red spectroscopy analysis of exopolysaccharide (EPS)

The bacterial exopolysaccharides were also characterized using Fourier transform infrared spectrophotometer. IR spectroscopies of bacterial EPS along with a standard, dextran sulfate were tested using Perkin-Elmer FT-IR instrument, which helped to analyze different sulfate, carboxyl and hydroxyl groups of these sample molecules (Wang et al., 2004). One part of extract was mixed with ninety nine parts of dried potassium bromide (KBr) separately and then compressed to prepare a salt disc of 3 mm diameter. These discs were subjected to IR- spectrophotometer. The absorption was read between 400 and 4000 cm^{-1}.

Agarose gel electrophoresis

The EPS samples (10 µg) were subjected to electrophoresis on a 0.6% agarose gel (Mauro et al., 1998). This experiment helps to find out the nature of the isolated product. The presence of EPS was analyzed by electrophoresis, using two different buffer systems such as acetate buffer (pH 3.6) and citrate buffer (pH 9) for 1hour at 100 v. After the electrophoresis, the gel was then fixed with 0.1% N-Cetyl-N,N,N-trimethyl ammonium bromide for 12 h. The gel was then dried and stained with toluidine blue solution 0.1% toluidene in acetic acid, ethanol and water in the ratio of (0.1:5:5 v/v). After staining, the gel was washed in destaining solution acetic acid, ethanol and water in the ratio (0.1:5:5 v/v) and result was documented.

High performance liquid chromatography (HPLC) analysis of exopolysaccharide (EPS)

The EPS were analyzed with a high performance liquid chromatography (HPLC) C18 system column (LC- 10VP Shimadzu) and eluted with distilled water at a flow rate of 1.0 ml/min at 20°C. The separated components were monitored by a refractive index detector. The EPS after being hydrolyzed and derivatives with methanol was analyzed for its sugar composition by HPLC. The column was calibrated with different molecular mass standard and a standard curve was then established.

Gas chromatography–mass spectrometry (GC-MS) analysis of exopolysaccharide (EPS) extract

0.1 g sample was mixed with 1.25 ml of 92% of sulfuric acid with a glass sticks and incubated for 60 min at 30ºC. The mixture were distilled with 13.5 ml of distilled water and incubated in a boiling water bath for 4 hours. After incubation, mixtures were cooled and 3.1 ml of 32% of NaOH (w/v) was added. At the end of hydrolysis, 0.2 ml of sample was taken in a separate tube and 2 ml of 2% sodium borohydride in dimethyl sulfoxide was added. The mixtures were then stirred for 90 min at 40ºC after which 0.2 ml of glacial acetic acid was added to decompose excess of sodium borohydrizde. After cooling, 4 ml acetic anhydride and 0.4 ml of 1-methyl immidazole were added to the solution. The mixture was then incubated for 10 mints at room temperature and then 20 ml of distilled water was added to decompose the excess of acetic anhydride. After cooling, 8 ml of dichloromethane was added and mixture was vigorously shaken for total alditol acetate extraction.

Alditol acetates were separated on a 30 m x 0.25 mm ID x 0.25 µm film thickness column DB 5 ms (agilent) attached to the GC-2010 (GCMS-QP 2010 SHIMADZU) chromatography equipment with a flame-ionization detector and a split injector. High purity hy-

Table 1. Biochemical characteristics of the *Lysinibacillus* sp.

Biochemical test	Result
Gram's staining	Gram positive
spore staining	Sub-terminal spores
Motility	+
Carbohydrate fermentation test	
a. D-glucose	+
b. Mannitol	+
c. Lactose	-
d. Sucrose	+
Indole Production	-
Methyl red test	-
Voges – Proskauer test	-
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	+
Catalase test	+
Oxidase	+
Nitrate utilization test	-

+ Positive Results. - Negative Results

drogen was used as the carrier gas at a flow rate of 1.40 ml/min. the column temperature was maintained at 200 and 240^{o}C respectively, and 1µl sample in dichloromethane was injected through a glass-lined splitter, set at 1/90 ratio. The absorption was read between 40 m/z and 800 m/z.

RESULTS

Screening of exopolysacharied producing bacteria

The bacterial isolate was identified by standard morphological and biochemical characterization in accordance with Bergey's manual of determinative bacteriology. The morphology of the isolate was Gram positive, rod shaped, spore forming and motile aerobes and biochemical characterization of the isolate was carried out and the results (Table 1).

The 16S rRNA gene of the EPS strain was PCR amplified using the 16S rDNA universal primers and both the strands were sequenced. The sequences were compared with the 16S rDNA sequences available in the RDP database (http: //rdp.cme.msu.edu/). Sequence analysis revealed that the strains were phylogenetically closely related to the genus *Lysinibacillus*. BLAST analysis of the 16S rDNA sequence of EPS producing isolate revealed that it is more score bit with *Lysinibacillus fusiformis*. Though the isolates had a close similarity, the dendrogram was constructed based on their phylogenetic relationship revealed that all the isolates were distinctly placed under separate clusters. 16S rRNA gene sequencing confirmed that the isolate was *Lysinibacillus fusiformis* (Figure 1). The *Lysinibacillus fusiformis* strain ME was submitted in the Genbank with Accession number JF906500.

Free radical scavenging activities of exopolysaccharide (EPS) extract from *Lysinibacillus fusiformis*

Total antioxidant capacity

The total antioxidant capacity of EPS extracts of *L. fusiformis* along with standard ascorbic acid (Figure 2). The activity of extracts was calculated based on inhibition percentage. The total antioxidant capacity of EPS extracts of *L. fusiformis* was found to be maximum in malt medium (80.13 ± 0.26%).

Reducing power

The reducing capacities of various concentration of EPS extracts from *L. fusiformis* in basal and malt medium is compared with standard compound which implies that as the concentration increases the reducing power of the extracts also increases *L. fusiformis* EPS extracts (Figure 3).

Hydrogen peroxide scavenging assay

The activity observed in hydrogen peroxide radical assay was directly proportional to change and as found to be concentration gradients to change. The hydrogen peroxide inhibition activity for the *L. fusiformis* of basal and malt medium EPS extracts. Which indicate higher activity EPS extracts of *L. fusiformis* was found to be maximum in malt medium (78.3 ± 0.26%) (Figure 4).

Picrylhydrazyl free radical (DPPH) scavenging assy

The activity observed in DPPH radical scavenging assay of EPS extracts of *L. fusiformis* along with standard Gallic acid (Figure 5). Which indicate higher activity for EPS extracts of *L. fusiformis* was found to be maximum in malt medium (48.39 ± 2.15%).

3-Ethylbenzothiazoline-6-sulfonic acid) (ABTS) inhibition assay

The activity observed in ABTS inhibition assay of EPS extrats of *L. fusiformis* along with standard Gallic acid (Figure 6). Which indicate higher activity for crude EPS extracts of *L. fusiformis* was found to be maximum in malt medium (50.75 ± 3.85%).

