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ARTICLES

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Shefali Mishra, K. S. Jagadeesh, P. U. Krishnaraj and M. Valan Arasu

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Histological and histochemical changes induced by the amendment of chitosan and inoculation of *Pseudomonas* sp. against Tomato leaf curl virus (ToLCV) in tomato (*Lycopersicon esculentum* Mill.)

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This study was conducted to evaluate the comparison between the histological and histochemical changes in leaves of tomato due to tomato leaf curl virus (ToLCV) with or without plant growth-promoting rhizobacteria (PGPR) and chitosan treatments. The combined treatment of chitosan and *Pseudomonas* sp. induced a significant (P=0.05) increase in the activities of polyphenoloxidase (PPO), peroxidase (POD), chitinase, phenylammonialyase (PAL) and enhanced the content of phenolic compounds in tomato leaves. Furthermore, the combined application reduced the disease severity (%) of ToLCV in tomato plants. Although, the histopathological and histochemical studies revealed more destruction in the diseased plants, these changes could be reduced to the level of the healthy plants due to combined treatment of chitosan and *Pseudomonas* sp.

Key words: *Pseudomonas* sp., Tomato leaf curl virus (ToLCV), induced systemic resistance, chitosan, biocontrol, *Bemisia tabaci*, histopathology.

INTRODUCTION

Tomato (*Solanum lycopersicon* L.) is one of the important and most widely grown vegetable crops of both tropics and sub tropics in the world and ranks second in importance among vegetables. Recently, there has been more emphasis on tomato production not only as a source of vitamins, but also as a source of income and food security in many countries including India. There are many production constraints and it is affected by several diseases leading to substantial losses in yield. Of all the viral diseases reported on tomato, tomato leaf curl virus *Tomato leaf curl virus* (ToLCV), a Geminivirus (family *Geminiviridae*, genus *Begomovirus*) is the most important and destructive viral pathogen in many parts of India (Saikia and Muniyappa,1989; Harrison et al., 1991). The disease is transmitted by whitefly (*Bemisia tabaci*) (Muniyappa and Veeresh, 1984).

The investigation of plant response to elicitors is one of the most rapidly developing lines of inquiry in plant
physiology.

The elicitors stimulate the contact between plants and phytopathogens and, thereby, trigger defensive mechanisms that constrain the invasion of pathogenic fungi, bacteria and viruses. Chitosan is one of the most studied elicitors, and it regulates the expression of resistance genes and induces jasmonate synthesis (Doares et al., 1995). These molecules have been shown to display toxicity and inhibit fungal growth and development. They were reported to be active against viruses, bacteria, and pests as well (Abdelbasset et al., 2010). Based on these proprieties that help strengthen host plant defenses, interest has been growing in using them in agricultural systems to reduce the negative impact of diseases on yield and quality of crops.

Histopathological and biochemical changes occur in tomato leaves after infection by ToLCV resulting in external manifestation in the form of different symptoms. The plants inoculated with Tobacco mosaic virus (TMV) in tomato exhibited the disintegration of palisade tissue with wider space in spongy parenchyma (Alok et al., 1986). There was reduction in the amount of reducing sugars, non-reducing sugars, total sugars, starch and total chlorophyll in the plants infected with yellow mosaic virus as compared to healthy plants (Thind et al., 1996). In case of papaya leaves infected with the Papaya ring spot virus (PRSV), the palisade layers and spongy cells became smaller, and the leaves have been shown with broad inter cellular spaces (Singh, 1971).

Biocontrol using plant growth-promoting rhizobacteria (PGPR) agent represents a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops (Jetiyanon and Kloepper, 2002). A number of plant growth promoting rhizobacteria have been implicated in the biocontrol of viral diseases in many crop plants such as Tomato spotted wilt virus (Kandan et al., 2003), Sunflower necrosis virus (Srinivasan et al., 2005), Banana bunchy top virus (Kavino et al., 2003) and TMV in tomato (Kirankumar, 2007). These viruses have been controlled essentially through induced systemic resistance (ISR) by activating defense genes encoding chitinase, beta-1, 3 glucanase, peroxidase, phenylammonia lyase and other enzymes (Srinivasan et al., 2005; Kirankumar, 2007) and chemicals (M’piga et al., 1997). Several antagonistic microorganisms such as Pseudomonas, Bacillus, Streptomyces, Gliocladium and Trichoderma spp. have the potential to control a variety of crop diseases. In other studies, beneficial microorganisms were combined with a natural compound such as chitin or chitosan to improve their biocontrol efficacy (Sid-Ahmed et al., 2003). The aim of the present study was to compare the histochemical changes in leaves of tomato due to ToLCV, with or without PGPR amendments along with chitosan treatments; and to study the elicitation of Induced systemic resistance (ISR) molecules the defense responses of plants induced by them.

MATERIALS AND METHODS

Collection and preparation of viral inoculums

The culture of ToLCV was obtained from the virus infected tomato plants in the field. Agricultural College, UAS, Dharwad, India, and inoculated to healthy tomato plants using whiteflies (Bemisia tabaci) as the vector and the infected plants were maintained in the glasshouse throughout the period of study.

Vector culture rearing

Whiteflies were collected from cotton and tobacco plants in the fields with the help of an aspirator by turning the leaves slightly upwards. Whiteflies were released onto the ToLCV diseased tomato plants grown in insect proof rearing cages which make the insects viruliferous after sucking of whiteflies on diseased leaves and their population were maintained by introducing younger tomato plants into the rearing cages.

Release of viruliferous insects

The viruliferous insects were collected from the diseased plants with the help of an aspirator, and released onto the leaves of healthy PGPR treated tomato seedlings. Immediately, the seedlings were placed in an insect proof rearing cage and insects allowed to feed on them for a week to cause infection by the virus. Thirty days old seedlings were used for release of the viruliferous insects. Thus, it was ensured that all seedlings were infected with ToLCV.

Field study

A field experiment was conducted to assess the effect of the selected PGPR strain and chitosan on reduction of disease severity and growth promotion in tomato plants. It was carried out at main agricultural research station, UAS, Dharwad, India, during summer (Jan.-May, 2012). Five-week-old seedlings of variety pusa ruby, raised in a glasshouse were transplanted in the main field with plot size 20 m × 10 m and 75 cm × 60 cm spacing.

Rhizobacterial treatment

The bacteria were cultured on nutrient broth (Peptone, 5 g; Beef extract, 3 g; NaCl, 5 g; pH 6.8-7.2/Lit) and incubated at 28°C for 48 h with shaking at 150 rpm. Bacteria were pelleted after centrifugation for 5 min at 10000 × g, and the pellet was mixed with sterile 1% carboxy methyl cellulose (CMC) suspensions (Himedia, Mumbai, India). The seeds were surface sterilized with sodium hypochlorite solution, placed in CMC-cell suspensions, air dried inside a laminar flow chamber and the biocoated seeds were sown in the plastic pots (10" diameter) having 10 kg capacity of soil. For soil application, the lignite based culture (1:3) was applied to soil @ 5 kg/ha before sowing seeds and mixed well. For foliar application, the lignite based culture was filtered through a muslin cloth and sprayed @ 1% (w/v) at 10 and 20 days after sowing (DAS). Control plants in pots without application of rhizobacteria were also maintained. All treatments were replicated five times and arranged in a randomized complete block design (RCBD).

Chitosan treatment

Bacteria were grown in nutrient broth medium on a shaker (150 rpm) at 28°C for two days and centrifuged at 10000 × g for 5 min.
Chitosan was dissolved in 100 mM acetate buffer (pH 4.5) and the pH adjusted to 6.5 using 1 N NaOH. The cell pellet was mixed with chitosan solution (5%). Crab-shell chitosans (deacetylation of about 90%) from Sigma-Aldrich were used in this work. The surface sterilized tomato seeds were soaked in chitosan-cell suspensions and kept on a shaker for 3 h at 28°C and 100 rpm. The seeds were shaken in chitosan solution until they became fully coated. The biocoated seeds were dried inside a laminar flow chamber. At 25 DAS, both upper and lower surfaces of the leaves were sprayed with the chitosan solution (1 mg/ml) prepared in 100 mM acetate buffer (pH 4.5) and the pH was adjusted to 6.5 with 1 N NaOH.

Sample collection, Enzyme and phenol estimation

Leaf samples were collected at 45 DAS and 75 DAS from both inoculated and uninoculated (control) tomato plants. They were frozen immediately in liquid nitrogen, ground to a powder and stored at -80°C until determination of phenylammonia lyase, chitinase, polyphenol oxidase and peroxidase activities. The peroxidase activity was assayed spectrophotometrically following the method described by Mahadevan and Sridhar (1986). The phenylammonia lyase activity was determined using the method described by Ross and Sederoff (1992). The polyphenol oxidase activity in leaves was estimated at 45 and 75 DAS following the method of Mayer et al. (1965). The chitinase activity was estimated at 45 and 75 DAS, following the method described by Miller (1959). The total phenol content in leaves was estimated at 45 and 75 DAS by following Folin Ciocalteu method (Sadasivam and Manickam, 1991).

Preparation of samples for histochemical changes using various staining techniques

Affixing, deparaffinising and hydrating the sections onto the slides

PGPR treated plants with or without amendment of chitosan and diseased plants were taken for the histochemical analysis. Tomato leaves after 45 days after inoculation (DAI) were used for histopathological studies. Leaf pieces (1.0 x 0.5 cm) were harvested following the paper boat technique (Jensen, 1962). The sections of 10 µm size embedded samples were taken using Leica microtome. An adhesive was prepared using gelatin at 1.5 g/100 ml distilled water, and a little quantity of potassium dichromate was added to prevent fungal growth. A few drops of gelatin were added on to the surface of clean microslide. Sections were carefully placed on adhesive and slides were warmed slightly over hot plate maintained at 50°C for 1-2 min to facilitate flattening and stretching of section ribbons. The excess adhesive was drained off and the slides were dried for 24 h at room temperature. The sections were deparaffinised using xylene and were then treated with different grades of alcohol for gradual dehydration. Later, the sections were subjected to staining either directly or after hydration depending on the requirement. After each step, the slides were blot dried to remove excess chemical adhered to the slides.

Staining, dehydration and mounting the sections for various biochemical analyses

The sections were subjected to histological and histochemical staining for localization of different cellular chemical compounds namely insoluble polysaccharides, proteins and nucleic acids. To observe anatomical changes in diseased leaves, sections were passed through safranin and fast green stains. Sections were stained in 1% safranin (1 g of safranin in 100 ml of 50% alcohol) for 2 h and then dehydrated in 50, 70 and 90% alcohol for 5 min, stained with 0.5% fast green (0.5 g of fast green in 100 ml of 95% alcohol) for 5 min and again dehydrated with 95% alcohol for 5 min, cleaned in xylene and mounted in DPX. The assessment of total insoluble polysaccharides was followed by Periodic acid Schiff’s (PAS) method (Hatchkiss, 1948). The reagents were prepared as described by Longley (1952). For the assessment of total proteins, the method developed by O’Brien and Mc Cully (1981) was used. For the staining of nucleic acids, the method of Toludine Blue O was used (Chayen et al., 1973). The dye Toludine blue was used for the purpose of detecting the richness of RNA and DNA in cells. The slides were photographed using an Axiosstar plus ("Carl Zeiss") Bright field microscope with Canon power shot G2 digital camera attachment.

Statistical analysis

The data obtained from field experiments were subjected to Randomized Complete Block Design analysis (Gomez and Gomez, 1984). The level of significance used in the ‘F’ test was P=0.05. The critical difference values were calculated whenever the F test values were significant.

RESULTS AND DISCUSSION

Structural staining for anatomical changes

Histopathological studies were conducted to study the changes occurring due to ToLCV infection as well as to study to what extent these changes are reduced by inoculation with biocontrol agents. The histopathological studies revealed more anatomical destruction in leaves of diseased plants as compared to treated and healthy plants. There were more intercellular spaces. The palisade cells were markedly reduced in size, and lost their columnar compact nature (Figure 1). The reduction in size of leaf tissues and their destruction may be due to metabolic changes in tissues causing hypotrophy in palisade and spongy parenchyma cells. Similar observations were recorded in infected papaya leaves (Singh, 1971), Cucumber mosaic virus (CMV) affected chilli leaves (Mishra and Singh, 1973), CMV infected tobacco leaves (Ehara and Mishawa, 1975), TMV infected tomato (Alok et al., 1986) and pigeonpea infected by sterility mosaic virus (Singh and Rathi, 1996a).

Effect on polysaccharides content in leaves

The studies also revealed reduction in insoluble polysaccharides in palisade and spongy parenchyma cells of diseased plants as compared to healthy plants, which showed rich concentration of polysaccharides in the cells. The treatments receiving Pseudomonas sp. 206(4)+chitosan and Pseudomonas sp. B-15+Chitosan showed almost the same concentration of polysaccharides as seen in healthy cells whereas, Pseudomonas sp. 206(4) and Pseudomonas sp. B-15 showed a slight reduction in
concentration of polysaccharides in palisade and spongy parenchyma cells (Table 1 and Figure 2). The reduction in polysaccharides may be due to varied metabolism in infected tissues. Several workers have reported signifi-
Table 1. Histochemical changes in healthy, treated and ToLCV infected leaves of tomato plants (At 45 DAI).

<table>
<thead>
<tr>
<th>Status</th>
<th>Histochemical</th>
<th>Different regions of leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Epidermis</td>
</tr>
<tr>
<td>Healthy</td>
<td>Polysaccharides</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++</td>
</tr>
<tr>
<td>Diseased</td>
<td>Polysaccharides</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++++</td>
</tr>
<tr>
<td>Pseudomonas sp.206(4)+Chitosan treatment</td>
<td>Polysaccharides</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++</td>
</tr>
<tr>
<td>Pseudomonas sp.206(4) treatment</td>
<td>Polysaccharides</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++++</td>
</tr>
<tr>
<td>Pseudomonas sp.B-15+Chitosan treatment</td>
<td>Polysaccharides</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++</td>
</tr>
<tr>
<td>Pseudomonas sp.B-15 treatment</td>
<td>Polysaccharides</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Nucleic acids</td>
<td>++++</td>
</tr>
</tbody>
</table>

++++, Very rich; +++ rich; ++ Medium; +, Low.


