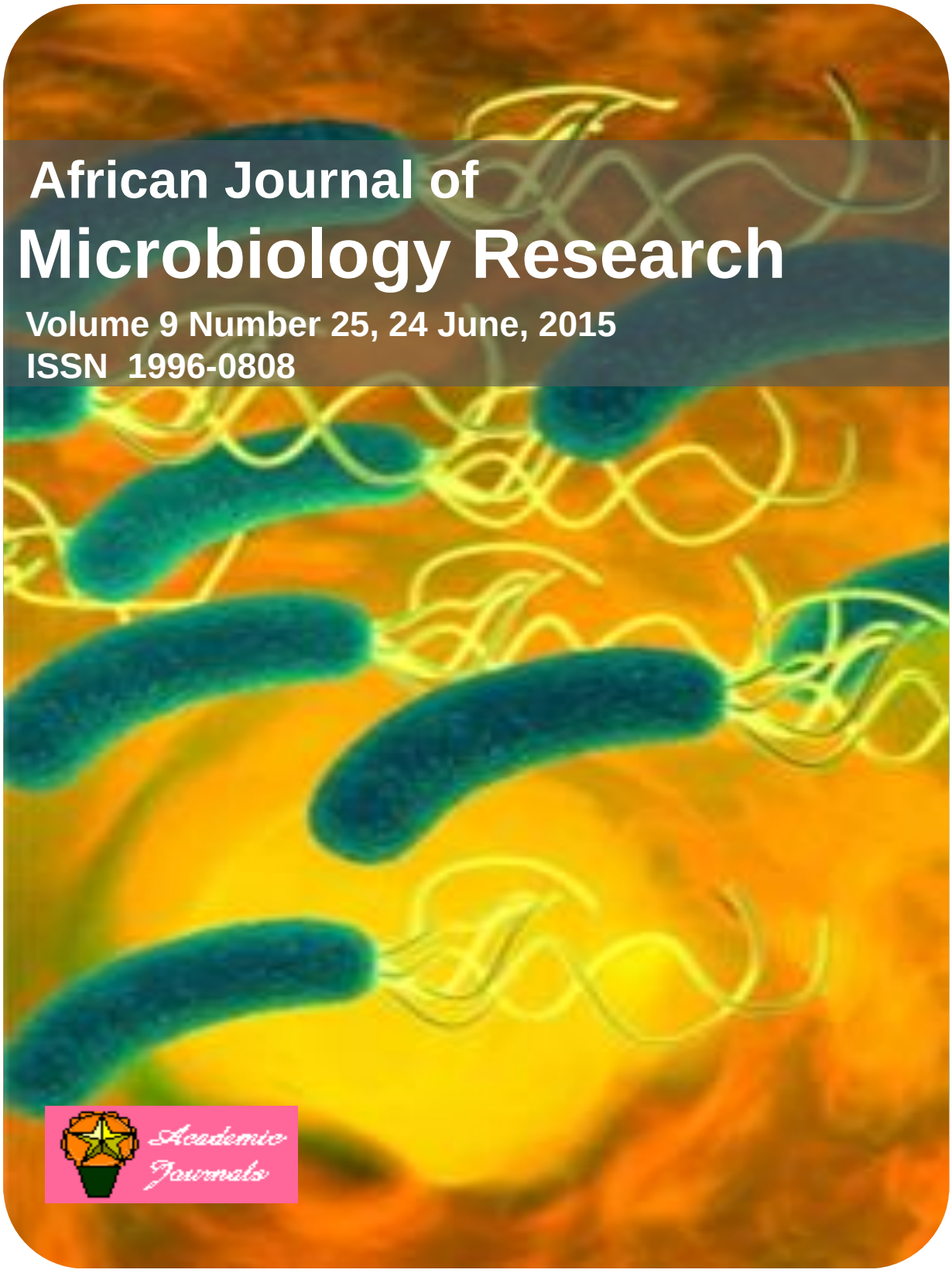

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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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

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Full Length Research Paper

In vitro antagonistic activity of *Pseudomonas* spp. against *Rhizoctonia soloni*

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The present research work deals with the *in-vitro* study of antagonistic activity of *Pseudomonas* spp. against plantpathogenic fungi *Rhizoctonia soloni.* The bacterial strains were isolated from rhizospheric soil of Korea district of Chhattisgarh. A total of 28 bacterial cultures were isolated from 25 representative soil samples collected from five blocks of Korea district of Chhattisgarh, out of which four were identified as *Pseudomonas* spp. (PKS10- *Pseudomonas syringae*, PKM11- *Pseudomonas syringae*, PKJ25- *Pseudomonas alcaligenes* and PKB27- *Pseudomonas alcaligenes*) from Plant Pathology Division, IARI Delhi. These four *Pseudomonas* spp. were examined for their ability to antagonize *R. soloni* in *in vitro* plate assay by dual culture inoculation along with the standard check (Pmtcc- *Pseudomonas* isolate from IMTECH Chandigarh). The antagonistic activity was interpreted by restricted growth zone of the fungal pathogen in dual culture. The diameter of hyphal growth of the fungi in dual culture with isolates PKS10, PKM11, PKJ25, PKB27, Pmtcc and control were 3.82, 3.68, 2.73, 3.41, 3.25 and 7.3 cm respectively. All the four *Pseudomonas* isolates PKS10, PKM11, PKJ25, PKB27 and Pmtcc (standard check) inhibited the hyphal growth of *Rhizoctonia* spp. by 47.67, 49.58, 62.60, 53.28 and 55.47% respectively. One among four *Pseudomonas* isolates, (PKJ25) *P. alcaligenes* was found to suppress the growth of fungal pathogen significantly in dual culture by 62.60% and was more effective than other isolates. This study suggests that *P. alcaligenes* isolates might be used as potential biological control agents against plant pathogenic fungi *Rhizoctonia soloni*.

Key words: Antagonism, biological control, *Pseudomonas alcaligenes, Rhizoctonia soloni*.

INTRODUCTION

Rhizoctonia solani has been encountered as one of the potent soil-borne fungal pathogens, which develops in both cultured and non-cultured soils. *R. solani* are highly destructive phytopathogens (Curtis et al., 2010), known to cause symptoms of damping- off and root rot diseases to wide range of vegetable and crop plants including tomato (Abu-Taleb et al., 2011; Karima et al., 2012). Various methods have been reported for controlling of damping-off disease but biological control is an efficient and ecofriendly methods. Many microbial species are involved in the biocontrol of phytopathogens such as *Trichoderma viride* (Hafez et al., 2013), *Pseudomonas*

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Table 1. Occurrence, identities, sources and abbreviation of *Pseudomonas* in Rhizospheric soil of Korea district of Chhattisgarh.

S/N	Bacterial genus identified from Rhizospheric soil	District	Block	Field	Crops
1	*Pseudomonas syringae* (PKS10)	Korea	Sonhat Block	Kailashpur	*Oryza sativa*
2	*Pseudomonas syringae* (PKM11)	Korea	ManendragarhBlock	Barbaspur	*Abelmoschus eculentus*
3	*Pseudomonas alcaligenes* (PKB27)	Korea	Baikunthpur Block	Dumaria	*Lycopersicon esculentum*
4	*Pseudomonas alcaligenes* (PKJ25)	Korea	Janakpur Block	Umarwaah	*Oryza sativa*

fluorescens and *Bacillus subtilis* (Sivasakthi et al., 2014). Adhikari et al. (2013) reported the antagonistic nature of rhizospheric bacteria against *R. solani*.

Pseudomonas spp. is one of the most promising groups of rhizospheric bacterial inhabitants that are extensively investigated to be used as biocontrol of pathogens in agriculture (O'Sullivan and O'Gara, 1992; Ganeshan and Kumar, 2006; Maurya et al., 2014). They show antagonistic activity against diverse phytopathogens such as *Pythium* spp. (Leoper, 1988) and *Rhizoctonia* spp. (Howell and Stipanovic, 1979). Various species of *Pseudomonas* are thought to play an important role in plant growth promotion and disease suppression (Kloepper et al., 1980; Jayaswal et al., 1990). In particular *P. fluorescence* (Howell and Stipanovic, 1979; Weller and Cook, 1983) and *P.cepacia* (Hebbar et al., 1992; Jayaswal et al., 1990) have attracted a considerable attention on account of their potential for biological control. Shalini and Srivastava (2008) screened out antifungal activities of *P. fluorescence* against phytopathogenic fungi. Antifungal activity of fluorescent Pseudomonads against *R. solani* is correlated to the production of secondary metabolites (Mina et al., 2013; Sharma et al., 2014; Mezeal, 2014). *Pseudomonas aeruginosa* rhizobacterial isolates PTR-3 exhibited antagonism of over 68.9% by restricting *in vitro* mycelial growth of *R. solani* up to 1.9 cm (Kamei et al., 2014).

In the present investigation the *in vitro* biocontrol efficacy of *Pseudomonas* spp. isolated from Korea district of Chhattisgarh is reported against *R. soloni*; they are able to antagonize plant pathogenic fungi in *in-vitro* condition, hence can be used as potential biocontrol agent.

MATERIALS AND METHODS

Isolation and characterization of *Pseudomonas*

In the present investigation soil samples were collected from randomly selected locations in the field region from Korea district of Chhattisgarh by composite sampling method (Walworth, 2004).

Korea District is North-Eastern District of Chhattisgarh State of India. Geographically, state lies in Latitude between 23° 02' 42" to 23 deg. 44' 46" North and Longitude between 81° 46' 42" to 82 deg. 33' 43" East. Height from Sea Level is 700 Meters. The District is bound on the North by Shidhi District of Madhya Pradesh, on the South by Bilaspur Districts, on the East by its parent District Surguja and on the West by Shahdol District of Madhya Pradesh (Figure 1). Temparature (Average) is 32°C (Max) and 17°C (Min); land area covers 5978 Sq. Km and forest area is 59.03%. The Climate is ideal with a beautiful monsoon, a mild summer and a bearable winter. Average Rainfall is 1410.9 mm, Soil type red-yellow and major crop-paddy (http://korea.gov.in/glance.htm (National Informatics centre Korea *Chhattisgarh).* Korea district is divided into 5 blocks- Baikunthpur, Manendragarh, Khadgawan, Sonhat and Janakpur.

All total of 25 soil samples were collected from 5 blocks of agro based areas of Korea district of Chhattisgarh, five representative soil samples from each block (Baikunthpur, Manendragarh, Khadgawan, Sonhat, Janakpur), during May- June 2009, all these were drawn from post harvested fields (Venkateswarlu et al., 1984). Total of 28 bacterial cultures were isolated from 25 soil samples of 5 blocks of Korea district of Chhattisgarh.