Fourier transforms infrared spectroscopy (FTIR) analysis

IR spectroscopy of intact exopolysaccharides (EPS)

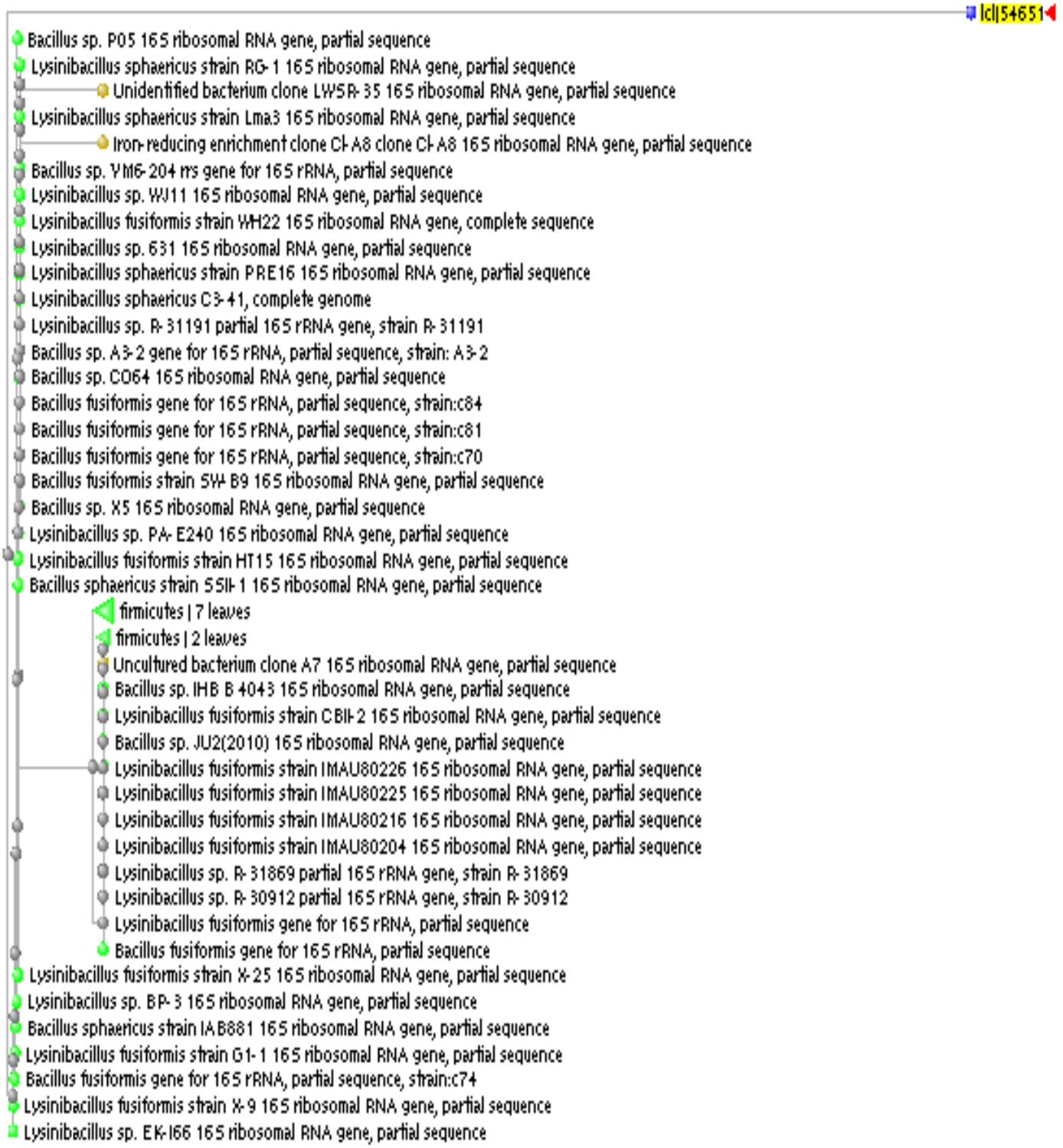

Figure 1. Phylogram of *Lysinibacillus fusiformis* strain ME.

showed the presence of hydrogen bonded compound, possible acid or amine salt. The *L. fusiformis* EPS extracts revealed characteristic absorption bands of EPS as observed in the reference compound dextran sulphate. The spectram of polysaccharide sample showed the band at 606.63, 929.72, 1036.77, 1077.28, 1404.22, 1456.30 cm^{-1} in spectrum of *L. fusiformis* malt EPS (Figure 7). In addition the spectrum showed the band around 1000, 1200, 1400, 1500 and 1600 cm^{-1} revealed the (1,3) – β – glucan linkages in addition to the bands in

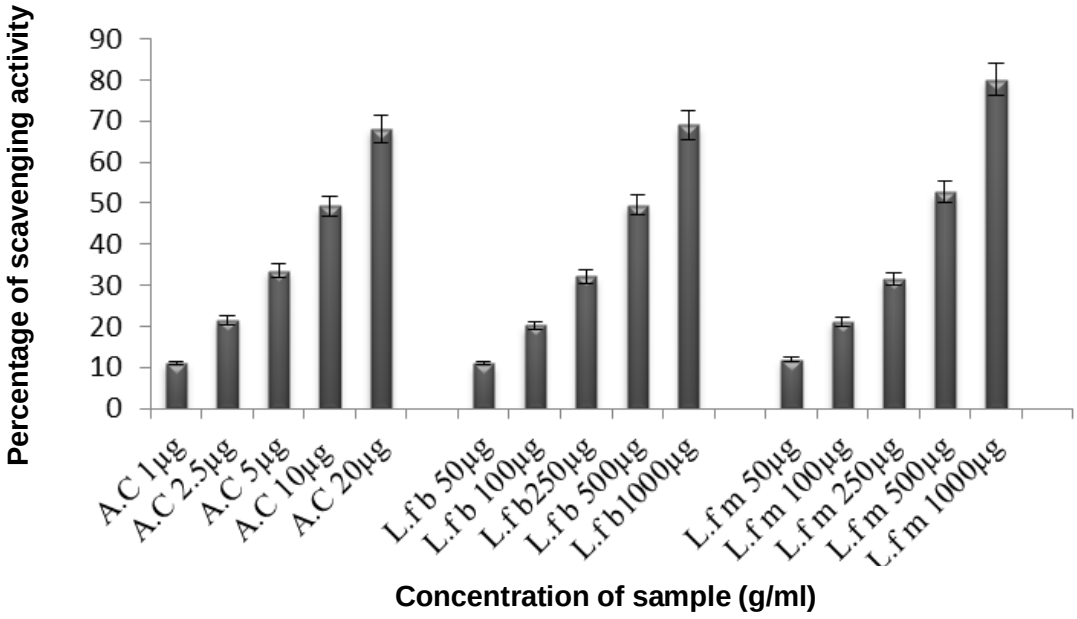

Figure 2. Total antioxidant activity of EPS *L. fusiformis* (basal – L.f b and malt – L.f m) compared with standard Ascorbic acid (A.C).

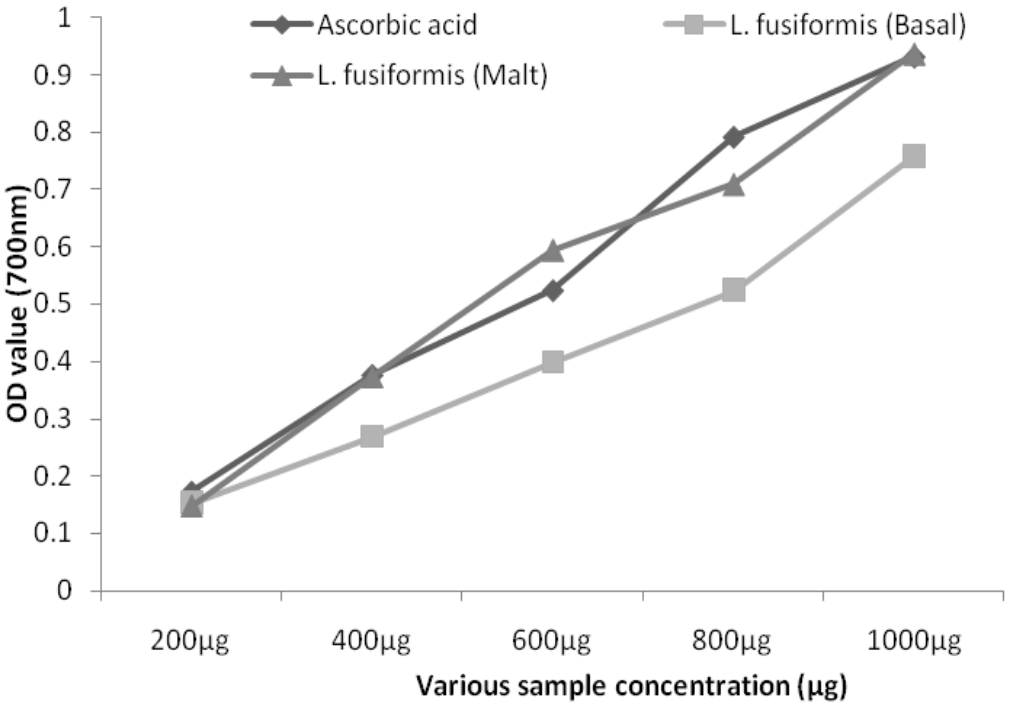

Figure 3. Reducing power of the EPS extracts.

the region of 2900 and 3400 cm^{-1} chemical bands were presented.