Effect on nucleic acid content in leaves

Nucleic acid content was found to be greater in palisade and spongy cells of diseased leaves when compared to healthy leaves. The manifestation of increase in nucleic acid content might be due to the combined effect of host and viral nucleic acid. Usually, concentration of virus in infected cells increases rapidly upon infection, thus, increasing their nucleic acid concentration in such cells. The treatments of *Pseudomonas* sp. 206(4)+ chitosan, and *Pseudomonas* sp. B-15 + chitosan showed almost similar nucleic acid quantity as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight increase in nucleic acid content in palisade and spongy parenchyma cells (Table 1 and Figure 3). Our results are corroborative with the results obtained by Joshi and Dubey (1974) for the CMV infected chili-pepper leaves, infected nuclei of tobacco (Russo and Martell, 1975), nucleic acids and chloroplasts of tomato cells infected by ToLCV (Channarayappa et al., 1992). Similar effect was also reported by Singh and Rathi (1996b), where the presence of foreign ribonucleo protein in cytoplasm and nucleus of virus infected pigeonpea and increase in total nucleic acid in virus infected host tissues of papaya (Johri, 1975).

Effect on protein content in leaves

There was a significant higher reduction of protein content in palisade and spongy parenchyma cells from infected plants when compared to healthy plants; this might be due to the degradation of host protein or reduction in synthesis of protein subsequent to viral infection since virus uses host cell contents for replication. The combined treatment of the plants with *Pseudomonas* sp. 206(4)+chitosan, *Pseudomonas* sp. B-15+chitosan showed almost the same quantity of proteins as seen in healthy cells; while, the individual application of *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a reduction in protein content in palisade and spongy parenchyma cells (Table 1 and Figure 4). The reduction of protein content by 41% in diseased papaya
Figure 3. Histochemical changes (nucleic acid) in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.

Figure 4. Histopathological changes (proteins) in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.
fruits was earlier reported (Singh et al., 1977; Sun, 1985). The histochemical analysis from our study revealed that there was an increase of nucleic acid and decrease of insoluble polysaccharides, and also protein content due to ToLCV infection.

**Effect of rhizobacteria and chitosan on disease resistance against ToLCV through induced systemic resistance in tomato plants**

Bio-control agents, mainly bacterial inoculants are believed to induce systemic defense responses in the plants besides other antagonistic mechanisms. Induction of defense responses by plant-growth-promoting rhizobacteria (PGPR) is largely associated with the production of pathogenesis related (PR) proteins like b-1,3-glucanase and the defense enzyme phenylalanine ammonia-lyase and oxidative enzymes like peroxidase and polyphenol oxidase (Compant et al., 2005). There was a greater level of phenolics in plants inoculated with *Pseudomonas* sp. and chitosan compared to uninoculated plants. These results are in agreement with previous results, where there was increased phenolics content in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus (Kandan et al., 2003). An increased chitinase activity resulted in preventing the damage caused by viral pathogen and, thus, increased the disease control percentage in all the rhizobacteria treated plants. There was induction in biosynthesis of defense molecules in all the treated plants. Combined application of *Pseudomonas* sp. 206(4) and chitosan on tomato plants resulted in the highest synthesis of phenol content, PO, PPO, chitinase and PALase activity which accounts to 30.55, 49.31, 38.79, 32.90 and 34.78% respectively; higher than the diseased control. The role of PPO in disease resistance is to oxidize phenolic compounds to quinones, which are more toxic to microorganisms than the original phenols and the enzyme itself is inhibitory to viruses by inactivating the RNA of the virus (Vidhyasekaran, 1988). Though all the treatments induced biosynthesis of phenolics, PAL, peroxidase, chitinase and polyphenol oxidase, the *Pseudomonas* sp. 206(4) + Chitosan treatment showed a greater defense activity compared to disease control (Figure 5). Cimammic acid, the product of PAL, is directly linked to cell lignification processes and the highest levels of PAL activity usually occur about one day after initial infection of pathogen (Podile and Laxmi, 1998). The PO and PPO activities are linked to lignification and generation of hydrogen peroxides at later stages of infection, which inhibit pathogens directly, or generation of other free radicals with antimicrobial activity, that restrict the development of pathogens (Silva et al., 2004).

Application of rhizobacteria in combination with or without chitosan reduced the ToLCV severity by 58-70%. The treatment receiving *Pseudomonas* sp. 206(4)+ chitosan reduced the disease severity by 68% whereas the treatment receiving *Pseudomonas* sp. B-15+ chitosan exhibited 68.30% ToLCV severity control under field condition (Figure 5f). Thus, the observed induction of enzymes and the corresponding reduction in ToLCV infection in tomato supports the hypothesis that the
resistance induced by isolates is systemic.

The increase in callose content as well as induction in ribonuclease and β-1,3 glucanase in potato plants as defense response against potato virus X (PVX) were observed when plants were sprayed with chitosan solution (1 mg/ml). In other observation, there was enhanced activation of catalase, PAL, Peroxidase, PPO and chitinase levels in sunflower when seeds were treated with chitosan (5%) for controlling downy mildew (Chirkov et al., 2001; Nandeeshkumar et al., 2008). Similar observations were reported with the potato virus X, tobacco mosaic and necrosis viruses, alfalfa mosaic.
virus, cucumber mosaic virus (Chirkov, 2002).

**Conclusion**

In the present research, we showed that *Pseudomonas* sp. 206(4) combined with chitosan is a potential biocontrol agent with strong and reproducible biocontrol effects on controlling disease severity of ToLCV in tomato plants. Histochemical studies revealed reduction in insoluble polysaccharides and proteins and increase in nucleic acid content in palisade and spongy parenchyma cells of diseased plant as compared to treated and healthy plant. Histochemical studies also clearly indicated...
that rhizobacteria-chitosan biopreparation is more effective in controlling ToLCV as compared to rhizobacteria alone. Although the histopathological and histochemical studies revealed more destruction in the diseased plants, these changes could be reduced to the level of the healthy plants due to combined treatment of chitosan and *Pseudomonas* sp.

Conflict of Interests

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

ACKNOWLEDGMENTS

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Figure 5f. Disease severity percentage exhibited by selected rhizobacteria in combination with or without chitosan after 75 days of pathogen (ToLCV) inoculation. Different letters on bars indicate statistical significance between treated and control according to LSD (P = 0.05). Each value represents the mean of three replications per treatment. Error bars indicate ± standard error (SE). Here, C represents chitosan.
In vitro antibacterial screening of methanolic extract of whole body tissue and ethylene diamine tetra acetate (EDTA) extract of cuttlebone of *Sepia pharaonis* (Ehrenberg, 1831) against selected clinical isolates

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The need for the discovery of new and novel antibiotics is imperative because evidence suggests that development and spread of resistance to any new antimicrobial agent is inevitable. In the present study, the *in vitro* antibacterial activity of methanolic extract of whole body tissue and ethylene diamine tetra acetate (EDTA) extract of cuttlebone (polysaccharide) of *Sepia pharaonis* was investigated against ten bacterial species including Gram-positive species (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative species (*Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Klebsiella oxytoca*, *Escherichia coli*, *Salmonella paratyphi*, *Vibrio parahaemolyticus* and *Proteus mirabilis*) with different concentrations such as 25, 50, 75 and 100% using disc diffusion method. The highest inhibition zone was recorded against *P. mirabilis* for methanolic extract (18.3±0.1 for 100% concentration) and against *S. pyogenes* for EDTA extract (polysaccharide) (15.5±0.06 for 100% concentration) of cuttlebone. But the activity was totally absent in negative control. For minimum inhibitory concentration (MIC) technique, various ranges of concentrations between 20 and 100 mg/ml were prepared and tested. MIC values were found ranging from 40 and 100 mg/ml. All assays were carried out in triplets. A wide spectral and concentration dependent antibacterial activity was recorded in both extracts.

Key words: Antibacterial activity, polysaccharide, cuttlebone, methanolic extract, ethylene diamine tetra acetate (EDTA), minimum inhibitory concentration (MIC).

INTRODUCTION

In nature, animals are provided with their own protective response against their predators and pathogens. Marine molluscs are exposed to microbial pathogens in their environment, which can number up to $10^8$ bacteria/ml of...
seawater (Ammerman et al., 1984). In order to defend themselves against such condition, molluscs have developed very effective mechanisms that are part of their innate immunity (Tincu and Taylor, 2004). Molluscs are widely distributed throughout the world and have many representatives in the marine and estuarine ecosystem such as slugs, whelks, clams, mussels, oysters, scallops, cuttlefishes, squids and octopods. Bioactive substances from marine biota have been used as special tools in pharmacological/biomedical research. Discovered bioactive compounds in molluscs were identified essentially as peptide, depsipeptide, steroids, sesquiterpene, terpenes, polypropionate, nitrogenous compounds, macroloides, prostaglandins and fatty acid derivatives, steroids, antimicrobial peptides (AMPs), miscellaneous compounds and algaloids; they all presented specific types of activities (Balcazar et al., 2006; Destoumieux et al., 1997; Jones, 1971). The presence of antimicrobial activity in Mollusca has been reported from the mucus of the giant snail Achatina fulica (Kubota et al., 1985; Iguchi et al., 1982) from the egg mass and purple fluid of the sea hare, Aplysia kurodai (Yamazaki, 1993; Kamiya et al., 1984) and from the body wall of the sea hare Dolabella auricularia (Iijima et al., 2003).

Among marine invertebrates, cephalopods belong to a molluscan group comprising of 700 species in which bacterial associations have been known for a long time (Pierantoni, 1917; Bloodgood, 1977; Romanenko et al., 1995) which include the reproductive organs (accessory nidamental glands) of myopsids, sepiliods and sepilids (Kaufman et al., 1998; Grigioni et al., 2000; Pichon et al., 2005) and the light organ of sepiloids (McFall-Ngai and Ruby, 1991; Nishiguchi, 2002) ink extracts of D. auricularia, O. vulgaris and S. aculeata (Vennila et al., 2011) polysaccharide extract of Sepia aculeata and Sepia brevimana and heparin and heparin-like glycosaminoglycans (GAGs) from the cephalopod Euprymna berryi was reported against the human pathogenic microorganisms (Shanmugam et al., 2008a, b). Antibiotics are one of the most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction.

However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illness not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. It is essential to investigate newer drugs with lesser resistance. In most of the findings concerning antimicrobial activity in molluscs, either single body compound alone, like haemolymph and egg masses, or extracts of whole body tissues have been tested for activity. The present study has been focused on the antibacterial activity of methanolic extract of the whole body tissue and also EDTA extract (polysaccharides) from the cuttlebone of pharaoh cuttlefish (Sepia pharaonis) on ten important clinically isolated human pathogenic bacteria.

MATERIALS AND METHODS

Sampling and identification

The cuttlefish, S. pharaonis was obtained from Thengaiittittu landing centre of Puducherry (Lat.11° 54’ 44” N; Long. 79° 49’ 13” E), South-east coast of India. The publications of Roper et al. (1984), Jothinayagam (1987) and Shanmugam et al. (2002) were used for identification.

Preparation of methanolic extract from body tissues

The methanolic extract of the body tissues was prepared by following the method of Ely et al. (2004). S. pharaonis was brought to the laboratory: skin, visceral organs, cuttlebone and ink sac were removed. Remaining edible body tissues were separated and cut into small pieces and homogenized (REMI, RQ-127 A) and extracted with 100% methanol for 24 - 48 h by incubating at room temperature. Then, the methanolic extract was centrifuged to collect the supernatant and concentrated under vacuum in a rotary evaporator (LARK, Model: VC- 100A). The crude methanolic extract of whole body tissue was assayed for antibacterial activity using standard disc diffusion method.

Preparation of EDTA extract from cuttlebone

The EDTA extract was obtained from the internal shell (cuttlebone) of S. pharaonis by following the method of Okutani and Morikawa (1978). The air-dried cuttlebones were pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered with Whatman No. 1 filter paper with hyflosuper cel. Then saturated barium hydroxide solution was added to the filtrate. The precipitate obtained after standing overnight was collected on a filter paper (Whatman No.1) with hyflosuper cel and washed with distilled water. The precipitate was dissolved in 10 mM EDTA solution and was dialyzed against deionised water. The dialyze solution present in the dialysis membrane was then freeze-dried and a pure white coloured powder was obtained. The lyophilized powder was used for assaying the antibacterial activities.

Microbial cultures

Ten bacterial species including Gram positive species (Staphylococcus aureus and Streptococcus pyogenes) and Gram negative species (Salmonella typhi, Klebsiella pneumoniae, Vibrio cholerae, Klebsiella oxytoca, Escherichia coli, Salmonella paratyphi, Vibrio parahaemolyticus and Proteus mirabilis) were used as test organisms. All the bacterial species were clinical isolates, obtained from Raja Muthiah Medical College Hospital, Annamalai University, Annamalai Nagar, South India.

Preparation of inoculum

Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. All the ten bacterial strains were individually inoculated in the sterilized nutrient broth and incubated at 37°C for 24 h. Mueller Hinton Agar (MHA, Himedia) was prepared, sterilized in an autoclave at 15 lbs for 15 min pressure and poured into sterile Petri dishes and incubated at 37°C for 24 h. The 24 h-old bacterial broth cultures were inoculated in the Petri dishes by using a sterile cotton swab.

Antibacterial assay

In vitro antibacterial activity was determined by disc diffusion
technique of Gunthorpe and Cameron (1987). The stocks for methanolic extracts were prepared in the concentration of 100 mg/mL. 60 mg of lyophilized cuttlebone powder of crude extract was dissolved in 0.6 mL of solvent (10 mM EDTA) to prepare stock solution. From this 0.24, 0.18, 0.12 and 0.06 mL of sample was taken and each was made up to 0.24 mL with respective (10 mM EDTA) solvent. The control with respective solvent (10 mM EDTA) was also prepared. These different concentrations (0.24, 0.18, 0.12 and 0.06 mL known as 100, 75, 50 and 25% respectively) of the extract were applied to 6 mm sterile disc, allowed to dry at room temperature and placed on agar plate seeded with bacterial strains. Positive control disc containing 50 µL of tetracycline (1 mg/mL) and as negative control containing 50 µL of methanol and 10 mM EDTA each were used. These impregnated discs were allowed to dry at laminar air flow chamber for 3 h, and were placed at the respective bacterial plates and incubated at 37°C for 24 h. Result was calculated by measuring the zone of inhibition in millimeters. Each extract was tested thrice for the confirmation of activity.