Isolation of rhizospheric bacteria was carried out by serial 10-fold dilutions technique (Pandey et al., 2006) on Nutrient agar and Pseudomonas agar base (all from Hi Media). Four out of 28 bacterial cultures were identified as *Pseudomonas* spp. by Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa campus, New Delhi, India - 12, for there confirmation through molecular marker (16s rRNA and 16s rDNA sequencing) (Kanimozhi and Panneerselvam, 2010). Isolated and identified bacterial cultures were characterized by studying cultural characteristics of individual isolates on nutrient agar, nutrient broth and Pseudomonas agar base medium. Morphological characteristics of bacterial cultures were studied microscopically. The bacteria were also tested for their biochemical reaction, antibiotic sensitivity and tolerance of pH (from 3 to 11 at intervals of 2 pH units), temperature (from 4.0 to 40°C) and salt concentration (from 0.5-20% NaCl) by growing them on Nutrient Agar Medium and broth.

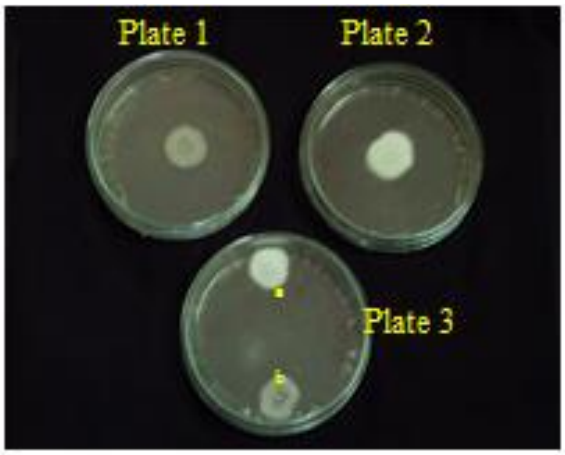
Isolation of fungal pathogen

Fungal pathogen *R. soloni* was isolated from stem of infected tomato plantlet with damping – off disease from local field. The fungal parthogen was characterized by microscopic examination (Ganesan and Gnanamanickam, 1987) and its pure cultures were maintained on PDA for further use (Devi et al., 1989).

Figure 1. Political map of Korea district of Chhattisgarh, India.

Assay for *in vitro* antagonism

A loopful of bacterial culture was placed (5mm in diameter) at one edge on the periphery of PDA plate and mycelial discs (5mm in diameter) were cut from actively growing fungal culture and placed opposite to the bacterial inoculation on PDA plate (Picture 1) (Ganesan and Gnanamanickam, 1987; Podile et al., 1988; Babu et al., 2000).



Picture 1. Pure culture plate of *Pseudomonas spp.* (plate 1) and *Rhizoctonia soloni* (plate 2). Dual culture plate of antagonism by *Pseudomonas* against *Rhizoctonia soloni* on PDA (plate 3). (a) *Rhizoctonia soloni* (b) *Pseudomonas* isolate PKJ25.

Zone of inhibition was recorded after 1 week of incubation, by measuring the restricted growth zone between the edges of fungal and bacterial colonies (Picture 2). Plate with pure *Pseudomonas* inoculum corresponding to pure fungal inoculum was taken as control. Inoculated Petri plates were incubated at 25±1°C for 07 days (Picture 3). The assays of dual culture interaction were conducted in triplicates in Completely Randomized Design and repeated twice. The per cent inhibition of mycelial growth of the pathogens was calculated using the following formula (Perveen and Bokhari, 2012):

$$I = (C - T/C) \times 100$$

Where, I = Inhibition (%) or antagonistic effect, C = colony diameter of test fungus in control plate and T = colony diameter of the same test fungus in dual culture against *Pseudomonas* as antagonist.

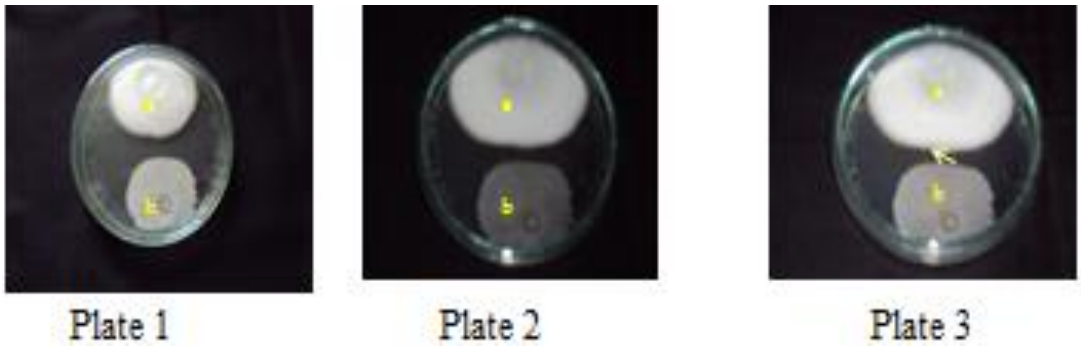
All four isolates of *Pseudomonas* (PKS10, PKM11, PKJ25 and PKB27) were tested *in vitro* for their antagonistic activity against test plant pathogens, *Rhizoctonia* spp. (causal agent of damping - off of tomato) along with standard check Pmtcc (*Pseudomonas* isolate from MTCC Chandigarh used as standard) in comparison to control (pure cultures of fungal pathogen taken as control for in vitro assay).

RESULTS AND DISCUSSION

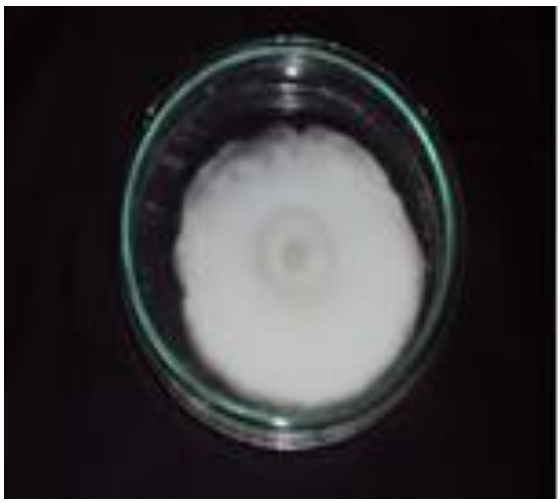
Two among four *Pseudomonas* isolates belong to *spp. P. syringae* (PKS10 and PKM11) and *P. alcaligenes* (PKJ25 and PKB27). These four isolates of *Pseudomonas* were from rhizosphere of *Oryza sativa* (Kailashpur, Sonhat Block), *Abelmoschus esculentus* (Barbaspur, Manendragarh Block), *Oryza sativa* (Umarwah, Janakpur Block) and *Lycopersicum esculentum* (Dumaria, Baikunthpur Block) respectively. Table 1 explains the *Pseudomonas* isolates, identities, sources and abbreviation.

Characterization of different *Pseudomonas* isolates

Results of morphological, biochemical and physical



Picture 2. Dual culture plate of antagonism by *Pseudomonas* spp. against *Rhizoctonia soloni*. (a) *Rhizoctonia soloni* (b) *Pseudomonas* isolate PKJ25: Plate 1(after 3 days of incubation), Plate 2 (after 5 days) and Plate 3 (after 7 days of incubation).



Picture 3. Pure culture plate of *Rhizoctonia soloni* on PDA.

characterization of *Pseudomonas* spp. are given in Table 2. *Pseudomonas* isolates PKS10 and PKM11 formed creamy colonies on Nutrient agar Medium and yellow colonies on Pseudomonas agar medium whereas isolates PKJ25 and PKB27 formed white colonies on NAM and PAB, PKS10 and PKM11 showed surface growth while PKJ25 and PKB27 showed turbidity in liquid medium. These isolates had the morphological features like singly arranged, flagellated, non-endospore forming, non –capsulated, motile, Gram negative rods. The isolates PKS10 and PKM11 showed growth from 25-37□C (optimum temp. 35□C), could tolerate pH range 5–11 (optimum pH for PKS10- 7 and PKM11-9) and salt concentration up to 5%. Isolates PKJ25 and PKB27 were able to withstand temperature as high as 45□C, high concentration of NaCl upto 5% and a wide range of initial pH from 5 to 11 (optimum pH for PKJ25-5 and PKM11-5). Similar characteristics were described for bacteria belonging to genus *Pseudomonas* and species *fluorescens* (Sharma et al., 2007; Malviya and Singh, 2012) and *putida* (Pandey et al., 2006; Malboobi et al., 2009b) respectively. The result interpreted by Malboobi et al. (2009a) in their investigation on PSB tolerance to extreme climates supported our findings. They intensively examined the isolates for tolerance toward high temperature, high concentration of NaCl and wide range of pH and found that all PSB strains survived at high temperature and could tolerate concentration of 2.5% NaCl and alkaline pH. All four isolates were sensitive to gentamicin (<10 µg ml^{-1}), polymyxin B (<300 µg ml^{-1}), chloremphenicol (<5 µg ml^{-1}) but isolate PKS10 was resistance to Ampicillin (<10 µg ml^{-1}). All the isolates tested positive for amylase, catalase, cellulase and chitinase activity but isolate PKJ25 and PKB27 showed negative test for protease and urease activity while all isolates were negative for gelatinase. Production of chitinolytic enzyme is an important means for identification of fungal inhibitors (Malviya and Singh, 2012; Kamei et al., 2014).

Result of *in vitro* antagonistic effect of different *Pseudomonas* isolates against plant pathogenic fungi under dual culture technique is depicted in Table 3. Among 4 of the *Pseudomonas* isolates, isolate PKJ25 inhibited the mycelial growth and was inhibitory to the *Rhizoctonia soloni* as compared to the other three and Pmtcc. The diameter of hyphal growth of the fungi with isolates PKS10, PKM11, PKJ25, PKB27, Pmtcc and control were 3.82, 3.68, 2.73, 3.41, 3.25 and 7.3 cm respectively. All the four *Pseudomonas* isolates PKS10, PKM11, PKJ25, PKB27 and Pmtcc inhibited the hyphal growth of *Rhizoctonia*by 47.67, 49.58, 62.60, 53.28 and 55.47% respectively.

in-vitro antagonism *Pseudomonas* isolate against *R. soloni*

Our result suggests *Pseudomonas* isolate PKJ25 *(P. alcaligenes)* was efficient and significantly suppressed the vegetative growth of the test fungi *Rhizoctonia* by

Table 2. Characterization of different *Pseudomonas* isolates.