The broad peak at 3442 cm^{-1} was OH stretching peak, the carbonyl (C=O) stretching was at 1647 cm^{-1}, the peak between 1900 and 2800 cm^{-1} was C-H stretching peak. N-H deformation was at 1404 cm^{-1}.

Polysaccharides C-O-C and C-O-P was at 1037 cm^{-1}, absorption at 1000 cm^{-1} was typical for glucose in pyranose

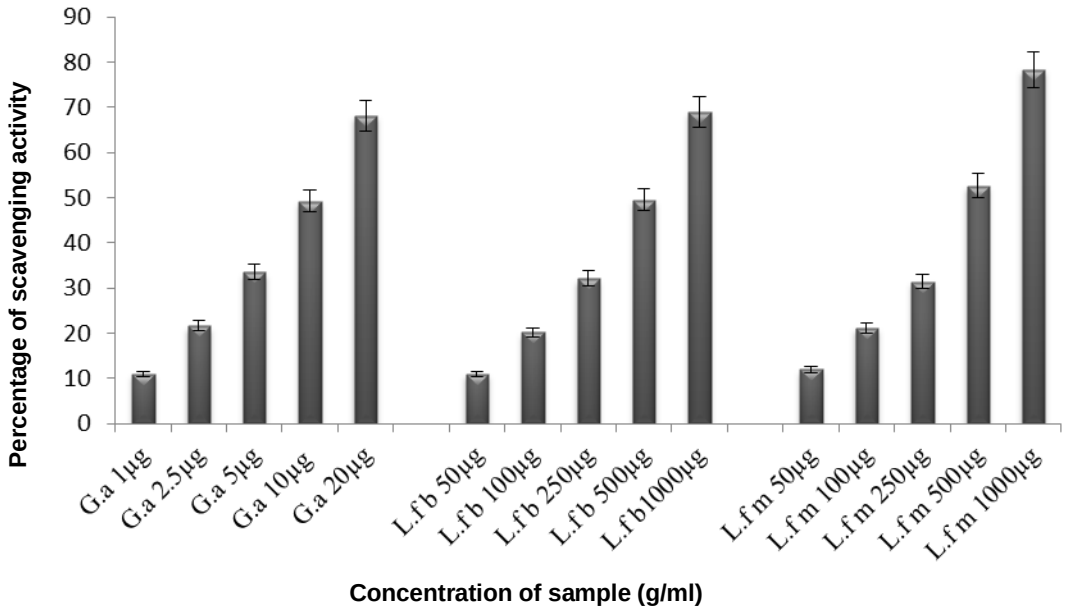

Figure 4. Hydrogen peroxide scavenging activities of *L. fusiformis* (basal - L.f b and malt – L.f m) compared with standard Gallic acid (G.a).

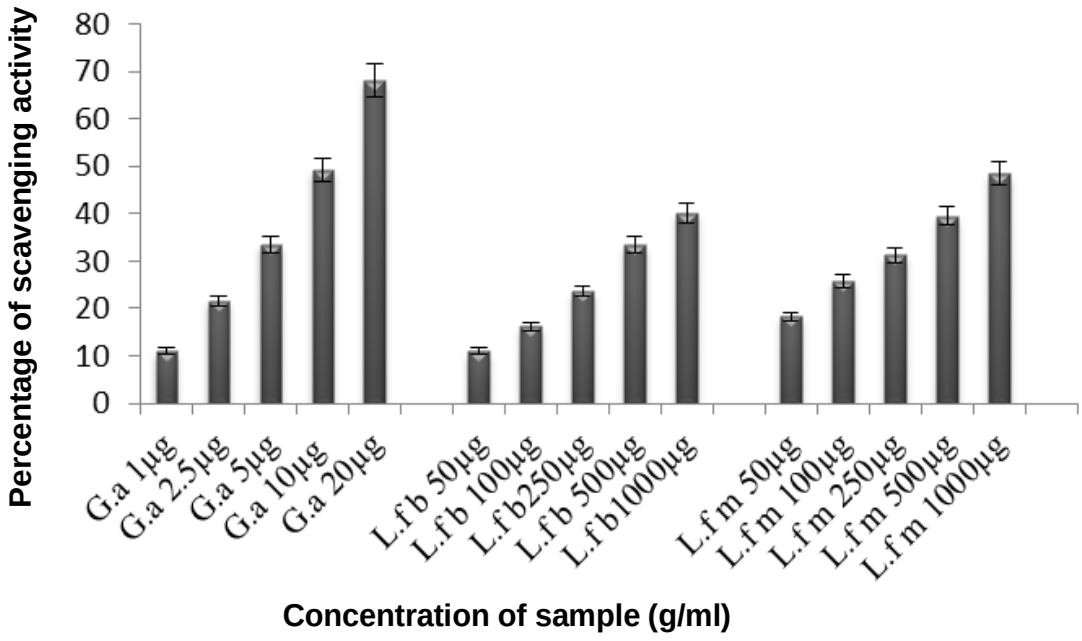

Figure 5. DPPH scavenging activities of crude EPS from *L. fusiformis* (basal - L.f b and malt – L.f m) compared with standard Gallic acid (G.a).

form. In the anomeric region (1000-1600 cm^{-1}) the polysaccharides exhibited the obvious characteristic absorption at 1037 cm^{-1}.

Agarose gel eletrophoresis

The mobility of the exopolysaccharides extracted from both the *L. fusiformis* was checked using agarose gel electrophoresis by applying various pH for two different buffer systems. The highest mobility was observed for higher pH values (pH 3.6 acetate buffer and pH 9.0 citrate buffer in the two buffer systems, especially the citrate buffer (pH 9.0) showed highest mobility for both the EPS extracts when compared with dextran sulfate (Figure 8 a and b).