### Determination of the minimum inhibitory concentration (MIC)

The methanolic extract and EDTA (polysaccharide) extract that showed significant antibacterial activity was selected for the determination of MIC followed by the turbidimetric method of Rajendran and Ramakrishnan (2009). A stock solution of 100 µg/mL was prepared and was serially diluted to obtain various ranges of concentrations between 20 and 100 µg/mL. To 0.5 mL of each of the dilutions of different concentrations was transferred into sterile test tube containing 2.0 mL of nutrient broth. To the test tubes, 0.5 mL of test organism previously adjusted to a concentration of 10⁵ cells/mL was then introduced. A set of test tubes containing broth alone was used as control. All the test tubes and control were then incubated at 37°C for 24 h. After the period of incubation, the tubes were studied for visible signs of growth or turbidity. The lowest concentration of methanolic and EDTA extract that inhibited the growth of bacteria was taken as the minimum inhibitory concentration. All assays were carried out in triplicates and the control test was carried out with the broth alone.

### Statistical analysis

Data on the inhibitory effect of methanolic extract and the EDTA extract of *S. pharaonis* were analyzed by one-way analysis of variance (ANOVA) using SPSS-16 version software followed by Duncun’s multiple range test (DMRT). *P* values <0.05 were considered as significant.

### RESULTS

The methanolic extract of whole body tissue and the EDTA extract of polysaccharides from cuttlebone of *S. pharaonis* showed wider activity against pathogenic organisms. In general, the activity was higher in 100% concentration and lower in 25% concentration but activity was totally absent in negative control whereas the positive control showed activity against all pathogenic organisms (Table 1).

In 100% concentration, the maximum inhibition zone was observed against *P. mirabilis* (18.3±0.1 mm) for methanolic extract and against *S. pyogenes* (15.5±0.06 mm) for EDTA extract of polysaccharide. The minimum inhibition zone of 9.9±0.36 mm was recorded against *S. aureus* for methanolic extract and against 8.3±0.12 mm *V. parahaemolyticus* for EDTA extract; 75% concentration of methanolic extract showed highest activity against *P. mirabilis* (16.5±0.06 mm), and against *S. pyogenes* (15.47±0.12 mm) for EDTA extract. The lowest activity against *S. aureus* was 8.9±0.36 mm for methanolic extract and 7.4±0.17 mm against *V. parahaemolyticus* for EDTA extract. Whereas, in 50% concentration of methanolic extract, highest activity of 13.8±0.06 mm was found against *P. mirabilis*, and 14.5±0.21 mm against *S. pyogenes* for EDTA extract. The lowest activity of 8.3±0.12 mm was recorded against *S. aureus* for methanolic extract and 9.23±0.06 mm against *S. typhi* for polysaccharides; for 25% concentration, the methanolic extract showed maximum activity of 12.47±0.06 mm against *P. mirabilis*, and 13.27±0.2 mm for EDTA extract against *S. pyogenes*. The minimum activity was 7.9±0.36 mm against *S. aureus* for methanolic extract and 7.2±0.2 mm against *S. typhi* for EDTA extract.

There was no activity in all concentrations for methanolic extract against *S. paratyphi* and *V. parahaemolyticus* and for EDTA extract against *K. pneumoniae*.

### MIC of the active extract against the test organisms

The MIC results found ranging from 40 and 100 mg/ml are given in Tables 2 and 3. MIC values for methanolic extract of *S. pharaonis* against bacterial species such as *S. aureus*, *K. pneumoniae*, *S. typhi*, *V. cholerae*, *K. oxytoca*, *E. coli*, *P. mirabilis* and *S. pyogenes* showed 100, 100, 80, 80, 60, 100, 60 and 80 mg/mL respectively. Whereas for EDTA extract of polysaccharides, the MIC values were found to be 100, 80, 80, 100, 80, 60 and 40 mg/mL against bacterial species such as *S. aureus*, *S. typhi*, *V. cholerae*, *K. oxytoca*, *E. coli*, *S. paratyphi*, *P. mirabilis* and *S. pyogenes*, respectively.

### DISCUSSION

This study dealt with the antibacterial activity of crude methanolic extract of a whole body tissue and EDTA extract (polysaccharides) of cuttlebone of *S. pharaonis* and compared them with different concentrations (0.24, 0.18, 0.12, and 0.06 mL of samples made as 100, 75, 50, and 25% concentration, respectively). The activity was recorded for both extracts in all the four concentrations for majority of bacterial species. The effects of the extracts were different for different bacterial species.

Many studies on bioactive compounds from mollusks exhibiting antitumor, antileukemic, antibacterial and antiviral activities have been reported worldwide (Hochlowski et al., 1983). Antibacterial activity has previously been described in a wide range of molluscan species such as oyster (*Crassostrea virginica*), mussel (*Mytilus edulis* and *Geukensia demissa*), muricid mollusks (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) (Anderson and Beaven, 2001; Benkendorff et al., 2001; Gunthorpe and Cameron,
Table 1. Zone of inhibition showed by the methanolic extract of whole body tissue and EDTA extract (polysaccharides) from cuttlebone of *S. pharaonis*.

<table>
<thead>
<tr>
<th>Name of the species/concentration</th>
<th>Methanolic extract zone of inhibition (mm)</th>
<th>EDTA extract–polysaccharides zone of inhibition (mm)</th>
<th>Positive (tetracycline) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7.9±0.36</td>
<td>8.9±0.2</td>
<td>8.9±0.36</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>9.2±0.2</td>
<td>10.3±0.12</td>
<td>11.1±0.15</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>10±0.2</td>
<td>10.1±0.36</td>
<td>12.23±0.25</td>
</tr>
<tr>
<td><em>V. cholera</em></td>
<td>10.27±0.12</td>
<td>12.23±0.15</td>
<td>12.47±0.06</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>10.5±0.1</td>
<td>13.4±0.2</td>
<td>15±0.2</td>
</tr>
<tr>
<td><em>S. paratyphi</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>9.5±0.06</td>
<td>10.7±0.1</td>
<td>12.6±0.06</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>12.47±0.06</td>
<td>13.8±0.06</td>
<td>16.±0.06</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>10.47±0.12</td>
<td>11.4±0.2</td>
<td>12.5±0.06</td>
</tr>
</tbody>
</table>

*The statistical significance: P values ≤0.05 (DMRT).

Table 2. MIC of the methanolic extract of whole body tissue from *S. pharaonis*.

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Concentrations (%)/activity</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>V. cholera</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*MIC concentration, -No growth, +Cloudy solution (slight growth), ++Turbid solution (strong growth), +++Highly turbid solution (dense growth).

Antibacterial peptides have been isolated and characterized from the hemocytes of *M. edulis* (Charlet et al., 1996) and from sea hare, *D. auricularia* (Iijima et al., 2003). Patterson and Murugan (2000) spectrum of antibacterial activity for aqueous ink extract reported broad of the cephalopods *L. duvacieli* and *S. pharaonis* against nine human pathogens. However, majority of marine organisms are yet to be screened for discovering useful antibiotics. The hypobranchial gland extracts of *Chicoreus ramosus* was found inhibiting the growth of ten bacterial strains; out of this, the broad inhibition zone was formed against *Streptococcus faecalis* and *S. aureus* (Emerson and Ayyakkannu, 1992b).
In the broad spectrum (7 species of gastropods, 1 bivalve and 5 cephalopods) study of Rajaganapathy (2001), the methanol and the saline extracts of ink gland, salivary gland, body mucus and internal shell of cephalopods such as Loligo duvaucelli, S. pharaonis, Sepiella inermis, Octopus dollfusi and Cistopus indicus recorded varying antibacterial activity against different bacterial strains viz., B. subtilis, E. coli, K. pneumoniae, P. vulgaris, P. mirabilis, S. typhi, S. flexnari, S. faecalis and V. cholerae. All the cephalopod extracts exhibited activity against at least three bacteria and the highest activity of 10.5 mm was recorded in the ink gland extracts against P. mirabilis. The saline extract of salivary gland and the methanol extracts of body mucus of L. duvaucelli, S. pharaonis, S. inermis, O. dollfusi and C. indicus showed significant activities against K. pneumoniae, B. subtilis, S. flexnari, S. typhi and S. faecalis with the maximum activity (8 mm) recorded in the salivary gland extracts against S. typhi. The body mucus extracts were reported to have promising activities against S. typhi with the inhibition zones measuring as high as 6 mm (Rajaganapathy, 2001). The maximum zone of inhibition (19 mm) in antibacterial activity from the gill extraction of Perna viridis, against S. aureus and minimum activity (11 mm) was observed against S. paratyphi (Chandran et al., 2009).

So far, there are only a few studies carried out on the antibacterial activity of the internal bone of cephalopods. Barwin Vino (2003) for EDTA extract (polysaccharides) of Doryteuthis sibogae gladius recorded 10 mm inhibition zone against E. coli and K. pneumoniae, 9 mm inhibition zone against S. aureus and 7 mm against S. typhi. Whereas the EDTA extract of L. duvaucelli extract showed only low activity, that is, 5 mm against P. aeruginosa, 4 mm against S. typhi and E. coli. At the same time, the gladius extract of both species showed no activity against V. cholerae. The polysaccharide extract from the gladius of D. sibogae recorded potent antibacterial activity against the bacterial strains mentioned above and at the same time the polysaccharide of L. duvaucelli gladius extract recorded only low activity. Further, the methanol extracts of the cuttlebone of S. pharaonis showed activity against S. flexnari (5 mm), S. faecalis and V. cholerae (4.5 mm) and S. typhi (3.5 mm); whereas S. inermis extracts of cuttlebone showed activity only against K. pneumoniae and V. cholerae (3.5 mm). Such similar activities were found only in 50, 75 and 100% concentrations of S. aculeata, but the Sepia brevimana extracts showed highest activity against all the strains at all concentrations (Mahalakshmi, 2003). In comparison, the activity was predominant in the cuttlebone extract of S. aculeata than S. brevimana (Shanmugam et al., 2008b).

Ramasamy et al. (2011a) screened the antimicrobial activity of polysaccharide from cuttlebone and methanolic extract from body tissue of Sepia prashadi. The antibacterial activity was predominant in cuttlebone extracts (using EDTA) of the cuttlefish, (S. prashadi) against almost all the 10 pathogenic bacterial species tested viz., E. coli, K. pneumoniae, S. aureus, P. aeruginosa, V. cholerae, V. parahaemolyticus, V. alginolyticus, Streptococcus sp., S. pneumoniae and Salmonella sp. The activity was recorded in almost all the concentrations except in negative control. The highest inhibition zone of 13 mm was recorded against V. parahaemolyticus in polysaccharide extract and 13 mm inhibition zone was recorded against S. aureus in methanolic extract. Further Ramasamy et al. (2011b) assayed the antimicrobial activity (in vitro) of methanolic extracts from S. inermis, S. kobiensis, S. lessoniana, O. aegina, O. dollfusi and O. aerolatus against 10 bacterial species and fungal strains and reported good (10-15 mm diameter) microbial activity was seen in the extracts of S. inermis, S. lessoniana and O. dollfusi which indicates the presence of potent antimicrobial compounds in them. Similarly, Vairamani et al. (2012) studied the antibacterial activity of polysaccharide from cuttlebone and methanolic extract from body tissue of S. inermis against 9 bacterial strains and found activity against 8 strains except Vibrio alginolyticus.

In the present investigation, the crude methanolic extract from the whole body tissue and the EDTA extract of polysaccharides from cuttlebone of S. pharaonis were used.

Table 3. MIC of the EDTA (polysaccharides) extract of the cuttlebone from S. pharaonis.

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Concentrations (%)/activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>S. aureus</td>
<td>*</td>
</tr>
<tr>
<td>S. typhi</td>
<td>-</td>
</tr>
<tr>
<td>V. cholera</td>
<td>-</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>-</td>
</tr>
<tr>
<td>E. coli</td>
<td>*</td>
</tr>
<tr>
<td>S. paratyphi</td>
<td>-</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>-</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>-</td>
</tr>
</tbody>
</table>

*MIC concentration, -No growth, +Cloudy solution (slight growth), ++Turbid solution (strong growth), +++Highly turbid solution (dense growth).
to study the antibacterial activity against selected human pathogens. The methanolic extract showed prominent antibacterial activity against eight bacterial species except *S. paratyphi* and *V. parahaemolyticus* with the activity ranging from 7.9±0.36 mm of inhibition zone against *S. aureus* (25% concentration) to 18.3±0.1 mm against *P. mirabilis* (100% concentration). Among the tested ten species highest inhibition zone of activity (for all four concentrations) was found against *P. mirabilis*; also it was found noteworthy in ascending order (12.47±0.06 for 25%; 13.8±0.06 for 50%; 16.5±0.06 for 75% and 18.3±0.1 for 100%); for the lowest inhibition activity similar ascending attitude was followed against *S. aureus* (7.9±0.36 for 25%; 8.3±0.12 for 50%; 8.9±0.36 for 75% and 9.9±0.36 for 100% concentration).

Further the EDTA extract showed good activity against nine bacterial strains tested, except *K. pneumoniae* with the activity ranging from 7.2±0.2 mm against *S. typhi* (25% concentration) to 15.5±0.06 mm against *S. pyogenes* (100% concentration). Similarly, high inhibition zone of activity in ascending manner (for all four con-centrations) was recorded against *S. pyogenes* (13.27±0.21 for 25%; 14.5±0.21 for 50%; 15.47±0.12 for 75% and 15.5±0.06 for 100%), also low inhibition activity against *S. typhi* and *V. parahaemolyticus* was found. In general, the increased concentration showed increase in the activity of the extracts (Shanmugam et al., 2008b).

Emerson and Ayyakkannu (1992a, b), Shanmugam et al. (2008b) and Ramasamy et al. (2011a) reported a broad spectral activity in the hypobranchial gland extract of *Chicoreus ramosus* against 10 bacterial species; good activity of the cuttlebone (EDTA) extract of *S. aculeata* and *S. brevimana* and *S. prashadi* against all bacterial strains. Although different species and experimental procedures were used in the different studies, they indicated the high frequency of detectable antibacterial activity in marine molluscs. However, there exists a difference in the activity shown by the compounds present in the extracts in laboratory studies and natural environments, which may be due to their varying concentration present in the extracts used in both places (Kelman et al., 2006; Mirnijd et al., 2011).