Characteristics	Bacterial Isolates			
	PKS10	**PKM11**	**PKJ25**	**PKB27**
Colony characteristics in NAM				
Colony colour	Cream	Cream	White	White
Colony size (after 24 h of incubation)	6 mm	5 mm	5 cm	5.5 cm
Form	R	R	I	I
Margins	Undulate	Lobate	Undulate	Undulate
Elevation	Flat	Flat	Flat	Flat
Optical feature	Opaque	Opaque	Opaque	Opaque
Colony characteristics in PAB				
Colony colour	Yellow	Yellow	White	White
Colony growth (after 24 h of incubation)	+++	+	++	+
Form	I	C	I	I
Margins	Undulate	Undulate	Lobate	Lobate
Elevation	Flat	Flat	Flat	Flat
Optical feature	Opaque	Opaque	Opaque	Opaque
Growth in liquid media	Surface	Surface	Turbid	Turbid
Microscopic Examination				
Gram staining	Negative	Negative	Negative	Negative
Shape	Rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single
Flagella	Polar flagella	Polar flagella	Polar flagella	Polar flagella
Motility	+	+	+++	+
Endospore	Negative	Negative	Negative	Negative
Capsule	Negative	Negative	Negative	Negative
Biochemical Reactions				
Catalase	Positive	Positive	Positive	Positive
Amylase	Positive	Positive	Positive	Positive
Protease	Positive	Positive	Negative	Negative
Gelatinase	Negative	Negative	Negative	Negative
Urease	Positive	Positive	Negative	Negative
Cellulase	Positive	Positive	Positive	Positive
Chitinase	Positive	Positive	Positive	Positive
Lactose fermentation (acid fermentation)	Positive	Positive	Positive	Positive
Antibiotic sensitivity test				
Ampicillin	Negative	Negative	Positive	Positive
Ciprofloxacin	Positive	Positive	Positive	Positive
Polymyxin B	Positive	Positive	Positive	Positive
Gentamycin	Positive	Positive	Positive	Positive
Physical factors affecting bacterial growth				
Effect of molecular O_2 (Aerobic/ Anaerobic)	Aerobic	Aerobic	Aerobic	Aerobic
Effect of Osmotic pressure/salt tolerance Concentration of NaCl (0.5%-20%)	5%	5%	5%	5%
pH tolerance (3pH-11pH)	5pH-11pH	5pH-11pH	5pH-11pH	5pH-11pH
Optimum pH	7pH	9pH	5pH	5pH
Temperature tolerance (4 - 50°C)				
Temperature tolerance (4 - 50°C)	25- 37°C	25- 37°C	25-45°C	25- 45°C
Optimum temperature	37°C±2	37°C±2	37°C±2	37°C±2

R, Regular; I,Irregular; C,circular; +, slight; ++, medium; +++, abundant .

Table 3. *In vitro* antagonistic effect of different *Pseudomonas* isolates against plant pathogenic Fungi under dual culture technique.

S. No.	*Pseudomonas* isolates	Fungi pathogen	
		Rhizoctonia soloni	
		Colony diameter (cm)	Inhibition%
1	PKS10	3.82	47.67
2	PKM11	3.68	49.58
3	PKJ25	2.73	62.60
4	PKB27	3.41	53.28
5	Pmtcc	3.25	55.47
6	Control	7.30	00.00

Result represents the mean of three replications per strain. Pure culture of fungal pathogen was taken as control.

restricting the hyphal growth, in *in vitro* condition with 62.60% inhibition followed by Pmtcc and PKB27 with 55.47 and 53.28% inhibition respectively. In similar study, Podile et al. (1988) reported inhibitory effect of *P. aeruginosa* and *P. fluorecens* on plant pathogenic fungi *R. solani, Sclerotium rolfsii.* Devi et al. (1989) suggested that antagonistic bacteria *Pseudomonas fluorescent* isolates (Pfr1-14) obtained from rice rhizosphere suppressed the rice ShB pathogen, *R. solonii in vitro* by inhibiting mycelial growth and sclerotial germination. Similar report of antagonistic effect of Fluorescent *Pseudomonas* was reported by Khan and Zaidi (2002) for *R. soloni* and *Fusarium oxysporium.* Akhtar and Siddiqui (2009) suggested the use of plant growth promoting rhizobacteria for the biocontrol of root-rot disease complex of chickpea and their studies showed that the three *Pseudomonas* spp. had inhibitory effect on *Macrophomina phaseolina*; *P. alcaligenes was* one of the biocontrol agent. Jayaraj et al., (2007) tested 08 fluorescent Pseudomonads isolated from tomato rhizosphere and observed highest growth inhibition (15.5 mm) of *Pythium aphanidermatum* and controlled damping off of tomato by 68.5%.

The antagonistic nature of *P. aeruginosa* rhizobacterial isolates PTR-3 and PCF-3 against *R. solani* was also reported by Kamei et al. (2014). Their finding suggest that rhizobacterial isolates PTR-3 restricted mycelial growth of *R. solani* up to 1.9 (cm) and were found to exhibit antagonism of over 68.9%. Sharma et al. (2014) reported that *Pseudomonas* spp. isolates showed antifungal activity against *Rhizoctonia* spp. in the range of 7.27-53.84% inhibition. Also, *P. fluorescens* isolate restricted the linear growth of *R. solani* by 81.3% as reported by Mezeal (2014).

Thus, present study receives strong support from the above observations and the information generated through this study will help for future studies on the antagonistic affect of native microorganisms on soil in Chhattisgarh (India) and consequently for the maintenance of native microorganisms as microbial antagonists for enhancement of crop production.

Conflict of interests

The authors did not declare any conflict of interest.

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Phosphate solubilizing rhizospheric bacterial communities of different crops of Korea District of Chhattisgarh, India

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Occurrence of Phosphate Solubilizing Bacteria (PSB) in the rhizospheric soil of different crops of Korea District of Chhattisgarh, India was studied. Phosphate solubilizing bacteria colonize the plant roots, affect plant growth positively and add nutrients to the soil. A good number of PSB were isolated, purified and identified from twenty-five different representative soil samples collected from five blocks of Korea District of Chhattisgarh, India. The bacterial species were *Pseudomonas alcaligenes, Pseudomonas syringae* and *Bacillus subtilis*. The total rhizospheric bacterial (TRBP) population indicated that bacterial population in the rhizospheric soil of Korea District of Chhattisgarh ranged from 3.03×10^6 to 4.92×10^6 cfu. TRBP was affected greatly by different physico-chemical properties of soil (nitrogen, phosphorus, potassium, organic carbon %, pH and electrical conductivity = EC). All the parameters studied showed positive correlation with TRBP except EC that showed negative correlation. On the basis of correlation co-efficient values, positive significant values are grouped in three. Example – strong (r = >0.5), medium (r = < 0.4 but greater than 0.1) and weak (r < 0.1). The present study indicates 100% distribution of phosphate solubilizing bacteria in soil of Korea district of Chhattisgarh, India, indicating only 14% occurrence of *Pseudomonas* spp. while 86% bacterial inhabitants belong to genus *Bacillus*. P (Phosphorus) had strong positive significant correlation with TRBP. N, K & OC% had medium correlation and pH had weak positive significant correlation with TRBP.

Key words: Correlation, PSB, *Pseudomonas* spp., physicochemical property of soil, Rhizospheric bacteria, egression.

INTRODUCTION

Rhizospheric bacterial inhabitants have marked impact on soil ecosystem. Plant root exudates are good nutrient source available for microorganisms in soil that supports their rapid proliferation in the rhizosphere (Marilley and Aragno, 1999). The composition of microbial community is affected by the amount and composition of root exudates which in turn influences nutrient availability of soil. The variation in total rhizospheric bacterial population in near vanity of roots may be due to organic carbon substrates supplied from the roots exudates, dead organic matter (Baudoin et al., 2001) and plant variety (Dunfield and Germida, 2001). Bacterial count is higher

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in rhizosphere, considered as hot spot of bacterial diversity (Botelho et al., 2006; Kumar et al., 2011). Bacteria in rhizhosphere are beneficial to plants, have ability to colonize the roots and exert plant growth promotion, thus called as plant growth promoting rhizobacteria (PGPR). Strains with PGPR activity, belongs to genera *Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconacetobacter, Pseudomonas* and *Serratia.* Also the root nodules of leguminous plants are the habitat of many species of bacteria like *Bacillus megaterium, Bacillus aerophilus, Microbacterium laevaniformans* and *Staphylococcus xylosus* (Singha and Sharma, 2013). Among these, species of *Pseudomonas* and *Bacillus* predominates in the rhizhosphere and are most extensively studied (Sutra et al., 2000; Widawati, 2011; Kumar et al., 2011; Keneni et al., 2012; Sharma et al., 2014). The bacterial community in rhizosphere act as both biofertilizers and microbial antagonists. The principal mechanisms of growth promotion include production of growth stimulating phytohormones, solubilization and mobilization of phosphate, production of siderophore and antibiotics, inhibition of plant ethylene synthesis, and induction of plant systemic resistance to pathogens (Kumar et al., 2011). The phosphate solubilizing bacteria is present in all the soil types and their population is higher in the rhizosphere as compared to the non-rhizosphere. The solubilization of mineral phosphate is attributed by the production and release of organic acids and acid phosphatases that play a major role in the mineralization of organic phosphorous in soil with simultaneous decrease in pH (Park et al., 2009). The phosphate solubilizing ability of PGPR is greatly affected by various environmental parameters such as Temperature, ph, carbon and nitrogen sources (Mujahid et al., 2015). Also diversity in the phosphate solubilizing bacterial community is impacted by physico-chemical parameters of different soil samples such as high concentrations of sodium, alkaline ph and high Electrical Conductivity (Mohan and Menon, 2015). In present study; data on comparative population of phosphate solubilizing rhizospheric bacteria in the agricultural soil of Korea District of Chhattisgarh, India has been depicted and correlation-regression between physico-chemical property and total rhizospheric microbial population of soil has been studied.

MATERIALS AND METHODS

Soil sampling

The present study was conducted on agricultural land of Korea District of Chhattisgarh India, one of the four major districts of northern hilly zones of Chhattisgarh State with area of 5978 Km^2. The climate is ideal with a beautiful monsoon, a mild summer and a bearable winter. Average temperature ranges from maximum 32°C to minimum17°C, 59.03% land area is covered with forest, average rainfall is 1410.9 mm (http://korea.gov.in/glaonce.htm) , soil type is red-yellow and major crop is paddy (http://korea.gov.in/). District Korea is divided into 5 blocks: Baikunthpur, Manendragarh, Khadgawan, Sonhat and Janakpur. (http://korea.gov.in/).

In the present investigation soil samples were collected from randomly selected 05 locations of 05 agricultural fields from each blocks of Korea District of Chhattisgarh, India (Walworth, 2004**)**. A total of 25 composite soil samples were collected to a depth of about 6 to 8 inches from 05 blocks of agro based areas of Korea District of Chhattisgarh, five soil samples from each blocks (Baikunthpur, Manendragarh, Khadgawan, Sonhat, Janakpur), during the month of May - June, 2009, all these were drawn from post harvested fields (Venkateswarlu et al., 1984). Each sample weighing 200 g were obtained as representative samples of crop fields and analyzed for its physical and chemical properties and the occurrence of phosphate solubilizing bacteria.