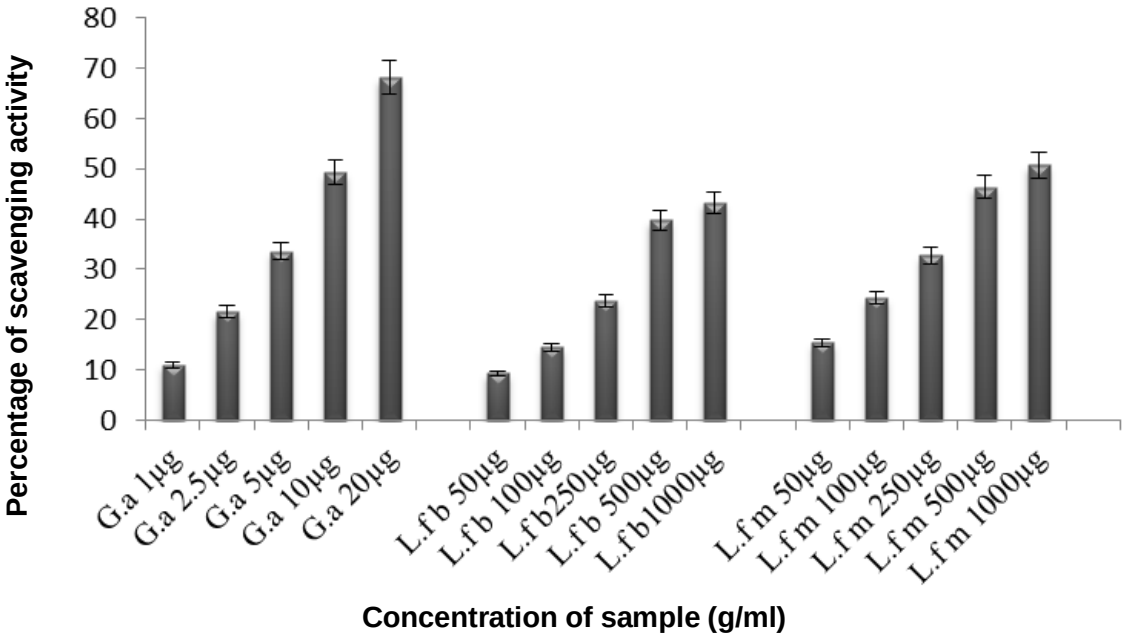

Figure 6. ABTS scavenging activities of crude EPS of *L. fusiformis* (basal - L.f b and malt – L.f m) compared with standard Gallic acid (G.a).

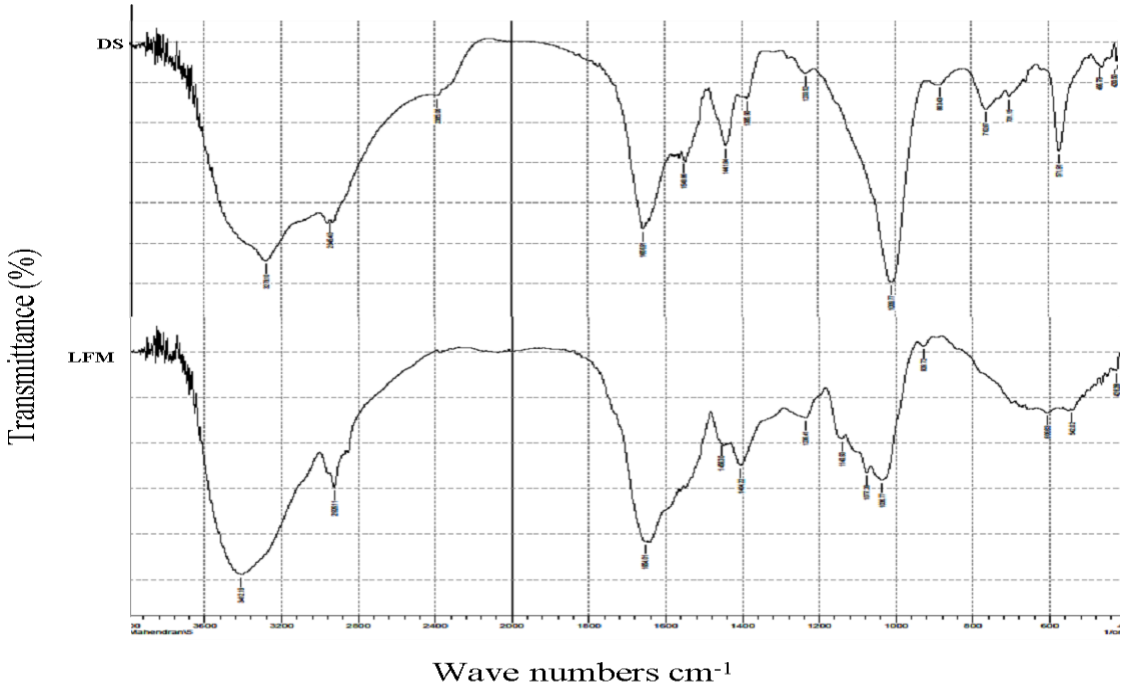

Figure 7. FT-IR spectrum of bound EPS in *L. fusiformis* (malt) compared with Dextran sulphate (DS).

High performance liquid chromatography (HPLC)

The obtained fractions were analyzed with a high performance liquid chromatography (HPLC) system C18 column. HPLC was applied to elucidate the relative molecular mass of the exopolysaccharides. It has been confirmed with the previous reference. The EPS production has been quantified through HPLC, in which for EPS production from *L. fusiformis* from potential EPS malt medium with the retention time as the EPS production was found to be higher for the EPS malt medium. Here independent peaks were identified with retention time (Figure 9).

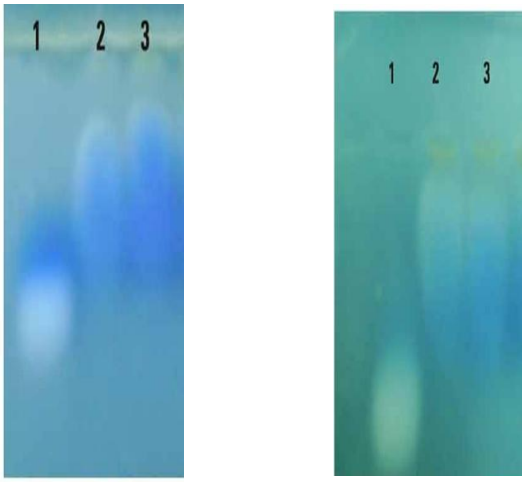

Figure 8. Agarose gel electrophoresis (a). Acetate buffer (pH 3.6): Lane 1 -Dextran sulfate; Lane 2-L. fusiformis (Basal). ; Lane 3-L. fusiformis (Malt). (b). Citrate buffer (pH 9): Lane 1-Dextran sulfate; Lane 2-L. fusiformis (Basal). ; Lane 3-L. fusiformis (Malt).

Gas chromatography–mass spectrometry (GC-MS) analysis of exopolysaccharide (EPS) extract

The electron impact fragmentation patterns of the mass spectra of derived alditol acetates were prepared from the hydrolyzed exopolysaccharides. In bacterial exopolysaccharide the peaks are corresponding to 1-methyl-2-formylimidazole (47.95%) with various retention times. The retention times and the percentage of monosaccharide concentration of the fractionated products of both the exopolysaccharide contained alditol acetate residues. In this result the exhibited proper and various peaks compared to *L. fusiformis* (Malt) (Figure 10).

DISCUSSION

In present study the total antioxidant capacity of EPS extracts of *L. fusiformis* was found to be maximum in malt medium (80.13 ± 0.26%). In present study of *L. fusiformis* EPS extract from malt medium showed better reducing power when compared with *L. fusiformis* basal medium. (Czochra and Widensk, 2002) it can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells. In present study the which indicate higher activity EPS extracts of *L. fusiformis* was found to be maximum in malt medium (78.3 ± 0.26%). The ABTS radical reactions involve electron transfer and the process take place faster rate when compared to DPPH radicals. In the present study the ABTS radical scavenging activity was more in higher activity EPS extracts of *L. fusiformis* was found to be maximum in malt medium (50.75 ± 3.85%). The radical scavenging ability using the same per weight basis the antiradical performance of polysaccharide fractions with respect to DPPH radicals was measured and compared. The order of effectiveness of polysaccharide fractions in inhibiting free radicals was as follows: AP-III>AP-II>AP-I. AP-III had the highest radical scavenging activity, followed by AP-II. After 60 min incubation, 63.01% of DPPH radical were quenched by fraction AP-III, followed by fraction AP-II which was able to quench 45.3%. Surprisingly, inhibition of DPPH radicals was only 21.9% pure dextran (Sigma) was assayed (Mohsen et al., 2007). In present study of DPPH radicals which indicate higher activity EPS extracts of *L. fusiformis* was found to be maximum in malt medium (48.39 ± 2.15%).