When compared with the above mentioned studies, whole body tissue methanolic extract as well as cuttlebone EDTA extract (polysaccharide) exhibited better activity and the activity was found dose dependent. The effect of extracts was different with different bacterial strains. The level of activity measured by disc diffusion assay is dependent on both the rate of diffusion of extract into the agar and the potency of extract. The deference in response may be due to species-specific characteristics. Extracts that contain highly active compounds (more potent), but have physical properties that generate a lower diffusion rate, may reappear to have low activity in the assay (Kelman et al., 2006). Further, the dose as well as the concentration of the active principle in the extract shows either ‘bactericidal’ or ‘bacteriostatical’ effects shows either ‘bactericidal’ or ‘bacteriostatical’ effects against the bacteria (Zarakolu et al., 1999).

**Conclusion**

The present study of *S. pharaonis*, exhibiting a wide spectral antibacterial activity has been recorded in both extracts, as compared to the studies of Emerson and Ayyakkannu (1992a), Shanmugam et al. (2008b) and Ramasamy et al. (2011a). It also revealed that the cuttlebone, thrown as a waste in the landing centers and processing plants, might be used for the extraction of polysaccharides that have antibacterial activity. The activity was found dose dependent and supporting the presence of bioactive principle. Further investigation on the purification and chemical elucidation of the bioactive principles present shall pave the way for the development of either the base or a new drug itself in the future.

**Conflict of Interest**

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

**ACKNOWLEDGEMENTS**

The authors are thankful to the Director and Dean, CAS in Marine Biology, Faculty of Marine Sciences, and Annamalai University for providing necessary facilities. The authors are also thankful to the Centre for Marine Living Resources and Ecology (CMLRE), Ministry of Earth Sciences, Cochin for the financial assistance.

**REFERENCES**


Full Length Research Paper

Evaluation of antimicrobial activity against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* and antioxidant activities of *Streptomyces* sp. TC1

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A *Streptomyces* sp. TC1 isolate was screened for antimicrobial activity against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* under *in vitro* condition. The crude extracts obtained from the culture filtrate by solvent extraction using ethyl acetate were possessed with moderate antimicrobial activity tested in agar well diffusion method. The crude extract is also analyzed for antioxidant activities by six different *in vitro* assays and found to possess good antioxidant potential. The characterization of crude extract by thin layer chromatography (TLC) showed the presence of coumarin and phenolic components. The fourier transform infrared spectroscopy (FT-IR) results also support the presence of functional groups pertaining to coumarin and phenolic components. These results indicate that the *Streptomyces* sp. TC1 can be utilized as a biocontrol organism for the management of bacterial leaf blight disease in rice.

**Key words:** Antimicrobial, antioxidant, thin layer chromatography (TLC), *Streptomyces*.

**INTRODUCTION**

The soil dwelling bacteria belonging to the genus *Streptomyces* are aerobic, Gram-positive in nature and majority of which are soil saprophytes (Ripa et al., 2010). The genus *Streptomyces* are widely recognized as sources of antibiotics and other important novel secondary metabolites including antibacterial, antitumour, antifungal, antiviral, antiparasitic and other properties having wider applications (Solecka, 2012). The *Streptomyces* sp. is widely studied for their biocontrol potential against plant pathogens because of their antibiotics producing nature. Many numbers of antibiotics have been isolated from genus *Streptomyces* and therefore this genus is one of several biological control agents which are widely studied and used to control various plant pathogens (Martinez, 2014).

Rice is a predominant crop cultivated all over the world and its product was affected by a number of fungal, bacterial and viral diseases. Bacterial blight (BB) of rice, a vascular disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was first noticed by the farmers of Japan in...
1884. Later the incidence of BB was reported from different parts of Asia, Northern Australia, Africa and USA. As a cost effective strategy, with the biological control having significance in the management of bacterial leaf blight thereby, the usage of synthetic chemical may be avoided thus the environmental pollution and induction of resistance among pathogen races can be prevented (Velusamy and Gnanamanickam, 2008). The bacterial blight of rice reduces grain yield to varying levels depending on the stage of the crop, degree of cultivar susceptibility and a great extent to the conduciveness of the environment in which it occurs (Akhtar et al., 2011). The observed crop losses caused by X. oryzae pv. oryzae ranging between 20 to 30% and can be as high as 50% in some areas of Africa (Verdier et al., 2012).

The present study describes the antimicrobial potential of Streptomyces sp. TC 1 against bacterial leaf blight pathogen Xoo. The antioxidant activity of the ethyl acetate crude extract from the culture filtrate of Streptomyces sp. TC 1 was reported. The chemical characterization of crude extract by TLC and FT-IR is also addressed.

MATERIALS AND METHODS

Microbial culture

The Streptomyces sp. TC1 (GenBank accession number: KC954629) was grown in Ken knight agar slants for five days at 28°C and maintained under refrigerated conditions. The stock culture of Streptomyces sp. TC 1 was maintained in Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The test culture X. oryzae pv. oryzae (Xoo) from various sources were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India.

Chemicals

The chemicals used in the present study were of analytical reagent grade. They were purchased from Himedia, Sigma, Qualigens, and SD Fine Chem., India.

Fermentation and crude extract preparation

A seed culture of Streptomyces sp. TC1 was prepared by inoculating a loop of biomass into a 200 ml Erlenmeyer flask containing 100 ml of Ken knight broth and then incubated at 28°C for 3 days. A 10% level of this inoculum was transferred into 1000 ml of production medium contained in 3 L Erlenmeyer flasks (15 in number). The production medium have the composition of soluble starch 1.0%, casein 0.03%, KNO₃ 0.2%, NaCl 0.2%, K₂HPO₄ 0.2%, CaCO₃ 0.002%, MgSO₄.7H₂O 0.005% and FeSO₄.7H₂O 0.001% with pH 8.0. The inoculated production flasks were incubated for 7 days at 28°C.

The fermentation broth of strain TC1 was centrifuged, and the supernatant was collected. The crude extract from the liquid culture medium was extracted through shaking with equal volume of ethyl acetate in a separating funnel. The ethyl acetate extract was evaporated to dryness by concentrating under rotary vacuum evaporator.

Screening of antimicrobial activity

In vitro antimicrobial activity of the crude extract from Streptomyces sp. TC1 was studied against various isolates of Xoo strains by agar well diffusion method (Perez et al., 1990). The crude extract was diluted in 100% ethanol at the concentration of 10 mg/ml. The antimicrobial activity was evaluated by loading 50 µl/well. The standard antibiotic tetracycline was used as a positive control at the concentration of 1 mg/ml. The antimicrobial spectrum of the crude extract was determined in terms of zone of inhibition. Ethanol was used as negative control. The control zones were subtracted from the test zones and the results were derived. Similarly, the antimicrobial spectrum of the culture filtrate is also evaluated by agar well diffusion method.

Antioxidant assays

Ferric reducing antioxidant power (FRAP)

The antioxidant capacities of crude extract were estimated according to the procedure described by Puluiod et al. (2000). FRAP reagent (900 µL), prepared freshly and incubated at 37°C, was mixed with 900 µL of distilled water and 30 µL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37°C for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L FeCl₃.6H₂O and 25 mL of 0.3 mol/L acetate buffer (pH 3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm using a spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranging from 100 to 2000 µmol/L (FeSO₄.7H₂O) were used for the preparation of the calibration curve. The parameter, equivalent concentration (EC), defined as the concen-tration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L FeSO₄.7H₂O EC1 was calculated as the concentra-tion of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1 µmol/L concentration of Fe (II) solution, determined using the corresponding regression equation.

Metal chelating activity

The chelations of ferrous ion by test sample were estimated by the method of Dinis et al. (1994). The test samples and standard EDTA were added to a solution of 2 mmol/L FeCl₂ (0.05 mL). The reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 mL) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm against the blank. The chelating activity of the crude extract was evaluated using EDTA as standard and results were expressed as µg EDTA equivalents/mg test sample.

ABTS⁺ radical scavenging activity

The total antioxidant potential was measured by bleaching of ABTS radical cations (Re et al., 1999). ABTS⁺ radical cations were prepared by incubation of 150 µM (50 ml) with 2 M potassium persulphate (1.25 ml) for 2 h at 50°C in phosphate buffer, pH 7.0 (0.02 M/L) (Campononico et al., 1998). To 996 µL of the ABTS⁺ radical cation, 4 µL of the test sample were added. The absorbance of the sample was measured after 15 min at 734 nm. Trolox was used as standard and the results were expressed as mg of Trolox equivalents.
Radical scavenging activity using DPPH* method

The DPPH\(^*\) radical scavenging activity of the crude extract obtained from Streptomyces sp. TC1 along with standards Rutin and butylated hydroxy toluene (BHT) were measured according to the method of Blois (1958). This method is based on the reduction of the free radical DPPH\(^*\) (2,2-diphenyl-1-picrylhydrazyl). The reaction takes place when 1 ml of DPPH\(^*\) (0.1 mM solution of DPPH\(^*\) in methanol) was mixed with 3 ml of the test sample containing 20 μg of crude extract at room temperature. After a reaction time of 30 min, absorbance values at 517 nm were measured. The percent inhibition of the DPPH\(^*\) radical by the samples was calculated according to the formula:

\[
\text{Percentage (%) inhibition} = \left(1 - \frac{A_s}{A_c}\right) \times 100
\]

Where, \(A_c\): absorbance of the control; \(A_s\): absorbance of the test sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the crude fraction was measured according to the method of Klein et al. (1991). The test sample at a concentration of 20 μg was added with 1.0 mL of iron-ErDA solution (0.13% ferrous ammonium sulphate and 0.28% EDTA), 0.5 mL of EDTA solution (0.018%) and 1.0 mL of DMSO (0.85% DMSO (v/v) in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1.0 mL of ice-cold TCA (17.5% w/v). Subsequently, 3.0 mL of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and the volume was made up to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm. The percent inhibition of the hydroxyl radical by the samples was calculated according to the formula:

\[
\text{Percentage (%) inhibition} = \left(1 - \frac{A_s}{A_c}\right) \times 100
\]

Where, \(A_c\): absorbance of the control; \(A_s\): absorbance of the test sample.

Superoxide anion radical scavenging activity

The superoxide scavenging ability of the test samples were assessed by the method of Nishikimi et al. (1972) with slight modification. About 1 ml of nitro blue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of test sample were mixed. The reaction started by adding 100 μL of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank sample. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The capability of scavenging the superoxide radical was calculated using the following equation:

\[
\text{Scavenging effect (%)} = \left(1 - \frac{A_s}{A_b}\right) \times 100
\]

Where, \(A_b\): Absorbance of the blank; \(A_s\): Absorbance of the test sample.

Thin layer chromatographic separation of crude extract and detection of chemical groups

Silica gel G was used for preparing TLC plates (20 x 20 cm). Different solvent systems (ranging from low polar to high polar) were tested for the effective separation of ethyl acetate crude extract from Streptomyces sp. TC1. A suitable mobile phase was standardized as chloroform : methanol (9:1) based on better separation.

In pre-coated silica gel sheets, ethyl acetate crude extract from Streptomyces sp. TC1 was run using the chloroform : methanol (9:1) as mobile phase and after TLC separation, the sheets were kept in room temperature for drying. The TLC sheets were sprayed with chemical reagents for detection of chemical groups viz., alkaloids, coumarins, flavonoids, organic acids, phenols, saponins, steroids, tannins, terpenoids. The spraying reagents used were aluminium chloride, p-anisaldehyde-sulfuric acid, bromocresol green, Dragendorffs reagent, ethanolic solution of KOH (5%), ferric chloride, folins Ciocalteau reagent and vanillin sulphuric acid.

FT-IR spectroscopic measurement

The Fourier transform infrared spectroscopy (FT-IR) measurement of sample was performed using the Perkin Elmer 1600 FT-IR spectrophotometer in a diffuse reflectance mode at a resolution of 4 cm\(^{-1}\) in KBr pellets and the absorption frequencies are expressed in reciprocal centimeters (cm\(^{-1}\)). The FT-IR analysis was carried out at Department of Chemistry, Bharathiar University, Coimbatore.

RESULTS

In this present study, the antimicrobial potential of Streptomyces sp. TC1 against Xoo was evaluated and the antioxidant capacity of the ethyl acetate extract of culture filtrate is also studied in detail.

Antimicrobial activity

The antimicrobial activities in terms of zone of inhibition against various isolates of Xoo were carried out for the culture filtrate as well as crude extract. The crude extract produced a zone of inhibition of 1.5-2.2 cm against the tested Xoo isolates (Table 1). The highest activity of 2.2 cm inhibition zone was found against the Xoo isolate obtained from TN1 variety. Whereas, the lowest activity of 1.5 cm zone of inhibition was observed for Xoo isolate obtained from CO50 variety. The ethyl acetate crude extract exhibited higher antimicrobial activity as compared to culture filtrate which produced an inhibition zone of 1.1-1.6 cm against the tested isolates. The tetracycline antibiotic produced an inhibition zone of 2.7-3.1 cm against the tested Xoo isolates. The variation observed in the antimicrobial activity against the Xoo pathogen is due to the existence of different resistance genes among the races of Xoo isolates.

Antioxidant activity

The antioxidant (FRAP and metal chelating) and radical
Table 1. Antimicrobial activity of culture filtrate and crude extract of *Streptomyces* sp. TC1.