Representative soil samples were air-dried at room temperature to reduce moisture content. Each sample was then analyzed for microbial content and tested for organic matter, potassium, phosphorous and nitrogen contents, pH and EC, for which the desired sample amount, 100 g was taken to laboratory for microbiological analysis (isolation of rhizospheric PSB) and another 100 g was used for physic-chemical analysis at Biotech Lab and Training Centre, Collectorate campus, Ambikapur (Surguja). Rest quantity of the soil samples, were stored in dry place for future use if needed.

Isolation of phosphate solubilizing soil bacteria

The isolation of Phosphate solubilizing rhizospheric bacteria was carried out by serial 10-fold dilutions technique (Pandey et al., 2006) and pour-plate method on Pikovskaya (PVK) agar (Pikovskaya, 1948) and Modified Pikovskaya media (Gupta et al., 1994; Dave and Patel, 1999) at Plant Pathology Laboratory, RMD College of Agriculture and Research Station, Ambikapur (Chhattisgarh), India. Phosphate solubilizing bacteria was screened by selecting the microorganisms which are capable of producing a clear zone on plate.

Enumeration and purification of phosphate solubilizing bacterial isolates

The population of individual phosphate solubilizing rhizospheric bacterial isolates was enumerated on basis of per gram of soil using following formula (Schmidt and Caldwell, 1967; Tripathi et al., 2013).

Number of bacteria per gram soil = No. of colony forming units × dilution / Dry weight of 1 g soil × aliquot taken

Different colonies showing clear zone around the line of growth on each plates were aseptically transferred on the surface of Nutrient agar media and Pseudomonas Agar base media to obtain pure sub cultures of each primary culture. The prepared pure cultures were then preserved in refrigerator at low temperature (4°C) in the Department of Plant Pathology, RMDCARS IGKVV Ambikapur for longevity of bacterial isolates for their further use.

Physiological markers tests like growth behavior in medium, color change on Modified Pikovskaya medium along with biochemical markers like gram staining reaction, starch hydrolysis test (Sharma et al., 2007) were carried out for the primary identification of the bacterium *Pseudomonas*. After primary identification, cultures were sent to Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa campus, New Delhi, India - 12, for their molecular identification based on 16s rRNA and 16s rDNA gene sequencing (Kanimozhi and Panneerselvam, 2010; Singha

Table 1. Physico-chemical Property and total rhizospheric microbial population of soil of Korea District (Chhattisgarh).

S/No.	Field area	Crops	Soil parameters							
			N	P	K	OC %	pH	EC	TRBP cfu (10^6)	Blocks
1.	Cheetajhor	*Oryza sativa*	166.6	34.9	250.2	0.67	6.5	0.11	3.58	
2.	Banjaridand	*Oryza sativa*	126.4	37.1	277.6	0.86	6.3	0.19	3.69	
3.	Akhradand	*Oryza sativa*	153.2	23.1	303.5	0.39	6.1	0.8	3.03	Khadgawan block
4.	Dubchhola	*Oryza sativa*	255.6	14.9	211.2	0.28	6.4	0.9	3.08	
5.	Bhukbhukii	*Oryza sativa*	277.8	40.9	207.5	0.76	6.2	0.4	3.81	
6.	Navgayi	*Oryza sativa*	126.2	37.1	297.1	0.67	6.3	0.18	3.69	
7.	Katgodi	*Abelmoschus esculentus*	126.3	42	336.2	0.79	5.95	0.7	4.01	Sonhat block
8.	Kachardand	*Oryza sativa*	161.1	47	266.1	0.61	6.3	0.1	4.8	
9.	Ghughra	*Oryza sativa*	171.5	31	259.4	0.44	6.2	0.3	4.09	
10.	Kailashpur	*Oryza sativa*	144.5	41	216.5	0.69	6.7	0.7	4.05	
11.	Barbaspur	*Abelmoschus esculentus*	129.5	39.6	325.5	0.66	6.4	0.11	4.3	
12.	Sarbhoka	*Triticum aestivum*	162.2	48.9	232.4	0.45	6.8	0.21	3.65	
13.	Pahad hanswahi	*Triticum aestivum*	278.2	41	291	0.68	6.9	0.4	4.92	Manendragarh block
14.	Kachhod	*Oryza sativa*	126.4	33.9	255.2	0.86	6.3	0.18	3.17	
15.	Belbehra	*Oryza sativa*	229.5	33.7	248.1	0.66	6.2	0.5	3.81	
16.	Kharwat	*Triticum aestivum*	163.2	43.5	336.2	0.36	5.95	0.3	3.99	
17.	Tendua	*Triticum aestivum*	129.1	32.9	243.1	0.64	6.01	0.7	3.03	
18.	Bisunpur	*Oryza sativa*	220.2	31	297.2	0.66	6.1	0.12	3.07	Baikunthpur block
19.	Dumaria	*Lycopersicum esculentum*	169.1	40.5	257.2	0.8	5.87	0.13	4.83	
20.	Nagar	*Cajanus cajan*	144.2	39.6	226.2	0.64	5.9	0.8	3.57	
21.	Khetawli	*Oryza sativa*	148.1	33.7	325.5	0.49	6.7	0.4	3.87	
22.	Barel	*Oryza sativa*	163.2	30	294.4	0.36	6.1	0.1	3.59	
23.	Dhobataal	*Oryza sativa*	146.5	24	211.2	0.49	6.5	0.7	3.06	Janakpur block
24.	Chutki	*Lagenaria vulgaris*	175.6	37.2	297	0.73	6.1	0.18	3.87	
25.	Umarwaah	*Oryza sativa*	254.6	53.1	330.2	0.89	5.86	0.3	4.2	

NPK (Kg/ha), EC (mmol/cm), OM-Organic matter, TRBP- Total rhizospheric bacterial population.

and Sharma, 2013).

RESULTS

Soil analysis

Soil testing refers to the chemical analysis of soil and is well recognized as a scientific means for quick characterization of the fertility status of soils and predicting the nutrient requirement of crops. It also includes testing of soils for other properties like texture, structure, pH, cation exchange capacity, water holding capacity, electrical conductivity, phosphorus (P), potassium (K), organic matter, sulfur (S), boron (B), zinc (Zn), and other micronutrients. Total 25 soil samples collected from Korea District were analyzed for N, P, K, EC, organic matter (Organic Carbon %), pH and total rhizospheric bacterial population (TRBP).

Results depicted in Table 1, indicates that nitrogen content of soil samples ranges from 126.2- 278.2 Kg/ha, phosphorus content is 14-55 Kg/ ha and potassium analysis indicates that 11 soils samples of the District belongs to high category of potash (> 280 Kg/ha) while rest of the 14 samples have potash in the medium range (110-280 Kg/ha). Table 1 also indicates the result data on EC, OC% and pH of soil. EC of all the samples from the district were less than 1 milimole cm^{-2} organic carbon percentage (OC%) of 6 soil samples were low (<0.5), other 6 samples have high OC% (>0.75) and rest samples are in the range of medium OC% (0.5-0.75). pH values of the soil ranged from 5.86-6.9 indicating soils are acidic to neutral suitable for cultivation.

Studies on the total rhizospheric bacterial (TRBP) population indicates that bacterial population in the rhizospheric soil of Korea District of C.G. ranged from 3.03 x 10^6 to 4.92 x 10^6 cfu. Highest rhizospheric bacterial population (4.92 x 10^6 cfu) was recorded from rhizospheric soil of *Triticum aestivum* (Pahadhanswahi, Sonhat block), followed by 4.83 x 10^6 cfu from rhizospheric soil of *Lycopersicum esculentum* (Dumaria, Baikunthpur block), 4.80 x 10^6 cfu from rhizospheric soil of *Oryza sativa* (Kachardand, Sonhat block) 4.30 x 10^6 cfu from rhizospheric soil of *Abelmoschus esculentus* (Barbaspur, Manendragarh Block) 4.20 x 10^6 cfu from rhizospheric soil of *Oryza sativa* (Umarvah, Janakpur

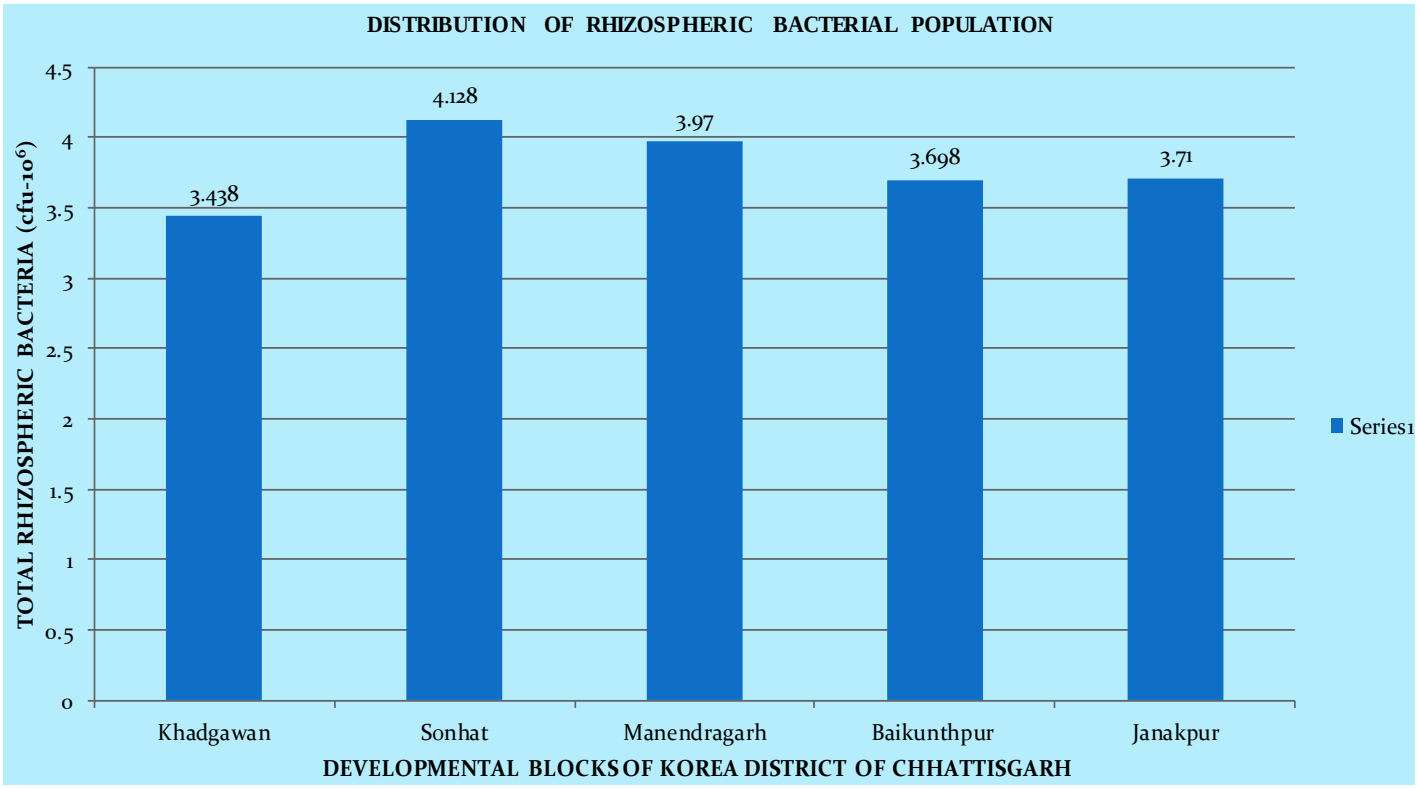

Figure 1. Distribution of total rhizospheric bacterial population in five blocks of Korea District of Chhattisgarh, India.