The bacterial EPS extracts gave characteristics bands for EPS. Here, carbonyl (C= O) stretching peak and OH stretching peak was at broad and the maximum peak and the band at 1000-1500 showed the presence of polysaccharide. The cultivation temperature by 10˚C below optimal level inhibits the EPS biosynthesis by microbial cells. However, under low temperature of the growth, environment profiles of the high productivity of extracellular polysaccharide occur by bacterial cells (Sutherland 2002). The IR spectrum of the polymer proved the presence of carboxyl group, which may serve as binding sites for divalent cations. The carboxyl group may also work as functional moieties to generate new or modified polymer variants using different approaches like novel. The time dependent increase in the Si-O stretching vibration (1200 to 1000 cm^{-1}) hindered our ability to extract IR data from this region of the EPS silica spectrum symmetric phosphate stretch of nucleic acid and C-O stretching modes of sugar/sugar phosphate (Omoike and Chorover, 2006).

The electrophoretic migration of exopolysaccharides in agarose gel, using various buffer systems at various pH depends on the structure of polysaccharide. The exopolysaccharides extracts of *L. fusiformis* in the gel depending upon the size of the molecules. These molecules were compared with standard dextran sulfate. The exopolysaccharides had different electrophoretic mobility for different buffer system depending on the structure of the polysaccharide (Dietrich et al., 1985).

The fully methylated products were hydrolyzed with as it, converted into the alditol acetate and analyzed by GC-MS. Methylation analysis of the polysaccharides glucan such as 2,3,4-tetra-Me.Glu. and 2,3,6-tri Me-Glu. When the oligosaccharide alditol contain hexoses. (Gal) or deoxyhexoses (Rha), the sequence of this saccharide may be determined on the basis of MS. The electron impact fragmentation patterns of the mass spectra of derived alditol acetates were prepared from the hydrolysed EPS (Vijayabaskar et al., 2011). In present study the EPS production has been quantified through HPLC, in which for EPS production from *L. fusiformis* from potential EPS malt medium with the retention time as the EPS production was found to be higher for the EPS malt medium.

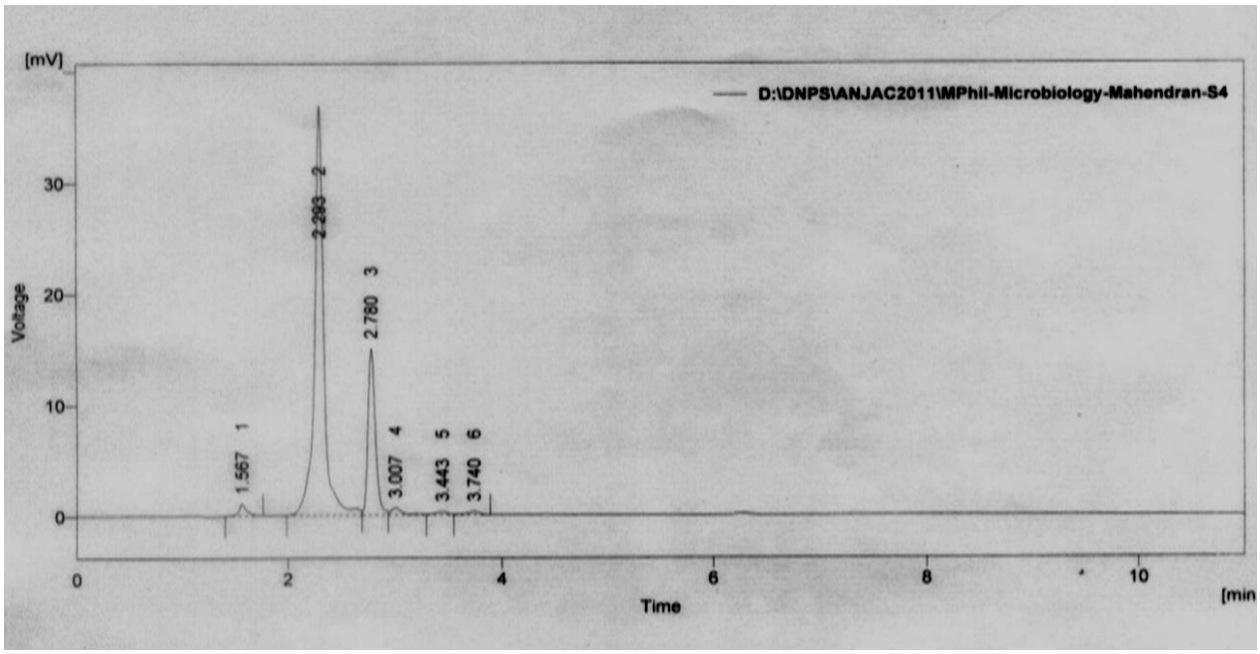

Figure 9. HPLC analysis of EPS from *L. fusiformis* (Malt).

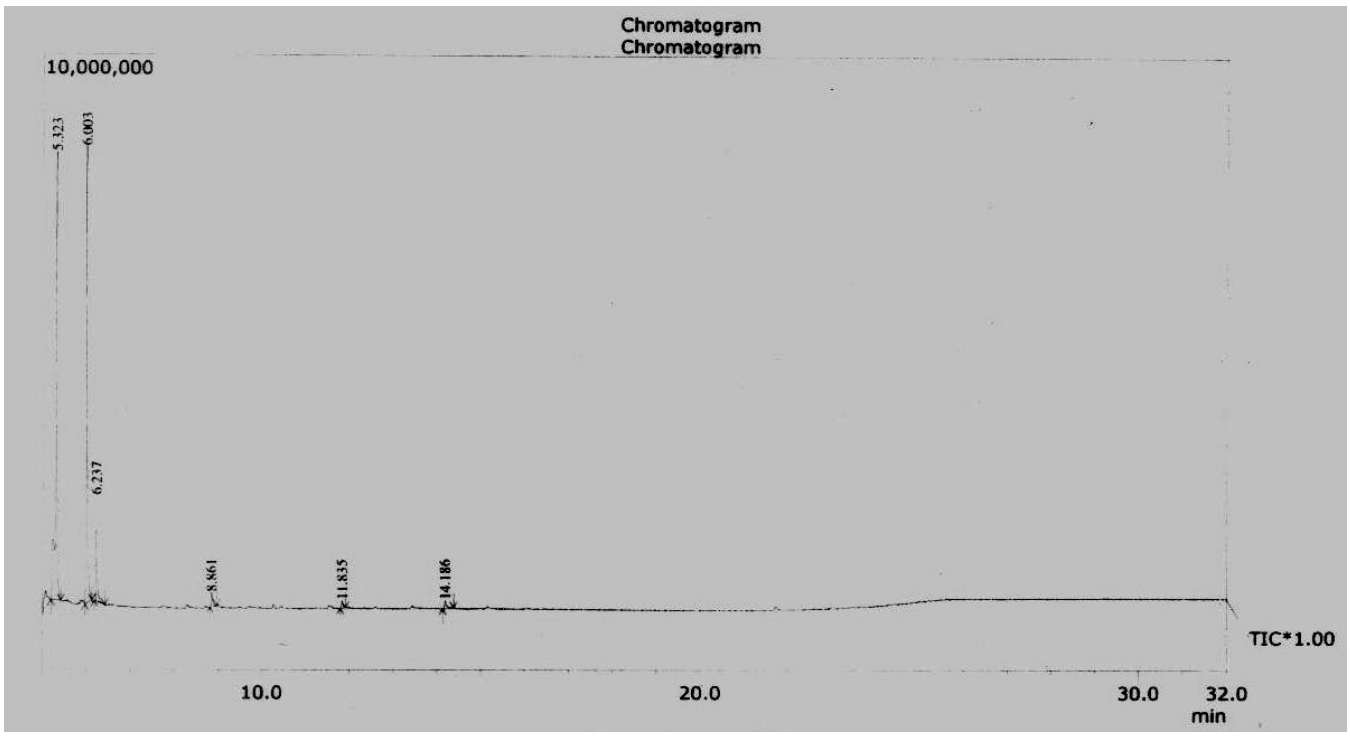

Figure 10. GC-MS analysis of EPS from *L. fusiformis* (Malt).