<table>
<thead>
<tr>
<th>Source of <em>Xoo</em> isolate</th>
<th>DIZ (cm)</th>
<th>Culture filtrate</th>
<th>Crude extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASI 16</td>
<td>1.6 ± (0.06)</td>
<td>2.1 ± (0.15)</td>
<td></td>
</tr>
<tr>
<td>ADT 39</td>
<td>1.5 ± (0.20)</td>
<td>1.8 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td>ADT 43</td>
<td>1.2 ± (0.10)</td>
<td>1.7 ± (0.12)</td>
<td></td>
</tr>
<tr>
<td>CO 43</td>
<td>1.5 ± (0.12)</td>
<td>2.0 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td>CO 47</td>
<td>1.2 ± (0.06)</td>
<td>1.6 ± (0.00)</td>
<td></td>
</tr>
<tr>
<td>CO 50</td>
<td>1.1 ± (0.12)</td>
<td>1.5 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td>TN 1</td>
<td>1.6 ± (0.12)</td>
<td>2.2 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td>TNRH 180</td>
<td>1.2 ± (0.06)</td>
<td>1.7 ± (0.15)</td>
<td></td>
</tr>
<tr>
<td><em>Oryza grandiglumis</em></td>
<td>1.4 ± (0.10)</td>
<td>1.9 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td><em>Oryza merdionalis</em></td>
<td>1.5 ± (0.06)</td>
<td>2.0 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td><em>Urochola mutica</em> (Paragrass)</td>
<td>1.5 ± (0.00)</td>
<td>2.1 ± (0.06)</td>
<td></td>
</tr>
<tr>
<td><em>Bouteloua dactyloides</em> (Buffalograss)</td>
<td>1.4 ± (0.06)</td>
<td>1.8 ± (0.12)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE of three replicates.

Table 2. Antioxidant activity of crude extract along with standards.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FRAP(^a)</th>
<th>Metal chelating(^b)</th>
<th>ABTS(^c)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>306.67 (± 1.76)</td>
<td>1.49 (± 0.01)</td>
<td>130.2 (± 1.3)</td>
<td>51.4 (± 1.28)</td>
</tr>
<tr>
<td>Rutin</td>
<td>291.09 (± 1.36)</td>
<td>0.55 (± 0.01)</td>
<td>86.6 (± 1.4)</td>
<td>86.6 (± 1.03)</td>
</tr>
<tr>
<td>BHT</td>
<td>325.43 (± 1.21)</td>
<td>1.54 (± 0.01)</td>
<td>101.5 (± 2.0)</td>
<td>74.2 (± 0.79)</td>
</tr>
</tbody>
</table>

Values are mean ± SE of three replicates, \(^a\)mmol Fe(II)/mg, \(^b\)µg EDTA equivalent/mg, \(^c\)mmol trolox equivalent/mg.

scavenging (ABTS\(^c\), DPPH\(^c\), OH\(^c\) and O\(_2^\cdot\)) properties of crude extract along with standards (rutin and BHT) were screened mainly by *in vitro* methods and their corresponding values are tabulated. In most of the antioxidant assays, the crude extract exhibited significant antioxidant activity as compared to standard rutin and BHT (Table 2). The crude extract exhibited good reducing power with the value of 306.67 mmol Fe(II)/mg, which is lower than the value observed for standard BHT. The good chelating effect would be beneficial and removal of free state iron from circulation could be a promising approach to prevent oxidative stress-induced diseases. The metal chelating activity of the crude extract is 1.49 µg EDTA equivalent/mg, which is more than that of value obtained for standard rutin. The total antioxidant activity of the crude extract is analyzed by ABTS\(^c\) radical scavenging activity. The isolated compound exhibited an ABTS\(^c\) assay value of 130.2 mmol trolox equivalent/mg, which was higher than the value observed for both standards. The model of scavenging the stable DPPH\(^c\) radical is a widely used method to evaluate the free radical scavenging ability of various samples. The effect of antioxidants on DPPH\(^c\) radical scavenging was thought to be due to their hydrogen donating ability. The crude extract exhibited a DPPH\(^c\) radical scavenging activity of 51.4%. Similarly, the radical scavenging ability of the crude extract is carried out for OH\(^c\) radicals. The percentage inhibition of OH\(^c\) radicals by the crude extract is observed as 61.87% and found to be higher as compared to standards. The superoxide radical scavenging activity exhibited by crude extract is 47.65%, which is lower than the value observed for standards rutin and BHT.

**TLC and FT-IR**

The TLC separation of the crude extract was carried out using different polar solvents as mobile phase. The better separation of crude extract was observed in mobile
Table 3. TLC characterization of crude extract.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
</tr>
<tr>
<td>Organic acids</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
</tr>
</tbody>
</table>

+Positive, - negative.

Figure 1. FT-IR spectrum of ethyl acetate crude extract.

phase chloroform: methanol (9:1). TLC spraying reagents were used to find out the functional groups present in the crude extract. The spraying reagents indicate the presence of coumarin and phenolic components in the crude fraction (Table 3). The appearance of spots under UV light after spraying with ethanolic solution of KOH (5%) denotes the presence of coumarin compound in the crude extract. Whereas the development of orange colour spot with Dragendorffs reagent confirmed the presence of phenolic compounds. Further, the appearance of blue colour spot with Folin Ciocalteau reagent confirms the presence of phenolic functional group.

The intense absorption band at 3372 (OH), 1653 (C=O) and 1618 (phenyl) cm$^{-1}$ in FT-IR spectroscopy indicate the presence of aromatic phenolic compound with carbonyl moiety (Figure 1). The IR spectrum further strengthens the presence of coumarin and phenolic compounds in the crude extract obtained from the culture.
DISCUSSION

In the present investigation, the antimicrobial and antioxidant properties of \textit{Streptomyces} sp. TC1 were extensively studied. The chemical characterization of crude extract by TLC and FT-IR spectroscopic studies were also carried out.

Various disease management practices, such as chemical control, host-plant resistance, modification of cropping systems and biological control have been employed to reduce damage caused by bacterial leaf blight. Chemical control and host plant resistance, two of the most common management practices, have their limitations. Chemical pesticides harm the environment, and host-plant resistance, which is based on a single gene, may not be durable in the field leading to frequent resistance breakdowns. It is imperative to develop environmental-friendly and sustainable control strategies.

Actinomycetes have been and remain the most fruitful source of microorganisms for all types of bioactive metabolites, including agroactive type. Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, β-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, antitumor and immunosuppressive activities (Mahajan and Balachandran, 2012). For instance, Prabavathy et al. (2006) reported that the effectiveness of \textit{Streptomyces} sp. PM5 inhibit the mycelia growth of rice blast fungus \textit{Pyricularia oryzae} and the rice sheath blight fungus \textit{Rhizoctonia solani} was related to the production of aliphatic antifungal compounds (SPM5C-1 and SPM5C-2).

In the present study, the crude extract of \textit{Streptomyces} sp. TC1 was evaluated against various isolates of bacterial leaf blight pathogen Xoo by agar well diffusion assay and found not to exhibit an inhibition zone of 1.5-2.2 cm. The wide variation in the antimicrobial activity is due to the degree of resistance between the Xoo races. The \textit{Pseudomonas} strain PDY7 exhibited strong antibacterial activity against the rice bacterial blight (BB) pathogen by the laboratory dual plate assays (Velusamy et al., 2013). The improved rice varieties released in India, though had a certain degree of resistance to bacterial blight, and became susceptible due to emergence of new races of the pathogen. Screening of rice germplasm for BB resistance resulted in existence of 6 different \textit{X. oryzae pv. oryzae} races in Pallisa and Kibuku region of Uganda (Habarurema et al., 2013). This may be due to existence of variability in the pathogen and emergence of new pathotypes (Goel et al., 1998). Each pathotypes posses varied levels of susceptibility to antimicrobial compounds due to their resistance genes (Leach et al., 1995).

Free radicals have been implicated in many disease conditions, the important ones being superoxide radical, hydroxyl radical, peroxyl radical and singlet oxygen. These highly reactive species have a potential for bringing about extensive damages, including lipid peroxidation, DNA lesion and protein fragmentation within the cells of biomolecules. There is production of reactive oxygen species (ROS) in bacteria attack, in almost all cell components, including DNA, protein and lipid membrane. Recent studies have indicated that a radical-based approach is needed for the treatment of free-radical-induced diseases (Cleveland and Kastan, 2000). Free radicals are chemical species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. In recent years, much attention has been devoted to natural antioxidant and their association with health benefits. There are several methods available to assess antioxidant activity of compounds (Ali et al., 2008).

In the present study, the antioxidant activity of the crude extract is analyzed by six different \textit{in vitro} assays. The antioxidant activity is also compared with the standard antioxidants rutin and BHT. The crude extract exhibited significant antioxidant activity in the tested assays.

Qualitative analysis of biochemical compounds present in crude extracts is mandatory for identifying a particular compound or a group of compounds responsible for furnishing antimicrobial action. To accomplish this, TLC studies were employed. The TLC studies suggest the presence of coumarin and phenolic components in the crude extract of \textit{Streptomyces} sp. TC1, which was observed by using spraying reagents. Similarly, FT-IR spectroscopy proved to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt et al., 2007; Hazra et al., 2007). The FT-IR analysis of ethyl acetate crude extract also strongly supports the presence of coumarin and phenolic components. The absorption peaks produced at 3372, 1653 and 1618 cm\(^{-1}\) indicate the presence of hydroxyl, carbonyl, phenyl groups, respectively, which are characteristics of coumarin and phenolic compounds.

The antimicrobial activity results strongly recommend that the \textit{Streptomyces} sp. TC1 can be exploited as a biocontrol organism for bacterial blight disease management in rice. Preliminary characterization by TLC and FT-IR suggested the presence of coumarin and phenolic components in the culture filtrate.

Further investigations are needed in order to determine the structure of active compounds for studying the mode of action. Further, the antimicrobial property of this organism can be screened against other plant pathogens of interest.
Conflict of interest

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

ACKNOWLEDGEMENTS

The authors thank Professor R. Rabindran, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for providing Xoo isolates to conduct antimicrobial activity studies.

REFERENCES


Seroprevalence of *Chlamydia trachomatis*, cytomegalovirus, herpes simplex virus 1 and 2 in Saudi women with normal and abnormal early pregnancy: A case control study

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³Department of Pharmacology, Faculty of Medicine, Assiut University, Egypt.

This work measured the seroprevalence of *Chlamydia trachomatis*, cytomegalovirus, herpes simplex virus-1 and 2 in Saudi women with normal and abnormal early pregnancy. This is a case-control study; serum samples were collected from 250 women as follows: 100 normal pregnancies, 70 ectopic pregnancies and 80 spontaneous abortions. IgG and IgM antibodies against candidate organisms were measured using enzyme linked immunosorbant assay (ELISA). The prevalence of *C. trachomatis* IgG, but not IgM, was significantly higher in ectopic pregnancy (18.5%) and abortion (10%) as compared to normal pregnancy (5%) (P < 0.05). The frequency of HSV-1 IgG, but not HSV-2, was also higher in ectopic pregnancy (94.3%) and abortion (87.5%) than in normal pregnancy (64%). However, the rate of detecting both IgM and IgG antibodies together either against HSV-1 or HSV-2 was higher in the ectopic (41.1 and 30%, respectively) and abortion groups (28.7 and 36.2%, respectively) as compared to the control (9 and 16%, respectively). Correspondingly, the frequency of cytomegalovirus (CMV) IgG was significantly higher in ectopic pregnancy (91.4%) and abortion (75%) as compared to normal pregnancy (58%). Similar results were also observed for CMV IgM antibodies either alone or with IgG antibodies in the ectopic and abortion groups as compared to control (P < 0.05). Candidate organisms were common by serology in Saudi females with abnormal early pregnancy. Additionally, the high frequency rate in normal pregnancy alarms the potential high risk of congenital infection. Further studies are needed to measure the true prevalence of these sexually transmitted infections (STIs) and their associated pregnancy complications in Saudi Arabia.

**Key words:** *Chlamydia trachomatis*, cytomegalovirus, herpes simplex virus, ectopic pregnancy, abortion, Saudi Arabia.

**INTRODUCTION**

Sexually transmitted infections (STIs) are major health problem and the World Health Organization (WHO) has estimated that 27 million people are infected in the Middle East (WHO, 2012). Infection with sexually transmitted...
organisms, particularly Chlamydia trachomatis, cytomegalovirus (CMV) and herpes simplex virus (HSV) is associated with acute illness, adverse pregnancy outcome, infertility and sometimes mortality (WHO, 2012).

C. trachomatis is the most common sexually transmitted bacterial infection worldwide and the majority of patients are asymptomatic (den Hartog et al., 2005). Untreated cases can result in pelvic inflammatory disease, ectopic pregnancy (EP), abortion and infertility in women, and transmission during labour could cause conjunctivitis and pneumonitis in the newborn (Ghazi et al., 2006).

CMV, HSV-1 and HSV-2 can be transmitted sexually and infection is usually latent, persistent and majority of the patients are asymptomatic. These viruses have also been associated with adverse effects on reproductive health and they can be transmitted during pregnancy/labour resulting in severe neonatal neurological manifestations (Ghazi et al., 2002; Obeid, 2007).

Serological screening against candidate organisms is mainly used for screening during routine clinical work-up and it is considered a useful tool to reflect on recent, previous and/or re-activation of infection with those pathogens (Gijsen et al., 2002; Land et al., 2003; den Hartog et al., 2005). However, serological tests are not useful in discriminating between clearance and persistence of infection in those subjects who are only positive for IgG antibodies against the organisms of interest, whereas persistence is an important risk factor for the development of abnormal early pregnancies (Gijsen et al., 2001; Land et al., 2003).

Therefore, serological results should be confirmed with a more sensitive technique (for example, nucleic acid amplification test) to confirm the results and to differentiate between clearance and current/persistent infections (Gijsen et al., 2002).

Information on STIs in Islamic countries is notably limited and currently only few studies reported their prevalence in Saudi Arabia. Hence, the objective of this study was to measure the seroprevalence of IgM and IgG antibodies against C. trachomatis, CMV, HSV-1 and HSV-2 in sera samples collected from Saudi females diagnosed with abortion or ectopic pregnancy and the results were compared with those obtained from normal pregnancy.

MATERIALS AND METHODS

Ethical approval

Ethical approval was obtained from the Faculty of Applied Medical Sciences Ethics Committee (AMSEC 10-15-9-2013) and all serum samples were collected following obtaining of informed written consent from all the participants.

Participants and study design

In this case control study, a total of 250 women were recruited consecutively from the Maternity and Children Hospitals in Jeddah and Makkah between January 2013 and April 2014, and for whom TORCH screening was not performed during their clinical examination. The participants were categorised into:

Control group (n = 100)

Consisted of women with normal pregnancy (NP) (mean age 30 ± 7.3 years) and who attended the antenatal clinic for routine follow-up.