Block) 4.09 x 10^6 cfu from rhizospheric soil of *Oryza sativa* (Ghughra, Sonhat Block) 4.05 x 10^6 cfu from rhizospheric soil of *Oryza sativa* (Kailashpur, Sonhat Block) 4.01 x 10^6 cfu from rhizospheric soil of *Abelmoschus esculentus* (Katgodi, Sonhat Block). Lowest rhizospheric bacteria population 3.03 x 10^6 cfu was recorded from rhizospheric soil of *Oryza sativa* (Akhradand, Khadgawan Block).

Figure 1 depicts the Distribution of Total Rhizospheric Bacterial population in five Blocks of District Korea. The occurrence of maximum TRBP is found in Sonhat followed by Manenedragarh and Janakpur, respectively. The lowest TRBP exists in Khadgawan followed by Baikunthpur, therefore according to the occurrence of TRBP the whole Korea District is grouped into two Zonal Blocks that is, First Zonal Block comprising of blocks Sonhat, Manendragarh, Janakpur and Baikunthpur with TRBP > cfu 3.5 × 10^6 and Second Zonal Block comprising of Block Kadgawan withTRBP < cfu 3.5 × 10^6. Data recorded in the present investigation indicates the highest Rhizospheric population (> cfu 3.5 × 10^6) is present in First Zonal Blocks- Sonhat, Manendragarh, Janakpur and Baikunthpur where OC% is also high. The Total rhizospheric population is usually influenced by organic carbon substrates supplied from the roots exudates and dead organic matter in near vanity of roots (Baudoin et al., 2001).

Correlation and regression studies between physico-chemical property and total rhizospheric microbial population of soil of Korea District (Chhattisgarh)

The present study of correlation and regression (Table 2) between physico-chemical property and total rhizospheric bacterial population of soil of Korea District (Chhattisgarh), indicates that the TRBP is affected greatly by different physico-chemical property of soil that is, N, P, K, OC%, pH and EC. All the parameters studied showed positive correlation with TRBP except EC that showed negative correlation. N, P, K, OC% and PH values have positive significant correlation with TRBP but EC have negative correlation with TRBP. On the basis of correlation co-efficient values, positive significant values are grouped in three. Example – strong (r = >0.5), medium (r = < 0.4 but greater than 0.1) and weak (r < 0.1). Thus the present study reveals that P has strong positive significant correlation with TRBP. N, K & OC% have medium correlation and pH has weak positive significant correlation with TRBP.

Isolation of Phosphate solubilizing bacteria from Rhizosphere of Korea District of Chhattisgarh

Total of 28 phosphate solubilizing bacteria were isolated

Table 2. Correlation and Regression studies between physico-chemical property and total rhizospheric microbial population of soil of Korea District (Chhattisgarh)

S/No.	Independent Character	Dependent Character	Correlation (r)	Calculated t- value	Regression Equation
1.	N	TRBP	0.184942	0.903*	**Y=** .002 **X=+**3.427
2.	P	TRBP	0.655487	4.163*	**Y=**.044 **X=+**2.201
3.	K	TRBP	0.21609	1.395*	**Y**=.004 **X**=+2.787
4.	OC%	TRBP	0.29935	1.505*	**Y=**.970 **X=+**3.188
5.	pH	TRBP	0.091674	0.442*	**Y=** .173 **X=+**2.706
6.	EC	TRBP	-0.38674	-2.011**	**Y**= -.807 **X**=+4.097

Significant (p=>0.05) = *Non-Significant(p< 0.05) =**

on Pikovskaya and Modified Pikovskaya medium from 25 soil samples (Table 3), collected from five blocks of Korea District of Chhattisgarh. Four bacterial isolates were identified as *Pseudomonas* spp. out of 28 bacterial cultures from division of plant pathology, IARI, Delhi and rest were identified as *Bacillus subtillis*.

DISCUSSION

Soil sampling and analysis

Soil is a medium that provides physical support to plants and supply plants with mineral nutrients that are essential for their growth and reproduction. Highly fertile soil contains >0.75% organic carbon, > 560 Kg/ha nitrogen, >55 Kg/ha phosphorus and > 280 Kg/ha potassium (Arora, 2002) and pH values between 6 and 7.5 and electrical conductivity values between 0 and 0.8 dS/m are optimal for crop growth (Arias et al., 2005). Sampling depth depends on the crop, cultural practices, tillage depth, and the nutrients to be analyzed. Because the greatest abundance of plant roots, greatest biological activity, and highest nutrient levels occur in the surface layers, the upper 12 inches of soil are used for most analyses. The analyses run on the surface sample include soil reaction (pH), phosphorus (P), potassium (K), organic matter, sulfur (S), boron (B), zinc (Zn), and other micronutrients. Sampling depth is especially critical for nonmobile nutrients such as P and K. The recommended sampling depth for nonmobile nutrients is 12 inches. The tillage zone, typically 6 to 8 inches deep, usually contains a relatively uniform, high concentration of nonmobile nutrients. Below the tillage zone the concentration is usually lower. Therefore, a sample from the tillage zone will usually have a higher content of nonmobile nutrients than a sample from the desired 0- to 12-inch sample depth.

The soil fertility levels influences plant growth and microbial population that itself is affected by tillage and nutrient mobility. A total of 25 soil samples, each weighing 200 g were obtained as representative samples (Mahler and Tindal, 1994) from 5 blocks of Korea District of Chhattisgarh, from rhizosphere region of crop fields and analyzed for Physico-chemical property at Biotech Lab Training and Demostration Center, Collectorate, Ambikapur (Chhattisgarh). Our results on the different parameters indicate that soils of Korea District of Chhattisgarh has pH (5.86-6.9), EC (0.1- 0.9 mmol/cm) and contained organic carbon (0.28-0.89%), N (126.2-278.2 Kg/ha), P (14.9-48.9 Kg/ha) and K (207.5-336.2 Kg/ha). The above soil test results are as per fertility rating interpreted by Arora, (2002) and Arias et al. (2005) suitable for cultivation of crops except nitrogen and phosphorus that were slightly less than optimum range.

Total rhizospheric bacterial population (TRBP)

The rhizosphere is a hot spot of soil organisms: microbial activity is stimulated by nutrient content of soil. Rhizosphere microorganisms have marked effect on plant performance in agricultural and marginal soils by influencing growth and development of root and improving nutrient availability in the rhizosphere.

Our results on the total rhizospheric bacterial (TRBP) population indicates that bacterial population in the rhizospheric soil of Korea District of C.G. ranged from minimum 3.03×10^6 to maximum 4.92×10^6 cfu. Highest rhizospheric bacterial population (4.92×10^6) was recorded from rhizospheric soil of *Triticum aestivum* (Pahadhanswahi, Sonhat block), followed by 4.83×10^6 from rhizospheric soil of *L. esculentum* (Dumaria, Baikunthpur block) and lowest rhizospheric bacterial population 3.03×10^6 was recorded from rhizospheric soil of *Oryza sativa* (Akhradand, Khadgawan Block). Marilley and Aragno (1999) reported that plant root exudates are good nutrient source for microorganisms that allow some microbial species to proliferate rapidly in the rhizosphere especially those with high growth rates and relatively high nutrient requirements such as pseudomonads. The composition of microbial community is affected by the amount and composition of root exudates which in turn influences nutrient availability of soil. The variation in total rhizospheric bacterial population in near vanity of roots may be due to organic carbon substrates supplied from

Table 3. Occurrence of Phosphate solubilizing bacteria in Rhizospheric soil of Korea District of Chhattisgarh.

S/No.	Blocks	Field	Crops	Bacterial genus identified from Rhizospheric soil	Presence (+) or absence (-) of PSB
1.	Khadgawan Block	Cheetajhor	*Oryza sativa*	*Bacillus subtilis*	(+)
		Banjaridand	*Oryza sativa*	*Bacillus subtilis*	(+)
		Akhradand	*Oryza sativa*	*Bacillus subtilis*	(+)
		Dubchhola	*Oryza sativa*	*Bacillus subtilis*	(+)
		Bhukbhukii	*Oryza sativa*	*Bacillus subtilis*	(+)
2.	Sonhat Block	Navgayi	*Oryza sativa*	*Bacillus subtilis*	(+)
		Katgodi	*Abelmoschus eculentus*	*Bacillus subtilis*	(+)
		Kachardand	*Oryza sativa*	*Bacillus subtilis*	(+)
		Ghughra	*Oryza sativa*	*Bacillus subtilis*	(+)
		Kailashpur	*Oryza sativa*	*Pseudomonas syringae*	(+)
3.	Manendragarh Block	Barbaspur	*Abelmoschus eculentus*	*Pseudomonas syringae*	(+)
		Sarbhoka	*Triticum aestivum*	*Bacillus subtilis*	(+)
		Pahadhanswahi	*Triticum aestivum*	*Bacillus subtilis*	(+)
		Kachhod	*Oryza sativa*	*Bacillus subtilis*	(+)
		Belbehra	*Oryza sativa*	*Bacillus subtilis*	(+)
4.	Baikunthpur Block	Kharwat	*Triticum aestivum*	*Bacillus subtilis*	(+)
		Tendua	*Triticum aestivum*	*Bacillus subtilis*	(+)
		Bisunpur	*Oryza sativa*	*Bacillus subtilis*	(+)
		Dumaria	*Lycopersicon esculentum*	*Bacillus subtilis* , *Pseudomonas alcaligenes*	(+)
		Nagar	*Cajanus cajan*	*Bacillus subtilis*	(+)
5.	Janakpur Block	Khetawli	*Oryza sativa*	*Bacillus subtilis*	(+)
		Barel	*Oryza sativa*	*Bacillus subtilis*	(+)
		Dhobataal	*Oryza sativa*	*Bacillus subtilis*	(+)
		Chutki	*Lagenaria vulgaris*	*Bacillus subtilis*	(+)
		Umarwaah	*Oryza sativa*	*Pseudomonas alcaligenes*	(+)

the roots exudates and dead organic matter (Baudoin et al., 2001). Rhizosphere soil of different plant species shows differential composition and abundance of microbial populations (Ponmurugan and Gopi, 2006).