In present study the *L. fusiformis* exopolysaccharide the peaks are corresponding to 1-methyl-2-formylimidazole (47.95%) with various retention times. The retention times and the percentage of monosaccharide concentration of the fractionated products of both the exopolysaccharide contained alditol acetate residues. In case of complex EPS neutral sugars are identified by their derivatives, alditol acetates by GC-MS (Hoebler et al., 1989).

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Short Communication

Helicobacter pylori and eye diseases in an Iranian hospital

Gholamhossein Yaghoobi[1], Mohammad Reza Rezvani[1]* and Elahe Heidari[2]

[1]Birjand University of Medical sciences, Ophthalmology Department, Valiasr Hospital, Southern Khorasan, Social Determinant Health Research center, Birjand, Iran.
[2]Mashad University of Medical Science, Pediatrics ward, Emmam Reza Hospital, Mashad, Iran.

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There is evidence of *Helicobacter pylori* infection that induces eye diseases. This study was done to find if there is any association between *H. Pylori* and ocular diseases, and if there is any difference in color of eye among them. Of all the 35 patients tested for biopsy with positive *H. pylori*, 23 (65.7%) were females and 12 (34.3%), males. The mean age of patients was 39.65±18.55 years. 20 (57.1%) patients were below 40 years old; 8 (22.9%) patients, 40 to 60 years and only 7 patients were more than 60 years old. Most of the patients were 20 years old and had no ophthalmic or systemic diseases in juvenile age group; most of them with the best correct vision were within normal measure, except the old patients who had normal tension glaucoma and two cataract cases. Brown eyes were the commonest but blue color was the least without any risk of ocular diseases or *H. Pylori* associated with more prevalent brown iris color in study group. Ophthalmic extragastric diseases do not show any association between eye diseases and *H. pylori* infection in juvenile age groups. Pesudoexfolitive glaucoma and two cases of cataract were among the old age related diseases which may be due to the late appearance of *H. pylori* effect. Best visual acuity, cup/disc character and brown color were the most associated finding.

Key words: Helicobacter pylori, extragastric manifestations, ophthalmic diseases.

INTRODUCTION

There is increasing evidence that *Helicobacter pylori (H. pylori)* is an important pathogen in human infections. It is confined to the stomach and induces a strong systemic immune host response. Therefore, plausible untoward effects of these responses may contribute to the development of disease in areas other than gastrointestinal tract (Feghhi et al., 2008). A possible association between *H. pylori* infection and eye diseases, including Sjogren syndrome, blepharitis, central serous chorioretinopathy and uveitis has been proposed (Izzotti et al., 2009). *H pylori* plays a potential role in the pathophysiology of many neurological and ophthalmological disorders also (cerebrovascular diseases, migraine, Alzheimer's disease, epilepsy, Parkinson's disease, multiple sclerosis, peripheral neuropathies, glaucoma, non-arteritic anterior ischemic optic neuropathy and lung cancer) (Deng et al., 2013).

H. pylori-specific IgG antibody levels significantly increase in the aqueous humor and serum of patients with primary open angle glaucoma (POAG) and Pesudoexfoliation glaucoma (XFG). These findings support a role of H. pylori infection in the pathobiology of glaucoma (Jannis et al., 2003).

Neither *H. pylori* infection nor seropositivity for virulent CagA-bearing *H. pylori* strains has significant association with the occurrence of glaucoma of any type (Kurtz et al., 2008). Eye color is one of the major racial and diagnostic features in anthropological studies and it is used to characterize differences between populations where certain authors have shown some associations between eye color

*Corresponding author. E-mail: Yaqubig@yahoo.com. Tel: (561) 4443001-9. Fax: (0561) 4445402.

Table 1. Patients' demography.

Parameter	Value
Female	23 (65.7 %)
Male	12 (34.3%)
Age 40 y ≥	20 (57.1%)
Age 40 y>	15 (42.8%)
Y= year	

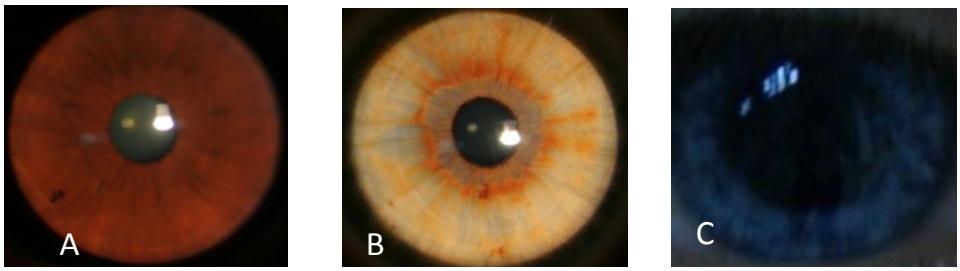

Figure 1. Iris colour classification; sample, A= brown, B= Green, C= Blue.

and the constitutional type (Dzintra and Vētra, 2011). Farooq et al. 's research demonstrated that current data are very limited for establishing any causal relationship between *H. pylori* and most of the above mentioned disorders (Farooq and Bhatt, 2008).

The possibility of long-term effects of *H. pylori* eradication therapy on the cause of these disorders could be a preventive parameter of human discomfort if this relationship could be established. So, this study was done to find extragastric eye diseases in biopsy established *H. pylori* infection.

MATERIALS AND METHODS

Thirty-five (35) subjects who participated in this study were recruited from 1st March, 2011 to 31 st December, 2011 from the Department of Ophthalmology. An ethics committee approval was obtained from the Institutional Review Board. Participants were enrolled after signing the consent form. Patients who had indication of endoscopic evaluation due to gastric disorders or peptic ulcer were enrolled in this study. Diagnostic approach used in this study was Rapid Urea Test (RUT) for all biopsy specimen of *H. Pylori* infection.

All of the 35 patients were made to complete ophthalmic examinations, including a slit-lamp examination, intraocular pressure (IOP) measurement with a Goldman applanation tonometer and fundus and optic disc examinations, using a 90 D biomicropscopy for iris color detection. Diagnosis of glaucoma was established based on IOP exceeding 29 mmHg and optic disc character at the initial visit or visual field examinations using a Humphrey visual field analyzer (Carl zeiss meditec, Dubin, CA, USA) to define suspected cases.

RESULTS AND DISCUSSION

Of the 35 patients, 23 (65.7%) were females and 12 (34.3%), males. The mean age of the patients was 39.65 ± 18.55 years. 20 (57.1%) patients were below 40 years old; 8 (22.9%) patients, 40 to 60 years and only 7 patients were more than 60 years (Table 1).

Majority of our patients (28) had no other systemic diseases except 7 (20%) who had history of ischemic heart diseases (two cases), asthema (one case), hypertensions (two cases) and anemia (two cases). Ophthalmic examinations including visual acuity were good in 80% of cases. Biomicroscopy of anterior segment and IOP mean (14.38 ± 4.37 m Hg) were within normal limits except one case of pseudoexfoliative glaucoma and two cases of cataract. There were no optic nerve or retinal diseases in these groups of 35 subjects with positive cases of *H. Pylori*. We could not find any other ocular inflammation or eye disease other than the ones mentioned above.