Case group (n = 150)

Included women with abnormal early pregnancy (AEP), who attended the Emergency Department with abdominal pain and/or vaginal bleeding (mean age 30.5 ± 6.6 years) and they were diagnosed clinically with either EP (n = 70) or abortion (n = 80).

Enzyme linked immunosorbant assay (ELISA)

ELISA was used for qualitative measurement of IgG and IgM antibodies against candidate organisms (Human Diagnostics, Germany). The detection of C. trachomatis IgM was performed using the NovaLisa kit (NovaTec Immunodagnostica, Dietzenbach, Germany). Additionally, the HSV IgM kit (Human Diagnostics, Germany) did not differentiate between HSV-1 and 2.

All samples were processed using a fully automated ELISA system (Human Diagnostics, Germany) according to the manufacturers’ instructions including the internal controls provided within each kit for quality control.

The sensitivity, specificity, inter and intra-assay coefficient of variation for each kit as reported by the manufacturers were as follow: C. trachomatis IgG kit (95.9, 96.2, 3.7 and 2.8%), C. trachomatis IgM kit (83.3, > 95, 3.1 and 12.1%), CMV IgG kit (100, 99.3, 3.4 and 3.6%), CMV IgM (93.75, 99.2, 5.6 and 7.8%), HSV IgM (90, 100, 4.7 and 9.8%), HSV-1 IgG kit (100, 99, 3.7 and 2.8%) and HSV-2 IgG kit (97, 100, 3.7 and 4.9%), respectively.

All the used kits in the present study were from the same batch and the validation criteria set by the manufacturers for each kit was checked and passed prior to the interpretation of the results. The threshold indices were calculated and the cut-off value was 0.64, 0.71, 0.4 and 0.6 for IgG antibodies against C. trachomatis, CMV, HSV-1 and HSV-2, respectively. For IgM antibodies, the cut-off values were 0.43, 0.51 and 0.55 for C. trachomatis, CMV and HSV, respectively. The samples were considered positive if the value of the sample was > cut-off + 10% cut-off value as recommended by the manufacturers.

Statistical analysis

Statistical analysis of the results was performed using SPSS version 16. Cross-tabulation followed by Chi square (X²) test.
was used for frequency analysis. P value < 0.05 was considered significant.

RESULTS

Seroprevalence of C. trachomatis IgM and IgG antibodies

IgM antibodies were detected in 1 case of normal pregnancy (1%), 2 cases of EP (2.8%) and 2 cases of abortion (2.5%) (P > 0.05). IgG antibodies were positive in 5 cases of normal pregnancy (5%), 13 cases (18.5%) of EP and 8 cases of abortion (10%), and the frequency was significantly higher in the 2 groups of abnormal pregnancy as compared to control group (P < 0.05). However, there was no significant difference between ectopic and abortion groups (Figure 1).

Seroprevalence of CMV IgM and IgG antibodies

IgM antibodies against CMV were detected in 18 cases of normal pregnancy and the seroprevalence was significantly lower as compared to EP (n = 51; 72.8%) and abortion (n = 41; 51.2%) groups (P = 0.0001 and 0.0003; respectively) (Table 1).

In the normal pregnancy group, 58% were positive for CMV IgG antibodies and it was significantly lower (P < 0.05) than EP (n = 64; 91.4%) and abortion (n = 60; 75%) groups (Figure 1). The seroprevalence was also significantly higher in EP as compared to the abortion group (P = 0.005).

The number of cases that were positive for both IgM and IgG antibodies in the normal pregnancy group was 14 (14%), 44 (62.8%) in EP and 26 (32.5%) in abortion groups. The frequency of detecting both types of antibodies was significantly lower in the normal pregnancy groups as compared to the other groups (P < 0.05).

Seroprevalence of HSV-1 and 2 IgM and IgG antibodies

The HSV IgM antibodies were detected in 20 females
with normal pregnancy (20%) and the seroprevalence was significantly lower as compared to ectopic pregnancy (n = 48; 56.1%) and abortion (n = 30; 37.5%) groups (P < 0.05) (Table 1).

IgG antibodies against HSV-1 were detected in 64 cases of normal pregnancy (64%), 66 cases of EP (94.3%) and 70 cases with abortion (87.5%). The frequency of HSV-1 was significantly lower in the control as compared to the other groups (P < 0.01).

HSV-2 IgG antibodies were positive in 32 normal pregnancies (32%), 29 ectopic pregnancies (45.7%) and 31 abortions (38.7%). There was no significant difference in the prevalence of HSV-2 IgG antibodies between the different study groups (P > 0.05).

The detection of IgM antibodies against HSV with either HSV-1 or HSV-2 IgG antibodies was found respectively in 9 (9%) and 16 (16%) cases of normal pregnancy, 29 (41.4%) and 21 (30%) cases of EP, 23 (28.7%) and 27 (36.2%) cases of abortion. The frequency of detecting HSV IgM antibodies with either HSV-1 or HSV-2 IgG antibodies was significantly higher in ectopic and abortion groups as compared to the control (P < 0.05).

**DISCUSSION**

Although STIs are major risk factors of EP and spontaneous early pregnancy loss, studies on the frequency of *C. trachomatis*, CMV, HSV-1 and HSV-2 in relation to reproductive health in KSA are limited in number. The current study is a pilot study measuring the seroprevalence of IgM and IgG antibodies against these organisms in Saudi women with normal and abnormal early pregnancy.

Our results showed a significant increase in the frequency of IgG antibodies against the candidate organisms, except for HSV-2, in the abnormal pregnancy groups as compared to the control. Additionally, the seroprevalence of HSV and CMV IgM antibodies were significantly higher in the abnormal pregnancy as compared to normal pregnancy and the number of cases that were positive for both IgM and IgG against these viruses were significantly higher in the case groups as compared to the control. The present study suggests that these organisms could represent a major risk factor for adverse pregnancy outcome during the first trimester in Saudi females. Moreover, the high prevalence of CMV, HSV-1 and HSV-2 IgG and IgM antibodies in the normal pregnancy group also suggests that these viruses could spread vertically to the neonates of infected mothers leading to congenital neurological disabilities.

Ectopic pregnancy has long been associated with *C. trachomatis* (den Hartog et al., 2005). Additionally, CMV and HSV 1 and 2 have recently been isolated from specimens collected from abnormal fallopian tubes (Medvedev et al., 2009; Qian et al., 2009). These organisms have also been detected in cases with spontaneous early pregnancy loss (el-Sayed Zaki and Goda, 2007; Al-Hindi et al., 2010). Our results correlate with the previous studies as high frequency rates of *C. trachomatis*, CMV and HSV-1 and 2 were detected in patients with EP and abortion. Hence, we plan to measure the effect(s) of these microorganisms on tubal ciliary beat frequency, and the expression of implantation and adhesion markers by the tubal/endometrial specimens collected from EP and abortion to identify the mechanism(s) by which they induce/promote EP and spontaneous abortion.

The significantly high number of positive cases observed in our study for both IgM and IgG against the designated viruses also suggests that the development of abnormal early pregnancy could be associated with re-exposure/reactivation of the viral infection. Our suggestion can be supported by several published reports that have shown an increase in the prevalence of viral reactivation during pregnancy and increase in the frequency of abnormal early pregnancy in patients with positive IgM and IgG antibodies (el-Sayed Zaki and Goda, 2007; Al-Hindi et al., 2010; Saraswathy et al., 2011).

Furthermore, the high frequency of IgM and IgG antibodies against the candidate viruses in the normal pregnancy group, which correlates with the previously published studies from Saudi Arabia (Ghazi et al., 2002; Obeid, 2007), merits further research especially since these viruses can be transmitted vertically to the foetus and could result in serious congenital neurological com-
plications. Large prospective screening studies should therefore be conducted to measure the prevalence of vertical transmission of these viral infections and the rates of subsequent congenital manifestations following birth.

In conclusion, IgM and IgG antibodies against \textit{C. trachomatis}, CMV and HSV-1 and 2 are common in pregnant females in the western province of Saudi Arabia and their frequency was significantly high in abnormal early pregnancy. Additionally, the high frequency rate for CMV, HSV-1 and 2 detected in women with normal pregnancy alarms the possible risk of vertical transmission to the newborn and subsequently the risk of congenital infection and the potential development of neurological manifestations. Therefore, the implementation of a national screening programme using TORCH test during the premarital screening and/or routine clinical follow-up of normal pregnancy by the health workers in the kingdom is needed for the early detection and/or prevention of the reproductive adverse outcomes associated with these organisms. Further studies are required to measure the true prevalence of these STIs and their associated pregnancy complications.

Conflict of Interest

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

ACKNOWLEDGEMENT

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REFERENCES


Maize (Zea mays) growth promotion by rock-phosphate solubilising bacteria isolated from nutrient deficient soils of Cameroon

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Rock phosphate is an alternative strategy for less expensive natural sources of plant nutrients. However, this can be efficiently used by plants only when associated with phosphate solubilising microorganisms. Phosphate solubilising bacteria (PSB) from soils of two agro ecological zones of Cameroon were screened for their phosphate solubilising ability on plates and in liquid cultures supplemented with Malian, Moroccan or Mexican rock phosphates. They were subsequently tested on maize grown in pots filled with unsterile soil amended with Malian rock phosphate for their aptitude in promoting maize growth. Under in vitro condition, Enterobacter sp. showed halo zone on plates supplemented with the different rock phosphates with an index of solubilization (IS) varying between 2.10 and 2.71. This strain also showed the highest concentration of mobilized P with all rock phosphates: 1075.17, 862.57 µg P/g for Malian, Moroccan and Mexican rock phosphates, respectively, followed by Klebsiella sp. with the concentrations of 862.57, 615.19, 426.29 µg P/g, respectively. The Malian (402.5 µg P/g) and Moroccan (403.7 µg P/g) rock phosphates appeared to be the easiest phosphates to be solubilised by the different strains. However, the Mexican rock phosphate (345.3 µg P/g) was less solubilised in broth. In general, all the strains in single and in consortia significantly increased the number of leaves, stem base diameter, total dry mass, shoot dry mass and root dry mass as compared to non-inoculated control. The effect of inoculation with single strain varied between 27.5 and 59.3% growth increase, while the effect of inoculation with consortia varied between 54.1 and 109.3% as compared to non-inoculated control. The findings of the current study suggest the potential use of rock phosphate and PSB that would enhance maize productivity in economically profitable and environmentally friendly ways.

Key words: Maize growth, phosphate solubilising bacteria, plant nutrient, rock phosphate.

INTRODUCTION

Phosphorus (P) is the second nutrient element after nitrogen mostly required by plant. It is the main component of nucleic acids, phospholipids and adenosine triphosphate (ATP). To overcome the specific P nutrient deficiency, various forms of P, varying from processed rock phosphates (P-fertilizers) to ground in the form of
phosphatic fertilizers, part of which is utilized by plants and the remainder converted into insoluble fixed forms. The effectiveness of using phosphate fertilizer is very low; only about 10 - 25% (Isherwood, 1998; Hosseini et al., 2010). Among the alternative P sources, the most important are locally available rock phosphate (RP) resources (Rajan et al., 1996). While the use of commercial P-fertilizers is not cost effective, rock phosphate as a source of P is not expensive but the availability of P is low (Jayadi et al., 2013). Not all the RP resources are readily plant available and agronomically reactive when applied directly to the soils. Reactivity is defined as the combination of RP properties that determine the rate of dissolution of RP in a given soil under field conditions. Solubility of RP can be increased by phosphate solubilising microorganisms (PSMs). Increasing RP solubility by microorganisms is due to the lowering of pH and or organic acid excretion (Vessey et al., 2004; Fankem et al., 2008). Microbial solubilisation of rock phosphate, especially low grade and its use in agriculture is receiving great attention. This process not only compensates for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil (Narsian and Patel, 2000).

PSMs include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion of soluble phosphates to be released in quantities in excess of their requirements. Among the bacterial genera identified to have phosphate solubilising capabilities are Pseudomonas, Azospirillum, Bacillus, Rhizobium, Burkholderia, Arthrobacter, Alcaligenes, Serratia, Enterobacter, Acinetobacter, Flavobacterium and Erwinia (Rodriguez et al., 2006). Seed or soil inoculation with PSMs is known to improve solubilisation of fixed soil phosphates in order to improve crop yields (Jones and Darrah, 1994). PSMs are low cost solutions that enrich the soil giving a thrust to economic development without disturbing ecological balance. The present study aims at characterizing bacterial strains in solubilising rock phosphate of different origins on plate and liquid culture media as well as assessing their impact on maize growth under pots grown conditions.

### Materials and Methods

**Microorganisms**

All the strains used in this study are from the culture collection of the Laboratory of Biotechnology, Faculty of Science, University of Douala. Strains were isolated from soils of two agro ecological zones in Cameroon; Pseudomonas sp. and Klebsiella sp. in zone I (The Sudano-Sahelian lowland region with savanna scrub and grass, an arid region with sparse rainfall and high median temperatures), Burkholderia sp., Enterobacter sp. in zone IV (The Monomodal Humid Forest, exceedingly hot and humid with a short dry season, densely forested and includes some of the wettest places on earth). They have been previously screened for their ability in solubilising sparingly soluble phosphates including calcium-phosphate (Ca₃(PO₄)₉), aluminium-phosphate (AlPO₄), iron-phosphate (FePO₄) and sodium-phytate, and could be recognized as inorganic/organic phosphate solubilisers.

**Rock phosphates**

Rock phosphates of different origins were used: the Tilemsi rock phosphate from Mali, rock phosphate from Gafsa in Mexico and rock phosphate from Morocco. They were analyzed for their chemical contents (Table 1).

To get rid of their soluble fractions, the different rock phosphates were washed four times with warm water following the cycle: 1 - 24 h - 1 - 24 h. They were then dried in an oven at 60°C until complete evaporation of water and homogenized before use.