Correlation and Regression studies between physico-chemical property of soil and total rhizospheric microbial population of soil of Korea District (Chhattisgarh)

Our result on the present study of correlation and regression between physico-chemical properties and total rhizospheric bacterial population (TRBP) of soil of Korea District (Chhattisgarh), indicates that the TRBP is affected greatly by N, P, K, OC%, pH of soil and showed positive significant correlation except EC, which showed negative correlation. Ross and Tate (1993) reported that microbial biomass and activities are coordinated by many soil and environmental factors such as soil organic matter quality, physic-chemical characteristics of soil. Pereira et al. (2006) observed that low pH values and high metal contents negatively affected the size and activity of soil microbial biomass. Also the soil external parameters such

as water content, temperature, pH, soil types (texture, organic matter, microaggregate stability, presence of key nutrients such as N, P, K, and Fe), composition of root exudates, presence of other microorganisms and plant species are major determinant of overall microbial diversity (Grayston et al., 1998; Dakora and Philipps, 2002). Plants grown with deficient or sufficient nutrient supply generaly have different microbial communities in the rhizosphere (Marschner et al., 2004; Marschner et al., 2005b; 2006; 2007). Rengel and Marschner (2005) told that nutrient deficiency can influence rhizosphere microorganisms. Microbial community composition is also influenced by soil properties as well as P addition (Marschner et al., 2006) and other management factors (Steenwerth *e*t al., 2003; Marschner et al., 2005b; Steenwerth et al., 2008), with agricultural intensification resulting in decreased microbial diversity and lowering of ecosystem function (Steenwerth et al., 2005).

Isolation of phosphate solubilizing bacteria from rhizosphere of Korea District of C. G.

Occurrence of phosphate solubilizing bacteria in rhizospheric soil of Korea District of Chhattisgarh depicted in Table 3. Our result on isolation of phosphate solubilizing *Pseudomonas* spp. from soil of Korea District of Chhattisgarh indicates 100% success for best distribution of phosphate solubilizing bacteria but poor distribution of *Pseudomonas* spp. indicating only 14% occurrence while 86% bacterial inhabitants belong to genus *Bacillus*. The number and type of bacterial inhabitants of rhizospheric soil is determined by the nutrient content of soil. The report of Glandorf *e*t al. (1993) supported our findings that the occurrence and composition o*f Pseudomonas* population differed between crops. Rhizosphere soil of different plant species shows differential composition and abundance of microbial populations (Ponmurugan and Gopi, 2006). Species of bacterial genus *Pseudomonas* and *Bacillus* predominates in the rhizhosphere are most extensively studied (Kumar et al., 2011).

In the present research work we were able to isolate phosphate solubilizing bacteria from agricultural soil of Korea District of Chhattisgarh, India. Korea District of Chhattisgarh is known for coal mines and mining is the main occupation of local residents. Nutrient status of agricultural land is poor and agricultural people generally depend upon single season crop. The information generated through the present investigation will help for the future study of the nutrient quality of soil, effect of soil pollution by pollutants in mining area and consequently for the maintenance of indigenous microorganisms in the soil through the correction of above studied soil parameters; however further research is needed to explore other potentials of these isolates and environmental parameters of Korea District of Chhattisgarh.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Characterization of phosphate solubilizing rhizobacteria isolated from lentil growing areas of Ethiopia

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Lentil (*Lens culnaris* Medik.) is an invaluable source of dietary protein for the majority of Ethiopian population. The low availability of phosphorus is one of the major soil related stresses that limit the production of this crop. This study was aimed at isolation and characterization of phosphate solubilizing bacteria from lentil growing areas of Ethiopia and evaluating their effects on growth of this crop under greenhouse conditions. In total, 41 phosphate solubilizing bacterial strains were isolated from lentil rhizosphere and characterized using biochemical tests and 16S rDNA sequence analysis. The results showed the presence of genera *Acinetobacter, Agrobacterium, Bacillus, Burkholderia, Chryseomonas, Enterobacter, Pseudomonas, Ralstonia* and *Sphingomonas.* Phosphate solubilzing efficiencies of these isolates were evaluated in liquid medium using different insoluble P-sources and the result indicated that they released considerable amount of phosphates from tricalcium phosphate, rock phosphate and bone meal. Besides, some of these isolates exhibited multiple plant growth promoting characteristics such as indole acetic acid production and growth inhibition of *Fusarium oxysporum* under *in vitro* conditions. The effects of inoculation of *Enterobacter kobei* (PSBL5), *Bacillus subtilis* (PSBL21), *Enterobacter* species (PSBL26), *Bacillus* species (PSBL31) and *Pseudomonas fluorescence* (PSBL37) on lentil growth, nodulation, N and P content of shoot were evaluated under a greenhouse conditions and the results showed that these features were significantly increased over uninoculated control plants. Based on the results of this study, we conclude that lentil rhizosphere soil from producing areas of Ethiopia harbor diverse phosphate solubilizing bacterial strains with excellent phosphate solubilization and plant growth promotion ability. Thus, most of the promising strains should be tested in the field conditions under different agroecology of the country.

Key words: Rhizosphere soil, PGPR, rock phosphate, bone meal.

INTRODUCTION

Lentil (*Lens culnaris* Medik.) is an important pulse crop grown widely in Ethiopia over an area of 105,956 ha with an annual production of 123,777 tones (CSA, 2010). It is an invaluable source of dietary protein for the vast majority of the Ethiopian population. In addition, lentil restores and maintains soil fertility through its nitrogen-fixing symbiosis. Thereby, it ensures the sustainability of the crop producing system. Thus, it has been grown in

rotation with other major cereals in traditional low input system. However, the yields of lentil has been 1.17 tones ha^{-1} (CSA, 2010) that could be low compared to its potential (Hailemariam and Tsige, 2006; Jida and Assefa, 2011) due to soil infertility stresses which prevail under Ethiopian soil and climatic conditions (Ahmed and Ayalew, 2006; Fikre and Bejiga, 2006).

Phosphorus (P) is one of the essential macronutrients that limit plant growth in tropical soils (Vessey, 2003; Khan et al., 2007). Most soils in tropical and subtropical areas are predominantly acidic and have strong P sorption (fixation) ability (Khan et al., 2007). This leads to wide spread available P deficiency which is particularly the case for the large parts of Ethiopian soils (Beyene, 1982; Mamo et al., 1988). These low levels of root available P are mainly due to high reactivity of soluble P with Fe and Al oxides in acidic soils (Gyaneshwar et al., 2002). Hence, this has been one of the stresses that constrain the production of lentil and other pulse crops in the country.

On the other hand, the application of chemical fertilizer is highly limited by its cost intensity. Hence, its use for pulse crops production such as lentil is very low in Ethiopia. Moreover, up to 75% of the P-fertilizers added to the soil could be converted into insoluble forms by reacting with free Ca^{2+} or Fe^{3+} and Al^{3+} ions in high and low pH soils, respectively (Goldstein, 1986). These cause the urgent need for economically feasible and ecologically acceptable P-fertilizer sources. Thus, using microbial biofertilizers for improving the availability of P in the vicinity of plant roots represents an alternative to the use of chemical fertilizers (Naik et al., 2008). It has been well documented that phosphate solubilizing bacteria (PSB) are able to convert insoluble phosphate to soluble primary and secondary orthophosphate ions (Pal, 1998; Peix et al., 2001a; Chen et al., 2006; Vyas and Gulati, 2009).

Rhizopshere bacteria that solubilize P and enhance plant growth are referred to as plant growth promoting rhizobacteria (Glick, 1995). Compared to the bulk soil, the proportion of PSB is high in the rhizosphere and such bacteria are metabolically more active (Vazquez et al., 2000). Earlier studies indicated that different species of PSB were able to solubilize naturally occurring insoluble inorganic P sources such as, rock phosphate and old bone (Ivanova et al., 2006; Keneni et al., 2010). Besides increased P uptake, the production of phytohormones such as indoleacetic acid (IAA) by PSB could also play an important role in plant growth promotion (Naik et al., 2008; Shahab et al. 2009). In addition, PSB could also enhance plant growth by suppressing different soil borne fungal pathogens of crops (Peix et al., 2001b).

Even though the majority of Ethiopian soils are characterized by low available P (Beyene, 1982; Mamo et al., 1988), most of the hitherto studies have focused on the rate of P-fertilizer application. Consequently, there is little information on native PSB isolates recovered from the rhizosphere of lentil grown in Ethiopian soils. However, an array of bacteria with phosphate solubilizing potential have been found associated and within the rhizosphere of agriculturally important crops (Chen et al., 2006; Vyas and Gulati, 2009; Muleta et al., 2013). Moreover, several studies indicated the potential biotechnological application of such native bacteria in promotion of plant growth (Peix et al., 2001ab; Shahab et al., 2009). Therefore, the objectives of this study were isolation and characterization of PSB from lentil growing areas of Ethiopia and evaluation of their effects on the growth of lentil under greenhouse conditions.

MATERIALS AND METHODS

Study sites and soil samples collection.

Soil samples were collected from lentil grown fields in Amhara, Oromia and Tirgay regional states of Ethiopia in October, 2009. These regions represent the major lentil producing areas of the country. The areas were distributed in Central and Northern parts of the country with an altitude range of about 1799-3101 m above sea level (masl) and soil pH values ranging from moderately acidic (5.58) to slightly alkaline (7.18) (Table 1). Thirty soil samples were excavated from the 15-20 cm depth and collected in sterile plastic bags. The samples were stored at 4°C in a refrigerator until the time of analysis. Further analysis of the soil samples was performed in the Laboratory of the Stream of Applied Microbiology, Addis Ababa University.