As shown in Figure 1, the commonest iris color was brown in 20 (57.1%), green iris color in 13 (37.1%) and 2 (5.7%) had blue eye (Table 2).

This study shows that *H. pylori* infection is associated with two cases of cataract and one case of pseudoexfoliative glaucoma. The 3 patients that had ocular diseases are old and it could be related to other possible risk factors other than *H. pylori* infection or may be a long time effect of *H. Pylori* infection. Our limitation in this study is descriptive and lacks control group because it is not logical to do biopsy in normal subject or do comparisons with other diagnostic approaches. There are studies that indicated ophthalmic diseases (Sergio et al., 2006; Kountouras et al., 2011; Choi et al., 2010). A report by Banić et al. (2012) among several diseases are associated with *H. pylori* infection; some of these, such as cardiovascular disease and open angle glaucoma have consistent evidence of a causative role; while for others, further studies are needed to verify the association. There

Table 2. Distribution of Ophthalmic manifestation and associated systemic diseases.

	Variable	Number (%)
Ophthalmic manifestation	Visual acuity more than 20/30	80%
	Mean intraocular pressure (mg Hg)	14m 38 ± 4.38
	Glaucoma	1
	Pseudo exfoliation	1`
	Retinal diseases	-
	Optic nerve diseases	-
	Intraocular inflammation	-
	Brown iris color	20 (50.17 %)
	Green iris color	13 (37.17 %)
	Blue iris color	2 (5.7 %)
Associated systemic diseases	Ischemic heart diseases	2
	Asthema	1
	Hypertention	2
	Anemia	2

is correlation between the iris color and certain conditions such as cataract and AMD with lighter eye colors. Several somatic or hereditary disorders can easily be recognized by the characteristic changes of the iris by just looking at it or the overlying cornea or intervening lens (Hashemi et al., 2010; Morrison, 2010).

We use the iris color to illustrate how recognition of some common and rare diseases that can be identified by direct visualization in the outpatient clinic can lead to prevention and management of underlying complications. The ocular diseases of our study were associated with brown color which was the commonest iris color 20 (57.1%) versus green iris color in 13 (37.1%) and 2 (5.7%) blue eye. The best approach to control these diseases is easy and cheap accessibility which can help to eradicate human discomfort. But to elucidate the degree *H. pylori* gets involved in the development of eye diseases and how to eradicate it as well as its cost, there will be need for future studies to clearly adjust various factors for comparing this infectious agent and other extra gastric diseases.

This descriptive study could not find any ophthalmic diseases among *H .pylori* positive of less than 60 years old patients. As there is a practical approach to treat H. Pylori infection, any of its casual in relation to extragastric diseases could also be prevented. Pesudoexfoliative glaucoma in one elderly with two cases of cataract in our study shows the important effect of infection duration or age related *H. pylori* infection. To find the long time effect of infestation or rule of age on *H. pylori* infection, there is need to do comparative or a large sample study.

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Short Communication

New record of twig blight on *Catharanthus roseus* in India

Pankaj Sharma*, P. D. Meena and Y. P. Singh

Directorate of Rapeseed-Mustard Research (ICAR), Bharatpur 321 303, India.

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During post rainy season 2011, twig blight disease symptoms were observed on *Catharanthus roseus*. Symptoms appeared as dark brown to black girdling lesions on the twigs. Acervuli containing masses of spores and dark setae were observed within lesions. On the basis of fungal morphology and completion of Koch's postulates, the pathogen was identified as *Colletotrichum gloeosporioides* (Penz.) Penz.& Sacc.

Key words: Twig blight, *Catharanthus roseus, Colletotrichum gloeosporioides*

INTRODUCTION

Periwinkle or Vinca [*Catharanthus roseus* (Linn.) G.Don. syn. *Vinca rosea* Linn.] belongs to the family Apocynaceae. It is an erect highly branched lactiferous perennial herb which is a chief source of patented cancer and hypotensive drugs. Flowers are borne on axils in pairs. It is one of the very few medicinal plants which have a long history of uses. *C. roseus* has more than 400 known alkaloids in its different parts. The alkaloids like antineoplastindimeric, vinblastin and vincristine are mainly present in aerial parts, whereas ajmalcine, vinceine, vincamine, raubasin and reserpine are present in roots and basal stem. The dimericindole alkaloids from *C.roseus* are mainly used for treatment of various human cancers. Pharmaceutical industry use it for the treatment of childhood leukemia, Hodgkin's disease, testicular cancer and cancerous tumors. *C. roseus* is one of the very few medicinal plants which have a long history of uses as diuretic, antidysenteric, hemorrhagic and anti-septic agent. It is known for use in the treatment of diabetes in Jamaica and India. Prevention of cancer, cancer treatment, anti-diabetic, stomachic, reduces high blood pressure, externally against nose bleeding, sore throat and mouth ulcers (Graf et al., 1996; Kirtikar and Basu, 1993; Narayana and Dimri, 1990).

After post-rainy season, an estimated 65-80% of the *C. roseus* surveyed in Bharatpur and Jaipur, India, were infected with twig blight. Symptoms first appeared as dark brown to black girdling lesions on the twigs. When lesions occurred at the ground line, the entire runner died. Where healthy twigs touched the soil or infected plant parts, new lesions were developed. Within a few weeks, the disease was spread to stems and leaves and caused large sections of the bed to die (Figure 1). As long as cool, damp conditions remain, the disease spreads in the planting. The spores of the fungus disseminate primarily by splashing and flowing water. Acervuli containing masses of spores and dark setae were observed within lesions.

MATERIALS AND METHODS

Collection of disease samples and isolation

Diseased samples were collected from *C. roseus* plants in different localities in Bharatpur (77°27'E, 27°12'N, 178.13 MASL) and brought to the laboratory for isolations. Infected tissues were cut into small (approximately 5 mm^2) pieces with sterilized scalpel and surface sterilized for 20 s in 95% ethanol followed by 60 s in 0.525% NaOCl. These tissues were washed thrice with sterilized distilled water and aseptically transferred onto 2% (w/v) autoclaved potato dextrose agar (PDA; Merck, Germany) containing 50 mg/l

*Corresponding author. E-mail: pksvirus@gmail.com. Tel: +91 9414239831. Fax: +91 05644 260565.

Figure 1. Stem blight symptoms on *Catharanthus roseus.*

streptomycin sulphate (Sigma, St. Louis, USA) medium in Petri plates. Inoculated Petri plates were incubated in the BOD at 25± 2°C for seven days in the dark (Sharma et al., 2011a). Microscopic examination was conducted by mounting fungal tissue in water and lactophenol, and dimensions of 50 each conidia and conidiophores were measured from 7 days old cultures with the help of Pro-image analyser attached with light microscope (Olympus, Japan BX 51).

Pathogenicity test

Pathogenicity was tested by completion of Koch's postulates (Sharma et al., 2012a, 2013). Conidia obtained from 7-day-old PDA culture were suspended in sterile distilled water (SDW) to 10^4 conidia/ml. 20 plants (45-days old) were sprayed with conidial suspension (50 ml on each) until runoff with hand sprayer. Plants were covered with plastic bags for 48 h and kept at 24± 2°C. After 7 days, lesions were developed on inoculated leaves. The fungus was re-isolated from acervuli that developed on the twigs and leaves, following previously outlined procedures. Inoculation tests were repeated once. The control plants sprayed with SDW did not show any symptoms. The morphological and cultural characteristics of the re-isolated pathogen were compared with the original pathogen. The fungus was identified from all infected twig samples.