**Preparation and evaluation of the concentration of the inoculum for P solubilisation and greenhouse experiments**

To prepare inoculums from each bacterial strain, pure bacterial colony was individually suspended into 50 ml Nutrient Broth (NB) (5 g peptone, 1 g beef extract, 2 g yeast extract, 5 g sodium chloride, 1000 ml distilled water, pH 7.0) and incubated at 28°C, 150 rpm, for three days. Cultures were then centrifuged at 10,000 g for 10 min at 4°C, followed by three washing with 0.85% sterile NaCl at the same conditions. Bacterial cells were re-suspended in 0.85% sterile NaCl and the optical density (OD) of the suspension adjusted to 0.2 at 620 nm wavelength. To assess the number of bacterial cells per milliliter, one ml of the bacterial suspension with OD 0.2 was serial diluted until 10⁻⁷. A 200 μl of dilutions 10⁻⁷ was used to inoculate nutrient agar (NA) (5 g peptone, 1 g beef extract, 2 g yeast extract, 5 g sodium chloride, 15 g agar, 1000 ml distilled water, pH 7.0) plates in duplicate. After incubation at 28°C, for 4 days, bacterial colonies were counted and the number of colony forming unit (CFU) per ml evaluated. Counting colonies allowed the determination of

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**Table 1.** Mineralogical composition of the different rock phosphates used (Magallón-Servín, unpublished).

<table>
<thead>
<tr>
<th>Origin</th>
<th>Total P₂O₅ (%)</th>
<th>Available P (%)</th>
<th>K (%)</th>
<th>Ca (%)</th>
<th>Mg (%)</th>
<th>Na (%)</th>
<th>Fe (%)</th>
<th>Al (%)</th>
<th>Mn (%)</th>
<th>Zn (%)</th>
<th>Cu (%)</th>
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<td>12.98</td>
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<td>28.19</td>
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<td>0.232</td>
<td>3.844</td>
<td>0.80</td>
<td>8360</td>
<td>87</td>
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</tr>
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<td>28</td>
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<td>0.219</td>
<td>25.94</td>
<td>0.222</td>
<td>0.358</td>
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</tr>
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<td>9.33</td>
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<td>1.93</td>
<td>0.552</td>
<td>0.267</td>
<td>0.42</td>
<td>96</td>
<td>219</td>
<td>38</td>
</tr>
</tbody>
</table>

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the concentration of the inoculum of 1.28 - 2.08×10^5 CFU/ml. Consortia were prepared by mixing equal volumes of the different strain suspensions.

Bacterial rock phosphate solubilising capacity on agar plate

The characterization of strains for their rock phosphate solubilising ability was assessed on plates filled with the National Botanical Research Institute’s Phosphate growth medium (NBRIP) (Nautiyal, 1999) with some modifications that contains (g/l): 20 g glucose, 5 g MgCl₂·6H₂O, 0.25 g MgSO₄·7H₂O, 0.2 g KCl, 0.1 g (NH₄)₂SO₄ and each rock phosphate type at a rate of 5 g l⁻¹ (Malian RP, Moroccan RP or Mexican RP) plus 0.5% BCG dye (Gadagi and Sa, 2002) for better observation, pH 7.5. Five microliters of each bacterial suspension OD 0.2 obtained as described above were transferred onto a single point of compartmented Petri dish. The plates were sealed with parafilm and incubated at 28°C for 5 days. The halo/yellow zone surrounding the bacterial colony indicates extension of phosphate solubilisation. The index of solubilisation (IS) as defined by Qureshi et al. (2012) was used as an indicator for the strain efficiency: IS = (Colony diameter + diameter of halo zone) / colony diameter.

Quantitative estimation of phosphate solubilisation by bacteria in liquid media

Bacteria were tested in liquid media to assess their capability in releasing phosphates from insoluble rock phosphate sources. A 50 ml NBRIP medium was distributed into 250 ml Erlenmeyer flasks. Individual rock phosphate types (Malian RP, Moroccan RP or Mexican RP) were added to the medium at the concentration of 5 g l⁻¹ and the pH adjusted to 7.5. After sterilization and cooling, 200 µl bacteria suspensions of 1.28 - 2.08×10^5 CFU/ml were used to inoculate flasks containing the different rock phosphates. Each treatment was replicated three times and non-inoculated flasks supplemented with different rock phosphates supplied with 200 µl of 0.85% sterile NaCl served as control. Incubation was made at 28°C, 150 rpm for 7 days. At end of the incubation time (7th day) and in all cases, the cultures were transferred into sterile falcon tubes, centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was taken for P determination following the method described by Murphy and Riley (1962).

Pot experiment

The pot experiment was conducted to assess the effect of a single strain or consortia of strains on maize (Zea mays) growth. The maize seeds consisted of the variety CMS8704 with a life cycle of 115 days and cultivated in the Nord, Centre, Littoral, South-East and Nord-West regions of Cameroon. The experiment was conducted in 3 L pots containing homogenised non-stereile soil with the following characteristics: pH H₂O 5.5; pH KCl 4.4; nitrogen, 0.06%; available phosphorus, 7.54 ppm; organic matter, 0.59%; organic carbon, 0.34%; iron, 1.75 ppm; aluminium, 0.29 mg/g; calcium, 1.04 mg/g; magnesium, 1.60 mg/g; potassium, 0.15 mg/g; sodium, 0.07 mg/g. The soil used was therefore very low in available phosphorus (7.54 ppm).

The experiment consisted of 12 treatments including 10 microbial treatments and two controls. The microbial treatments consisted of four single inoculations labeled A (Klebsiella sp.), B (Pseudomonas sp.), C (Burkholderia sp.) and D (Enterobacter sp.), and six consortia (AB, CD, ABC, ACD, BCD and ABCD). Consortia were obtained by mixing equivalent volumes of the bacterial suspension OD 0.2 of each strain. All the pots, except the positive control were amended with Malian rock phosphate at the rate of 80 kg ha⁻¹ (0.3625 g for 0.8836 dm³) to increase the amount of phosphate in soil. In all inoculated pots, one pre-germinated seed was placed in a pit, soaked with 1 ml of bacterial suspension and finely covered with soil. The control treatments consisted of a positive control (Cont+) supplemented with soluble KH₂PO₄ at the concentration of 350 mg P/g soil, and a negative control (Cont-) supplemented with Malian rock phosphate, both without bacterial inoculation.

The experimental design was a completely randomized block system with 12 treatments, 1 host plant and 4 replications, resulting in a total of 48 experimental units. Plant growth was followed during 6 weeks within which, each pot received 500 ml water three times/week and 200 ml of a solution of 200 mg l⁻¹ of N (1.4285 g l⁻¹ of 14:0:14 NK fertilizer) was added to each pot once a week. Growth parameters (number of leaves, plant height, stem base diameter) were taken every two weeks. At the end of the growth (six weeks after planting), the plants were harvested, the aerial part separated from the root part, and then dried at 60°C until the dry mass of materials became stable to determine shoot, root and total dry mass.

Statistical analysis

Statistical analyses were performed with Sigma plot 12.0. The analysis of variance (ANOVA) was run to find difference between factors and the HSD Turkey test to compare the different treatments means.

RESULTS

Strains characterization on plates supplemented with rock phosphates of different origins

In general, all the strains were able to solubilise at least one rock phosphate type but the index of solubilisation were different within strains and the different rock phosphates were not equally solubilised. Enterobacter sp. was the only strain able to solubilise the three rock phosphate types (IS>1), with the IS of 2.45, 2.10 and 2.71 for Malian, Moroccan and Mexican rock phosphates, respectively. The Malian rock phosphate was solubilised by all the strains (Table 2). However, Moroccan and Mexican rock phosphates were recalcitrant to Pseudomonas sp., Burkholderia sp. and Klebsiella sp. (IS<1). In Malian rock phosphate plate, Enterobacter sp. showed the highest value (IS=2.45) followed by Klebsiella sp. (IS=1.81), Burkholderia sp. (IS=1.65) and Pseudomonas sp. (IS=1.33). Additionally, Enterobacter sp. often showed the solubilising activity on the first day of incubation regardless of the rock phosphate type, while the rest strains showed the activity only on the second day. There is a significant difference between the different rock phosphate solubilisation on plates. The Malian rock phosphate is the easiest phosphate (IS=1.8) to be solubilised by the different strains, followed by Mexican rock phosphate (IS=1.43) and the Moroccan rock phosphate (IS=1.28) being the most recalcitrant phosphate on plates.

Quantification of mobilized phosphate in liquid medium

In general, all the strains were able to mobilise phosphate
Table 2. Rock phosphate solubilisation on plates and concentration of the solved P in liquid cultures amended with rock phosphates of different origins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Malian RP</th>
<th></th>
<th>Moroccan RP</th>
<th></th>
<th>Mexican RP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Index of Solubilisation (IS)</td>
<td>Concentration of the solved P (µg/g)</td>
<td>Index of Solubilisation</td>
<td>Concentration of the solved P (µg/g)</td>
<td>Index of Solubilisation</td>
<td>Concentration of the solved P (µg/g)</td>
</tr>
<tr>
<td>Control</td>
<td>6.67±0.0a</td>
<td>0±0.0a</td>
<td>156.24±14.7c</td>
<td>1.00±0.00a</td>
<td>95.4±8.7b</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>1.33±0.00a</td>
<td>17.24±1.2a</td>
<td>2.71±0.08b</td>
<td>156.24±14.7c</td>
<td>95.4±8.7b</td>
<td></td>
</tr>
<tr>
<td>Burkholderia sp.</td>
<td>1.65±0.11b</td>
<td>50.85±0.8b</td>
<td>86.03±5.7b</td>
<td>1.00±0.00a</td>
<td>112.32±6.0b</td>
<td></td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>2.45±0.05d</td>
<td>1075.17±9.4d</td>
<td>1161.04±10.2e</td>
<td>1.00±0.00a</td>
<td>862.57±18.8d</td>
<td></td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>1.81±0.04c</td>
<td>862.57±1.7c</td>
<td>1.00±0.00a</td>
<td>615.19±9.1d</td>
<td>426.29±13.3c</td>
<td></td>
</tr>
</tbody>
</table>

The different letters within the same column are significantly different (p < 0.05).

Figure 1. Efficiency of strains in solubilising rock phosphates of different origins in liquid media. The different letters indicate significant difference between strains regardless of the phosphate type (p < 0.05).

from insoluble sources, although no significant (p<0.05) difference was observed within Pseudomonas sp. (17.24 µg P/g) and control (6.67 µg P/g) regarding the Malian rock phosphate (Table 2). Enterobacter sp. showed the highest concentration of P in all rock phosphate types: 1075.17, 1161.04, 862.57 µg P/g for Malian, Moroccan and Mexican rock phosphates, respectively followed by Klebsiella sp. with the concentrations of 862.57, 615.19, 426.29 µg P/g for Malian, Moroccan and Mexican rock phosphates, respectively (Table 2). Pseudomonas sp. and Burkholderia sp. obtained the lowest concentration of P from the sparingly soluble rock phosphate sources. However, the strains that were not able to show any visible activity on plates amended with Moroccan and Mexican rock phosphates mobilised important amount of phosphate in liquid cultures containing the same rock phosphate types. For instance, Pseudomonas sp. solubilized 156.24 and 95.4 µg P/g; Burkholderia sp. 86.03 and 112.32 µg P/g and Klebsiella sp. 615.19 and 426.29 µg P/g for Moroccan and Mexican rock phosphates, respectively (Table 2).

There was a significant (p<0.05) difference between ability of strains in solubilising the different rock phosphate types. Enterobacter sp. showed the highest efficiency to solubilise the three types of phosphate in liquid cultures followed by Klebsiella sp., Burkholderia sp. and Pseudomonas sp. (Figure 1).

Regarding the facility of the different rock phosphates to be mobilised, there was a significant (p<0.05) difference between different rock phosphates solubilisation in liquid cultures (Figure 2). The Malian (402.5 µg P/g) and Moroccan (403.7 µg P/g) rock phosphates were the easiest phosphates to be mobilised by the different strains with no significant difference
between them, while the Mexican rock phosphate (345.3 µg P/g) was the most recalcitrant phosphate in liquid cultures.

**Effect of inoculation by strains on the maize growth**

**Number of leaves**

In general, the number of leaves varied within the treatments for each week (Table 3). Two weeks after planting, the number of leaves varied between 3 and 5/plant (Table 3), while the highest record (5 leaves/plant) was obtained from the positive control supplied with soluble phosphate and most of consortia (AB, ABC, ACD and BCD). Most of the treatments with single strain had 4 leaves/plant like the negative control, except strain A with 3 leaves/plant (Table 3). The highest number of leaves (7) was still obtained from the Cont, treatment (7) at the fourth week but the smallest observed in Cont. (5 leaves/
Table 4. Stem base diameter at second, fourth and sixth week after planting and root shoot and total plant dry mass at sixth week.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stem base diameter (cm)</th>
<th>Dry mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2(^{nd}) Week</td>
<td>4(^{th}) Week</td>
</tr>
<tr>
<td>Cont-</td>
<td>0.95±0.1ab</td>
<td>1.88±0.3a</td>
</tr>
<tr>
<td>Cont+</td>
<td>1.55±0.5b</td>
<td>5.88±0.3d</td>
</tr>
<tr>
<td>A</td>
<td>0.98±0.1ab</td>
<td>3.25±0.5abc</td>
</tr>
<tr>
<td>B</td>
<td>0.43±0.4a</td>
<td>2.75±0.5ab</td>
</tr>
<tr>
<td>C</td>
<td>0.78±0.5ab</td>
<td>4.03±0.2bc</td>
</tr>
<tr>
<td>D</td>
<td>1.00±0.0ab</td>
<td>3.25±0.5abc</td>
</tr>
<tr>
<td>AB</td>
<td>1.13±0.3ab</td>
<td>4.50±0.7cde</td>
</tr>
<tr>
<td>CD</td>
<td>1.48±0.4b</td>
<td>4.38±0.8bcd</td>
</tr>
<tr>
<td>ABC</td>
<td>1.40±0.7ab</td>
<td>4.20±1.1bc</td>
</tr>
<tr>
<td>ACD</td>
<td>1.18±0.6ab</td>
<td>3.73±1.0bc</td>
</tr>
<tr>
<td>BCD</td>
<td>1.25±0.5ab</td>
<td>3.75±1.0bc</td>
</tr>
<tr>
<td>ABCD</td>
<td>1.13±0.3ab</td>
<td>3.25±0.5abc</td>
</tr>
</tbody>
</table>

Cont- = negative control with rock phosphate without inoculation; Cont+= positive control with soluble phosphate (KH\(_2\)PO\(_4\)); A (Klebsiella sp.); B (Pseudomonas sp.); C (Burkholderia sp.); D (Enterobacter sp.); AB (Klebsiella sp. + Pseudomonas sp.); CD (Burkholderia sp. + Enterobacter sp.); ABC (Klebsiella sp. + Pseudomonas sp. + Burkholderia sp.); ACD (Klebsiella sp. + Burkholderia sp. + Enterobacter sp.); BCD (Pseudomonas sp. + Burkholderia sp. + Enterobacter sp.); ABCD (Klebsiella sp. + Pseudomonas sp. + Burkholderia sp. + Enterobacter sp.). The different letters within the same column indicate a significant difference (p<0.05) between treatments.