Isolation of phosphate solubilizing rhizobacteria

Each soil sample was thoroughly mixed, air-dried in a greenhouse and then filled into surface sterilized (96% ethanol) plastic pots (4 kg capacity). Lentil seeds (*Teshale* cultivar) were selected and surface sterilized with 96% ethanol and 3% sodium hypochlorite solutions and rinsed five times with sterilized distilled water. Surface sterilized seeds were allowed to germinate on sterile water agar (1%) surface for three days at 25°C and five pre-germinated seeds were planted on each pot, which were thinned down to three seedlings 5 days after emergence (DAE). All pots were situated in a greenhouse over the table in randomized block design and watered to a field capacity every three days for 45 days after planting (DAP). Fourty-five DAP lentil seedlings with good growth and healthy appearance were selected from each pot and uprooted carefully with their rhizosphere soil. Plant roots with adhered rhizosphere soil were carefully transferred into sterile Petri dishes and 10 g of it was transferred to flasks (250 ml) containing sterilized 90 ml saline solution (0.85% w/v NaCl). The flasks were incubated on a gyratory shaker at 120 revolutions per minute (rpm) at room temperature for 30 min and then serially diluted. Aliquots of the appropriate dilution were spread plated on Pikovskaya medium (Pikovskaya, 1948). The plates were incubated for 5 days at 30°C.

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Table 1. Summary of the isolation sites and abundance of PSB in lentil rhizosphere soils.

Name of isolation site	pH of isolation soil	Number of PSB isolates obtained	Designation of PSB isolates
Adanaba (T)	7.03	1	PSBL29
Ambo (O)	6.75	1	PSBL49
Asgori (O)	6.20	2	PSBL05,12
Asketema (A)	7.04	1	PSBL100
Chatawa (A)	5.12	1	PSBL116
Chole I (O)	5.58	1	PSBL54
Chole II (O)	6.05	1	PSBL52
Debra Selam (A)	6.00	1	PSBL57
Dagam (O)	6.36	1	PSBL94
Fala'a (T)	7.00	1	PSBL28
Fiche (O)	6.83	1	PSBL33
Fogera meda (A)	6.00	2	PSBL95,96
Geshana (A)	5.80	1	PSBL21
Ginchi (O)	6.60	1	PSBL120
Goha Tsion (O)	6.3	1	PSBL39
Gurura (O)	7.01	3	PSBL51,53,113
Keyit (A)	6.38	2	PSBL90,92
Korem (T)	6.93	1	PSBL28
Makesagnit (A)	6.39	1	PSBL97
Obbi (O)	6.61	1	PSBL31
Sandafa (O)	6.00	1	PSBL58
Sheno I (O)	5.58	2	PSBL26, 45
Sheno II (O)	5.58	1	PSBL20
Tefki (O)	6.55	6	PSBL02, 04,14,19,37,50
Teji (O)	6.70	1	PSBL101
Tikana (A)	7.18	1	PSBL93
Wajel (A)	6.12	1	PSBL55
Woliso (O)	5.58	1	PSBL13
Wuchale (A)	6.58	1	PSBL99
Yetinora (O)	6.21	1	PSBL112

Numbers in the same column followed by the same superscript letter do not differ significantly ($p>0.05$) by DMRT; ND: not determined; A: Amahara regional state; O: Oromia regional state; T: Tigray regional state

Colonies surrounded with a large halo zone and different morphologies were selected and transferred to Pikovskaya (PK) broth separately. The test tubes were incubated at 30°C for 48 h. The isolates were purified by re-streaking on PK agar plates and maintained on PK agar slants for short term storage at 4°C and in 50% (v/v) glycerol for long term storage at -20°C. Isolates were designated as PSBL (Phosphate Solubilizing Bacteria from Lentil) 01- PSBL120 (Table 1).

Characterization and identification of the PSB Isolates

Biochemical characterization and identification by the API kits system

All isolates were characterized using the following tests: Gram reaction using Gregorson's KOH (Gregorson, 1978), oxidase (Kovacs, 1956), Oxidative/Fermentative (O/F) (Huge and Leifson, 1953). Based on the result of these tests isolates were assigned to different groups and identified to genus and/or species level using the API identification system assisted by API Plus computer software (bioMèrieux-SA, France) following manufacturer instructtions. Gram positive rods were further identified to species level using API 50CH test kits. Gram negative rods and oxidase negative bacteria with fermentative reaction in the O/F test were identified using the API 20E test kits. Gram negative rods with oxidative reaction in O/F test were identified by using API 20NE test kits. Moreover, isolates were selected for 16S rDNA sequence analysis based on their phosphate solubilization activity, abundance and the result of API identification system.

Identification by 16S rDNA sequencing

Genomic DNA extraction, PCR amplification of 16S rDNA and sequencing: All isolates were streaked on LB agar media and incubated for 24 h at 30°C. A single colony of each isolate was suspended in 30 µl sterile H_2O. The DNA from Gram negative

bacteria was extracted using thermal denaturation method (Mohran et al., 1998). All tubes that contain colony suspensions were incubated at 95°C for 10 min. The DNA from Gram positive isolates was extracted by heat thawing at 65°C for 3 minutes followed by freezing at -70°C for 3 min; the cycles were repeated three times. All the tubes were centrifuged at 13,000 *g* for 2 min and stored at 4°C.

Two microliters of extracted DNA was used as a template for PCR amplification using a thermal cycler. In addition to the DNA, the PCR reaction consisted of 0.4 µl 10 mM dNTP, 2.5 µl 10x PCR(Taq) buffer, 2.5 µl 25 mM $MgCl_2$, 0.2 µl (5 units/µl) of Taq polymerase, 1 µl (10 mM) of forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1 µl (10 mM) of reverse primer1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR program included a denaturation step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 1.30 min, and an extension step of 10 min at 72°C. Amplified PCR products (1500 bp) were resolved on 1.0% agarose gel and stained with ethidium bromide (1 µg/ml). All amplified PCR products were sequenced with forward primer 27f by using Sanger sequencer (ABI-3730XL) at the Institute of Clinical and Molecular Biology, University of Kiel. The DNA sequences 850 bp were edited using Bioedit (www.mbio.ncsu.edu/BioEdit/) and analyzed by BLAST search (www.ncbi.nlm.nih.gov).

Phylogenetic analyses: A phylogenetic tree was constructed by using the neighbor joining method from distance matrices (Saitou and Nei, 1987). Bootstrap values (percentage of 500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Growth media and bacterial inoculum preparation

Bacterial isolates were grown in PK broth on a gyratory shaker at room temperature and 120 rpm for 72 h. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 g for 10 min. The pellets were re-suspended in normal saline solution to give a final concentration of 10^8 cells ml^{-1} using the viable plate count method and optical density measurement at 600 nm. All bacterial inoculums were prepared like this unless otherwise stated. The pH of all growth media was adjusted to 7.0 if not stated.

Determination of phosphate solubilization efficiency of the PSB isolates

Phosphate solubilization efficiency of the PSB isolates was evaluated both qualitatively and quantitatively. PK agar plates were spot inoculated with 20 µl of culture suspension of each isolate. After incubation at 30°C for 5 days formation of a clear zone around the spot was checked and their solubilization index (SI) was calculated as described in Muleta et al. (2013).

Based on their phosphate solubilizing activities and abundance of a particular group, isolates were selected and used for quantitative estimations of solubilization efficiency in PK broth. Hundred ml of PK liquid medium without phosphorus sources was dispensed in a flask (250 ml) and supplemented with the following insoluble phosphate sources which contained equivalent amounts of P: $Ca_3(PO_4)_2$ (TCP) (500 mg); Egyptian rock phosphate (RP) (600 mg) and bone meal (BM) (400 mg) were added separately as a phosphorus source. All flasks were autoclaved and inoculated with 100 µl culture suspension of each isolate separately. The flasks were incubated at room temperature on a gyratory shaker at 120 rpm for 12 days. Five ml of samples were withdrawn from each treatment on day 4, 8, and 12 and analyzed for phosphorus solubilized and pH changes. The sample was centrifuged at 15,000 g for 15 min and the clear supernatant was analyzed for P content following the phospho-molybdate method (Murphy and Riley, 1962).

Screening for *in vitro* antagonistic activity against *Fusarium oxysporum*

The *in vitro Fusarium oxysporumf.*sp. *lentis* (FOL) growth inhibition of the isolates was tested using the dual culture technique (Landa et al., 1997). Twenty microliters of culture suspension of each isolate was equidistantly spotted on the margins of nutrient agar (NA) plates amended with glucose (1.0%) and incubated at 30°C for 24 h. A 4-5 mm diameter agar disc from potato dextrose agar (PDA) cultures of the fungal pathogens was placed at the centre of the NA plate inoculated with the bacterial isolate and incubated at 30°C for 5-7 days. The radii of the fungal colonies towards and away from the bacterial colonies were measured and the percentage of mycelia growth inhibition was calculated according to Idris et al. (2008).

Assay for Indole-3-acetic acid (IAA) production

Indole-3-acetic acid production was detected by modified method as described by Brick et al. (1991). Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986). Cultures of the isolates were grown at 30°C in LB broth medium supplemented with 5 mM L-tryptophan for 48 h. The cultures were centrifuged (10,000 *g* for 15 min) and 2 ml supernatant was transferred to test tubes to which 100 µl of O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% $HClO_4$) were added. The mixtures were incubated at room temperature for 25 min. Quantification of the IAA was carried out using spectrometric analysis at 530 nm.

Effects of inoculation of PSB isolates on the growth of lentil under greenhouse conditions

Bacterial isolates that showed high phosphate solubilization efficiency in PK broth were selected for their *in vivo* growth promotion evaluation under greenhouse conditions. Lentil seeds were surface sterilized, germinated and transplanted as described before. Prior to seedling transplantation, germinated seedlings were flooded with PSB culture grown in PK broth adjusted to 10^8 cells $seed^{-1}$ for one hour on separate sterilized Petri plates. Five inoculated seedlings were transferred to each pot containing 3 kg vertisol collected from Dima Guranda, around Sebeta. This soil has the following physicochemical characteristics: pH; 6.0, total N; 0.025%, organic carbon; 0.975, total P; 1667 ppm, available P; 2.99 ppm, available K; 25.33 ppm, Cation Exchange Capacity; 50 Cmol/Kg and loamy texture.

The seedlings were thinned down to three 5 DAE. The pots were irrigated with distilled sterilized water every three days. Uninoculated P-fertilized (20 mg Kg^{-1} as KH_2PO_4) pots were included as positive controls (PC) and uninoculated non P-fertilized pots as negative controls (NC). At sixty DAP all seedlings were carefully uprooted and washed under gently flowing tap water. The numbers of nodules found on the roots were recorded, and shoot dry weight was measured after drying at 70°C for 48 h. Total N of the shoot was determined using Kjeldahl methodas described in Sertsu and Bekele (2000). The P concentration of the shoot was determined by phospho-molybdate method (Murphy and Riley, 1962) after ashing 0.5 g of oven dried and ground shoot dry matter.