RESULTS AND DISCUSSION

Developing colonies were extremely variable, effuse, grey to brown and reverse dark brown. The teleomorph was observed as small black dots (perithecia) immersed in the centers of older colonies (more than 35 days old). Conidia were borne on elongated phialides in acervular conidiomata. Conidia were straight, one-celled, hyaline, oblong, or cylindrical, slightly curved with truncate base and rounded apex and measured 15.0 to 22.5 × 3.0 to 5.5 µm in size. These morphological characteristics of the isolate were consistent with the description of *C. gloeosporioides* (Penz.) Penz. & Sacc. (Cannon et al., 2008). The Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, India (Accession No. NFCCI 2650) confirmed the identity.

Leaf spots and stem lesions on common periwinkle caused by *C. gloeosporioides* were reported by Koelsch et al. (1995). However, twig blight of *C. roseus* was also earlier reported to be caused by *Colletotrichum dematium* (Pers.) Grove. (McMillan and Graves, 1996). *C. gloeosporioides*, causing leaf blight on many plants has been reported from India (Jamaluddin et al., 2004). The pathogen causes anthracnose on *Saraca asoca* (Sharma et al., 2011b) *Jasminum grandiflorum* (Sharma et al., 2012b), onion (Sikirou et al., 2011) and olive (Ali et al., 2010). *C. gloeosporioides* also causes banana fruit rot (Jat et al., 2013). This is the new record of *C. gloeosporioides* causing twig blight of *C. roseus* as per the literature cited in India (Bilgrami et al., 1991; Jamaluddin et al., 2004). Since *C. roseus* foliage is a good medicinal value plant, the disease damaging its foliage deserves special attention.

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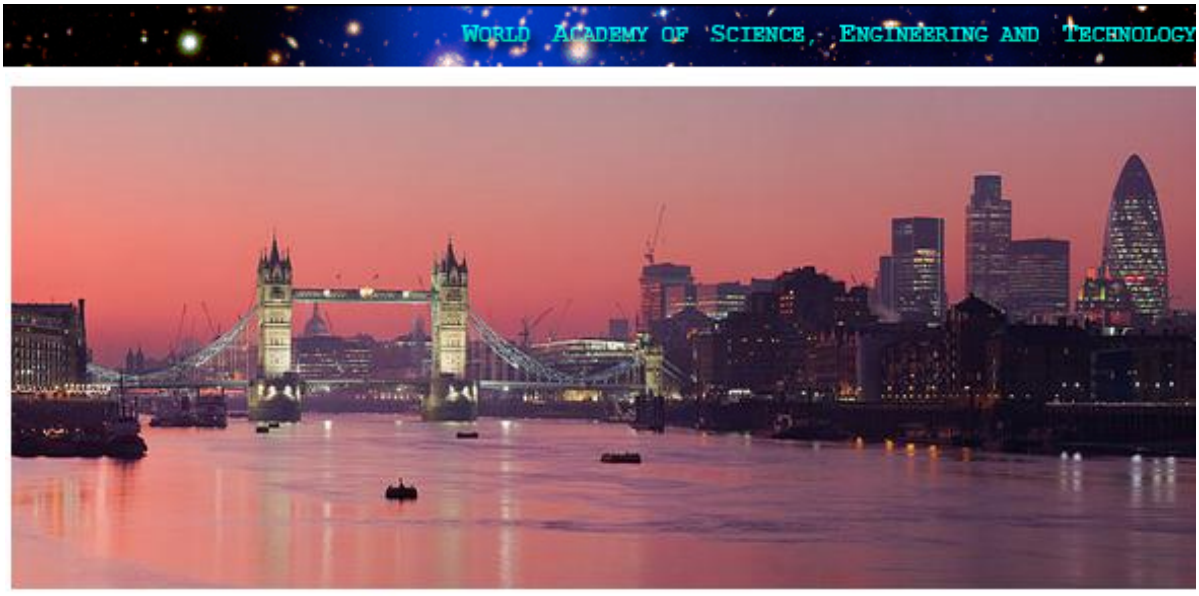

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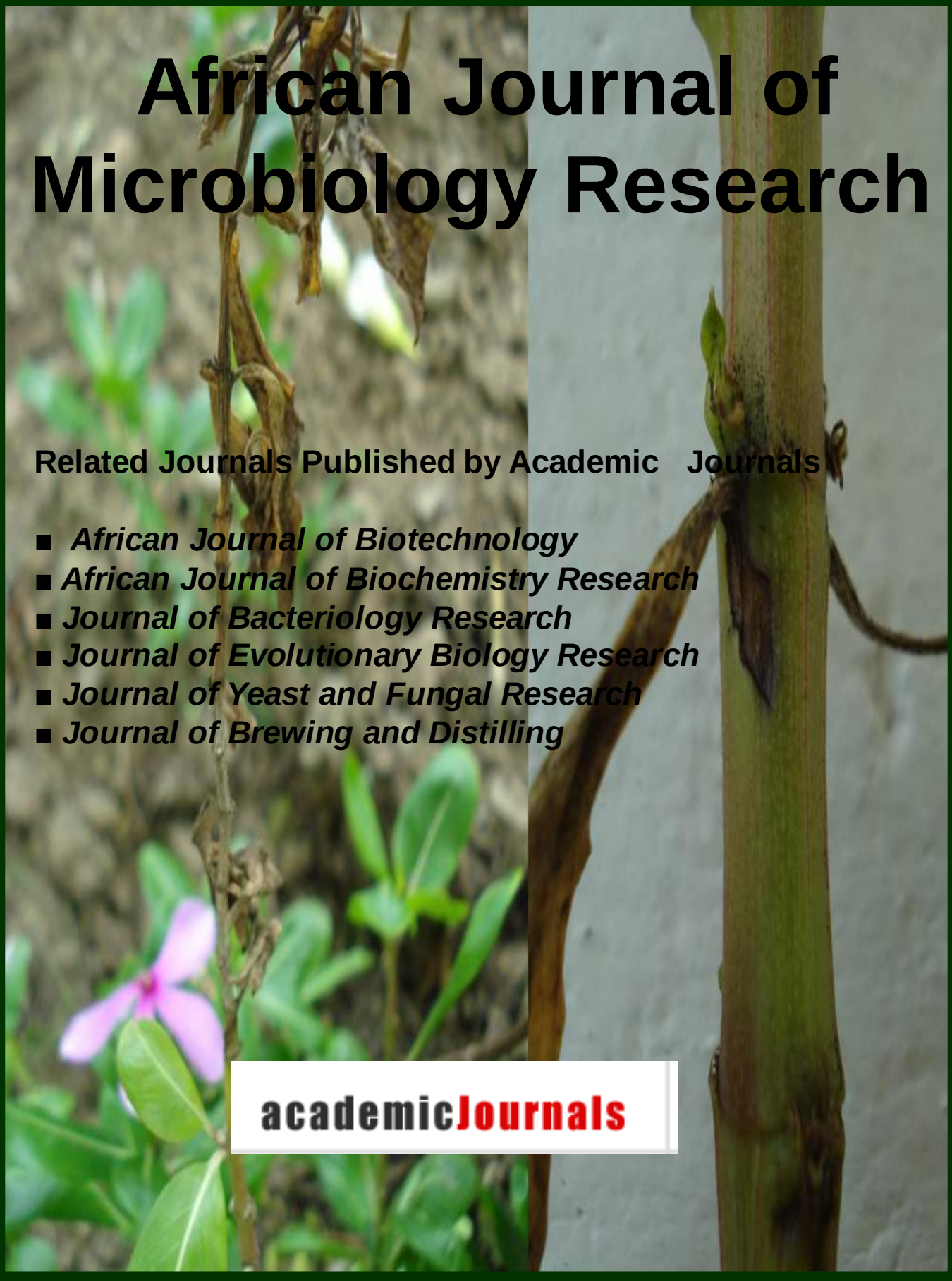

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