Plant height

In general, the plant height varied with treatments over time with significant (p<0.05) differences between treatments starting from two weeks after planting (Table 3). At the second week, the greatest height (8.33 cm) was observed in positive control but the smallest in negative control (5.18 cm). Treatment BCD was among the consortia that gave the highest (7.68 cm) value (Table 3). Similarly, treatment C was among inoculation with single strain that showed the highest height (6.75 cm). Four weeks after planting, the situation was identical with positive control (19.78 cm) (Table 3).

The greatest (16.63 cm) value for consortia was observed from CD and in A (13.73 cm) for treatments with single inoculation. Six weeks after planting, the negative control showed a height of 18.7 cm, while the positive control showed 24.28 cm. However, the highest score was obtained mainly by some consortia ABCD (27.75 cm) and BCD (26.91 cm). Treatment C had the highest (24.60 cm) height among those inoculated with single bacterium, while inoculation with single or with consortia gave an increase of 29.8% for Cont., 31.6% for C, 43.9% for BCD and 48.4% for ABCD. In general, except the B treatment, inoculation with bacterial strain either in single or in consortia had positive effect on plant with regard to height parameter.

Stem base diameter

In general, the stem base diameter varied with treatments over time, with significant differences between treatments starting from two weeks after planting (Table 4). In the second week, positive control had the biggest (1.55 mm) stem diameter, while the smallest (0.43 mm) value was recorded from treatment B. Treatment D (0.70 mm) had the greatest value among the single inoculation treatments but CD had the highest diameter (1.48 mm) among the consortia.

Four weeks after planting, the greatest (5.88 mm) diameter was recorded from positive control but the smallest (1.88 mm) was from negative control. The AB consortium showed the largest (4.50 mm) diameter among the consortia but C (4.03 mm) among the single
The highest (10.59 mm) diameter was observed at the sixth week on the positive control but the smallest (5.08 mm) value from the negative control (Table 4). After sixth week, ABC treatment showed the highest diameter (8.71 mm) among consortia but C (8.08 mm) among single inoculation treatments. Positive control inoculation in single or in consortia showed an increase of 108.5% for Cont., 59.1% for C, 71.6% for BCD and 66.2% for ABCD as compared to the negative control (Table 5). In general, except the B treatment, inoculation with strain either in single or in consortia had positive effect on plant stem base diameter.

**Root, shoot and total plant dry weight**

ABCD consortium had the highest (6.62 g/plant) value of root dry weight followed by the positive control with the mass of 5.88 g/plant (Table 4) six weeks after planting. The negative control had the smallest root dry weight (2.41 g/plant). Treatment C had the highest (4.03 g/plant) weight among single inoculation treatments. Concerning the shoot dry weight, the greatest value was obtained from positive control (7.10 g/plant), while the smallest (2.32 g/plant) recorded from negative control. Treatment BCD (6.10 g/plant) was the best among consortia, but C (4.24 g/plant) was the best among single inoculation treatments. Regarding the variation of the total dry weight, the greatest and the smallest values are, respectively obtained from positive control (12.98 g/plant) and C (4.74 g/plant). Treatment ABCD with a weight of 12.34 g/plant was the best treatment among consortia, but treatment C had the greatest weight of 8.24 g/plant among single inoculation treatments. There was an increase of 174% for positive control, 74.9% for C, 145.5% for BCD and 160.6% for ABCD as compared to the negative control (Table 5). In general, all the inoculation treatments, either in single or in consortia had positive effect on plant dry weight.

Based on the effect of inoculation on the different plant parameters measured as compared to the negative treatment without inoculation, the different treatments can be classified as follows: Cont+ > ABCD > BCD > AC = AB > CD > C > ABC = A = D > B six weeks after planting (Table 5). In general, consortia of strains had better effect than inoculation with single strain. Among the consortia, ABCD and BCD were the best in promoting maize growth, while C was the best among the single inoculations. *Pseudomonas* sp. (B) showed better performance when associated with any other bacteria.

**DISCUSSION**

The role of phosphate solubilising microorganisms capable of solubilising various forms of insoluble phosphates as well as rock phosphates is well known (Babana et al., 2013; Fankem et al., 2014). The activity of plates was assessed at least three times to confirm the real ability of strains in solubilising rock phosphates, since some bacteria can lose their solubilisation property after several successive subcultures (Kucey et al., 1989; Shankarrao, 2012). A halo zone on the plate is used to assess the P solubilisation activity of strains with the IS value as an indicator for the strain efficiency (Fernandez et al., 2007). Many bacteria strains isolated from Malian soils have been reported to solubilise the Malian (Tilemsi) rock phosphate and could be considered as rock phosphate solubilisers (Babana et al., 2013). Except *Enterobacter* sp., the three other bacterial strains did not

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of leaves</th>
<th>Plant height</th>
<th>Stem base diameter</th>
<th>Root dry weight</th>
<th>Shoot dry weight</th>
<th>Total dry weight</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont+</td>
<td>40</td>
<td>29.8</td>
<td>108.5</td>
<td>144.0</td>
<td>205.2</td>
<td>174.0</td>
<td>116.9</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>18.3</td>
<td>37.9</td>
<td>45.2</td>
<td>46.2</td>
<td>45.7</td>
<td>38.9</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>29.7</td>
<td>46.7</td>
<td>43.4</td>
<td>45.1</td>
<td>27.5</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>31.6</td>
<td>59.1</td>
<td>67.5</td>
<td>82.6</td>
<td>74.9</td>
<td>59.3</td>
</tr>
<tr>
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<td>40</td>
<td>18.6</td>
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<td>56.1</td>
<td>34.8</td>
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<td>38.5</td>
</tr>
<tr>
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<td>60</td>
<td>33.6</td>
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<td>130.3</td>
<td>124.4</td>
<td>127.4</td>
<td>87.1</td>
</tr>
<tr>
<td>CD</td>
<td>40</td>
<td>21.2</td>
<td>50.2</td>
<td>84.4</td>
<td>124.3</td>
<td>104.0</td>
<td>70.7</td>
</tr>
<tr>
<td>ABC</td>
<td>20</td>
<td>20.6</td>
<td>71.6</td>
<td>63.7</td>
<td>77.8</td>
<td>70.6</td>
<td>54.1</td>
</tr>
<tr>
<td>ACD</td>
<td>60</td>
<td>24.1</td>
<td>35.3</td>
<td>134.9</td>
<td>134.4</td>
<td>134.6</td>
<td>87.2</td>
</tr>
<tr>
<td>BCD</td>
<td>40</td>
<td>43.9</td>
<td>51.7</td>
<td>129.2</td>
<td>162.5</td>
<td>145.5</td>
<td>95.5</td>
</tr>
<tr>
<td>ABCD</td>
<td>60</td>
<td>48.4</td>
<td>66.2</td>
<td>174.8</td>
<td>145.9</td>
<td>160.6</td>
<td>109.3</td>
</tr>
</tbody>
</table>

The effect (%) is calculated according to the formula: \( \text{Effect} = \frac{\text{T} - \text{Cont}\_+}{\text{Cont}\_+} \times 100 \). Where, T is a given treatment and Cont- the negative control. A (Klebsiella sp.), B (Pseudomonas sp.), C (Burkholderia sp.), D (Enterobacter sp.), ABC (Klebsiella sp. + Pseudomonas sp. + Burkholderia sp.), ACD (Klebsiella sp. + Pseudomonas sp. + Enterobacter sp.), ABCD (Klebsiella sp. + Burkholderia sp. + Enterobacter sp.).
show any visible halo zone on plates amended with Moroccan and Mexican rock phosphates. This might be due to the low diffusion of the organic acids produced by these bacteria during their growth (Babana et al., 2013). However, the three strains conversely mobilized important amounts of P in liquid culture supplemented with the same rock phosphates and could be considered as potential biofertilizer strains. These contradictory results between plate halo detection and phosphate solubilisation in liquid cultures were found by Deubel and Merbach (2005) and Fankem et al. (2011). This indicates that liquid culture should be associated with halo zone for rock phosphate solubilisers characterization. However, the plate method is still a feasible way to pre-screen the isolates that possess phosphate solubilising ability. In line with the previous investigations (Azziz et al., 2012; Fernández et al., 2012), there was no clear correlation between solubilisation ability on plate and in liquid culture. Although all the strains showed good solubilisation in liquid cultures amended with the different rock phosphates, the amount of the solubilized P varied with the strain involved as well as the rock phosphate origin. Solubilisation of phosphate is commonly accompanied by a remarkable drop in pH (Kloeppe et al., 1999; Azziz et al., 2012; Fernández et al., 2012) as was the case for all of our strains. Regarding the efficiency of strains on plates and in liquid cultures, Enterobacter sp. showed the highest performance followed by Klebsiella sp. In agreement with this study, Park et al. (2011) found that Enterobacter cloacae could solubilise 17.5% RP in the growth medium and can be associated with RP as an alternative technique to soluble P compounds. Moreover, the ability of phosphate solubilisation by plant-associated Pseudomonas, Klebsiella, Enterobacter and Microbacterium species has been reported by Rodriguez and Fraga (1999).

Increasing the bioavailability of P in the soils with combined inoculation and rock materials has been reported by many researchers (Schilling et al., 1998; Lin et al., 2002; Han and Lee, 2005; Han et al., 2006; Marschner, 2009), which may lead to increased P uptake and plant growth (Han et al., 2006; Chen et al., 2006; Eweda et al., 2007; Jorquera et al., 2008; Sabannavar and Lakshman, 2009). The present study highlights the ease of the four strains in single or in consortia in promoting the maize growth. In general, all the strains in single and in consortia have significantly increased the number of leaves, stem base diameter, total dry weight, shoot dry weight and root dry weight as compared to non-inoculated control. The effect of inoculation with single strain varied between 27.5 and 59.3%, while the effect of inoculation with consortia is between 54.1 and 109.3% showing that bacteria are capable of promoting the plant growth when combined inoculation is done. While studying the response to the pots inoculation of corn seeds by bacteria, Hussain et al. (2013) found significant increases in plant height, shoot dry weight and root dry weight of 16, 42 and 29%, respectively. In the present study, the average values obtained for the same parameters are 26.03, 97.63 and 93.28%, respectively, with the average total plant dry matter of about 95.41%. However, the average total plant dry matter obtained with single strain is about 52.85%. This value remains greater than that of Abou-el-Seoud and Abdel-Megeed (2012) who reported the increase of dry weight of maize plants inoculated with single bacterium of 26% as compared to non-inoculated treatment. In addition, there are some similar reports on enhanced dry matter content of maize and groundnut due to inoculation of phosphate solubilising bacteria (Hameeda et al., 2008; Pandey et al., 2006; Walpola and Yoon, 2013).

Growth enhancement by bacteria may relate to its ability to produce extensive root length (Sheng and Huang, 2001) and can also improve root development and increase the rate of water and mineral uptake (Alexander, 1997; Saghiri et al., 2007). Many other researchers have found that phosphate solubilising microorganisms could increase maize growth and increase yield (Chabot et al., 1996; Yazdani et al., 2009; Hussain et al., 2013). This increase in growth may be attributed to auxin production (Gyaneshwar et al., 2002), ACC-deaminase activity (Naik et al., 2008; Jayadi et al., 2013), production of organic acids (Fankem et al., 2008) or phosphatases (Chabot et al., 1996) to solubilise/mineralize P, thereby increasing phosphate nutrition of inoculated plants. Collavino et al. (2010) testing E. aerogenes R4M-A and Burkholderia spp. R4M-F strains for IAA production in culture medium found that both produced and released free IAA. So, IAA produced by several bacteria can stimulate the development and proliferation of roots, with increases in uptake of water and nutrients (Bashan and De-Bashan, 2005). Therefore, the positive effect on growth with non-soluble P may result from the synergic combination of both bacterial capacities for IAA production and P mobilisation. The use of phosphate-solubilising bacteria as inoculants simultaneously increase phosphates uptake by the plant and crop yield (Mehta and Nautiyal, 2001). Moreover, bacteria belonging to genera Bacillus, Pseudomonas, Serratia and Enterobacter are reported to solubilise the insoluble phosphate compounds and aid in plant growth (Frey-Klett et al., 2005; Hameeda et al., 2008).

Burkholderia sp. is the best of the four used strains because of its efficiency in greatly increasing all the growth parameters. This is in agreement with the results of Linu et al. (2009) who found that Burkholderia sp. gave better results in improving growth of cowpea, and this strain had been previously evaluated by Pandey et al. (2005) to have phosphate solubilisation, auxin production, ACC deaminase activity and also nitrogen fixing ability. In addition to phosphate solubilisation, Burkholderia spp. and especially Burkholderia cepacia have potential for biological control and promoting plant growth (Babu-Khan et al., 1995; Kloeppe et al., 1999;
Rodríguez et al., 2000; Bhattacharyya and Jha, 2012). The growth and yield of maize increased when inoculated with *Burkholderia* sp. J62 in metal-polluted soil (Jiang et al., 2008).

**Conclusion**

The results obtained in the present study indicate that *Pseudomonas* sp., *Burkholderia* sp., *Enterobacter* sp. and *Klebsiella* sp. can effectively improve the solubility of rock phosphate applied as fertilizer, to increase the amount of available phosphate in the soil and increase the productivity of maize. This suggests that the use of rock phosphate combined with the co-inoculation of phosphate solubilising bacterial strains in soil with low fertility provides a sustainable alternative to the use of industrial fertilisers for maize production. This approach would ensure maize production in economically profitable and environmentally friendly manner.

**Conflict of Interest**

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

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