Data analysis

Statistical analysis of the data was carried out using analysis of variance (ANOVA) and the treatment means were compared relative to control following Duncan's multiple range test (DMRT) (Duncan, 1955). The relation between different parameters was evaluated by using the Pearson correlation coefficient using SPSS v.15 (SPSS Inc.2000).

RESULTS AND DISCUSSION

Isolation and Identification of PSB Isolates

Thirty soil samples were collected from different lentil producing areas found in central and northern parts of the country which represent major producing areas (Table 1). A total of 41 PSB isolates with higher phosphate solubilization activities (SI ≥1.34) were selected from the soil samples for further analysis (Table 1). The biochemical analysis revealed the presence of a wide array of Gram negative and Gram positive PSB in the rhizosphere of lentil. Gram negative rhizobacteria dominated the system accounting for 90% of isolates. Previous observation showed that the rhizosphere of many agriculturally important plants favor more Gram negative rhizobacteria than the Gram positives (Muleta et al., 2009). The results revealed the presence of 7 major genera: *Pseudomonas* (47.5%), *Burkholderia* (12.5%), *Bacillus* (10%), *Enterobacter* (10%), *Chrysomonas* (5%), *Sphingomonas* (2.5%) and *Agrobacterium* (2.5%). Members of the genus *Pseudomonas* were dominated by *Pseudomonas fluorescens* while all isolates of the genus *Burkholderia* were identified as *B. cepacia*. However, 12.5% them were left unidentified or showed low identification (<90%). This might be due to the limitations of using biochemical characteristics to identify the environmental isolates.

The partial 16S rDNA sequence analysis was also employed to identify the isolates to the genus and species level. The result showed the presence of genera such as, *Pseudomonas, Bacillus, Enterobacter, Acinetobacter, Ralstonia, Burkholderia and Agrobacterium.* Gram negative strains exhibited more diverse species compared to the Gram positive isolates. There were some disagreements between API kit identification and 16S rDNA gene sequence analysis which could be due to several reasons such as inoculum size and culture conditions. Moreover, genera *Ralstonia* and *Acientobacter* were identified only by sequences analysis indicating that this method is more powerful in identifying the environmental isolates.

Of the Gram negative strains three of them showed the highest similarity to different species of genus *Enterobacter*. The sequence analysis revealed that PSBL04 and PSBL92 were highly similar (100% sequence similarity) to *Enterobacter ludwigii* (FG859683), phosphate solubilizing bacteria isolated from tea rhizosphere and *Enterobacter cloacae* (GQ421477), antagonists of *Aspergillus flavus* from fermented beans, respectively while PSBL05 showed 99% identity similarity with *Enterobacter kobei* (NR 028993) (Table 2).

Two strains showed the closest match with species of *Pseudomonas*. PSBL57 showed 100% sequence similarity to *Pseudomonas costantinii* (EF111123) bacteria isolated from river while PSBL58 showed 99% sequence similarity with *Pseudomonas corrugate* (HQ242748) phosphate solubilizing bacteria from P-rich soil. According to API test kits identification of members of the *Pseudomonas* were dominated by *P. fluorescens* which was also confirmed by 16S rDNA sequences analysis since PSBL57 and PSBL58 showed good similarity with fluorescent pseudomonad species (*P. fluorescens (100%)* and *P. putida* (99%)).

Strain PSBL45 showed 100% sequence similarity with *Burkholderia cepacia* (JN208904) N-fixing symbionts of common bean and *P. fluorescens*, (HE610859) a drought resistance-promoting bacterium. Strain PSBL19 showed the highest (99%) sequence similarity to *Agrobacterium tumefaciens* (AB 681363) isolated from soil. PSBL39 showed the highest (99%) similarity to *Acinetobacter calcoaceticus* (JQ579640) phosphate solubilizing bacteria in paddy fields and endophytic bacteria in peanut, respectively.

Three members of Gram positive strains showed closest similarity to different species of genus *Bacillus*. PSBL12 showed 99% similarity with *Bacillus tequilensis* (JF 411313), bacteria isolated from disused river while PSBL21 and PSBL49 showed 99 and 100% sequence similarity, respectively with *Bacillus subtilis* (JN230358) bacterial endophytes from *Nicotiana attenuta*. The partial sequences of 16S rDNA genes of the isolates were multiple aligned with the nearest neighbors and other relevant bacterial sequences, and their identity and evolutionary relation was assessed by constructing phylogenetic tree (Figure 1). The phylogenetic tree showed the clustering of PSBL isolates with their respective genus with good bootstrap support values.

Phosphate solubilizing efficiency of the isolates on agar plates

The SI of each PSB isolate is presented in Table 2. Phosphate solubilizing activities of these isolates were assessed qualitatively on PK agar medium using SI as measure of their efficiency. The result showed a statistically significant variation among the isolates. The SI of the isolates ranged from 1.34 to 2.25 which were noted for isolate *Pseudomonas* sp. (PSBL101) and *A. tumefaciens* (PSBL19), respectively. This result indicates that *A. tumefaciens* (PSBL19) was the most efficient in phosphate solubilization on PK plates. Though it is not statistically significant, the result showed that there was positive correlation (r=0.5) between SI and the amount of soluble P released by the isolates in PK broth. Consequently, we should not rely only on SI to select efficient PSB isolates.

Table 2. Solubilization index (SI), identification and PGP characteristics of PSB isolated from lentil rhizosphere.

Isolate	Gram reaction	Solubilization Index (SI)±SD	O/F test	Oxidase	API identification	16S rDNA sequences identification
PSBL02	-	1.77±0.06^{e-h}	F	+	*Aeromonas hydrophila*	ND
PSBL04	-	1.82±0.08^{e-h}	F	-	*Pseudomonas fluorescens*	*Enterobacter ludwigii* (JX979114)
PSBL05	-	1.95±0.22^{c-e}	F	-	UI	*Enterobacter kobei* (JX979115)
PSBL12		1.95±0.21^{c-e}	O	+	*Bacillus sp.*	*Bacillus tequilensis* (JX979116)
PSBL13	+	1.86±0.10^{d-h}	O	+	*Bacillus sp.*	ND
PSBL14	-	1.79±0.07^{e-h}	F	+	*Aeromonas hydrophila*	ND
PSBL19	-	2.25±0.13^{a}	O	+	UI	*Agrobacterium tumefaciens* (JX979117)
PSBL20	-	1.87±0.13d^{e-h}	O	+	*Chryseomonas sp*	ND
PSBL21	+	1.88±0.05d^{e-g}	O	+	*Bacillus sp.*	*Bacillus subtilis* (JX979118)
PSBL26	-	1. 90±0.09^{de}	F	+	*Aeromonas hydrophila*	ND
PSBL28	-	1.85±0.09de^{f-h}	F	+	*Aeromonas hydrophila*	ND
PSBL29	-	1.81±0.06^{e-h}	O	+	*Burkholderia cepacia*	ND
PSBL30	-	1.80±0.07^{e-h}	F		*Chryseomonas luteola*	ND
PSBL31	+	1.88±0.08^{d-g}	O	+	*Bacillus sp.*	ND
PSBL33	-	1.84±0.03d^{e-h}	F	+	*Pseudomonas fluorescens*	ND
PSBL37	-	1.90±0.08^{de}	O	+	*Pseudomonas fluorescens*	ND
PSBL39	-	1.87±0.10^{d-h}	O	+	*UI*	*Acinetobacter calcoaceticus* (JX979119)
PSBL45	-	1.82±0.08^{e-h}	O	+	*Pseudomonas fluorescens*	*Pseudomonas koreensis* (JX979120)
PSBL49	+	2.21±0.10^{a}	O	+	*Bacillus sp.*	*Bacillus subtilis* (JX979121)
PSBL50	+	1.64±0.13^{g-i}	O	+	*Pseudomonas putida*	ND
PSBL51	-	1.87±0.17^{d-h}	O	+	*Sphingomonas paucimobils*	ND
PSBL52	-	1.86±0.20^{d-h}	O	+	*Pseudomonas sp.*	ND
PSBL53	-	1.60±0.15^{i}	O	+	*Pseudomonas fluorescens*	ND
PSBL54	-	1.65±0.14^{f-i}	O	+	*Pseudomonas sp.*	ND
PSBL55	-	1.45±0.18^{ij}	O	+	*Pseudomonas fluorescens*	ND
PSBL57	-	2.16±0.9^{a-c}	O		*Pseudomonas fluorescens*	*Pseudomonas costantinii* (JX979126)
PSBL58	-	2.13±0.12^{a-c}	O	+	*Pseudomonas fluorescens*	*Pseudomonas corrugate* (JX979127)
PSBL90	-	2.17±0.15^{a-c}	O	+	*Burkholderia cepacia*	ND
PSBL92	-	2.17±0.14^{a-c}	F	-	UI	*Enterobacter cloacae* (JX979128)
PSBL93	-	2.07±0.08^{a-d}	O	+	UI	*Ralstonia picketti* (JX979122)
PSBL94	-	2.16±0.06^{a-c}	O	+	*Pseudomonas sp.*	ND
PSBL95	-	2.14±0.15^{a-c}	O	+	*Pseudomonas sp.*	ND
PSBL96	-	2.14±0.15^{a-c}	O	+	*Burkholderia cepacia*	ND
PSBL97	-	1.86±0.11^{d-h}	O	+	*Pseudomonas sp.*	ND
PSBL99	-	1.88±0.10^{d-g}	O	+	*Burkholderia cepacia*	ND
PSBL100	-	2.18±0.12^{ab}	O	+	*Pseudomonas fluorescens*	ND
PSBL101	-	1.34±0.12^{j}	O	+	*Pseudomonas sp.*	ND
PSBL112	-	1.84±0.08^{d-h}	O	+	*Burkholderia cepacia*	ND
PSBL113	-	1.98±0.13^{b-e}	O	+	*Pseudomonas sp.*	ND
PSBL116	-	2.13±0.17^{a-c}	O	+	*Agrobacterium radiobacter*	ND
PSBL120	-	1.84±0.08^{d-h}	O	+	*Pseudomonas sp.*	ND

Numbers in the same column followed by the same superscript letter do not differ significantly (p> 0.05) by DMRT; **+**: the character present/gram positive; -: the character absent/gram negative; SD: standard deviation; FOL: *Fusarium oxysporum f.sp. lentis*; UI: unidentified; ND: not determined, O:Oxidative; F:Fermentative; parenthesis: accession number of the sequence in NCBI gene bank

Different insoluble P sources solubilization efficiency of the PSB isolates

The solubilization efficiencies of selected isolates were estimated in PK broth using TCP, RP and BM as insoluble P sources. Nine isolates were selected for this experiment based on their SI and abundance. Most tested isolates showed excellent TCP solubilization ability as they released considerable amounts of soluble phosphates in the medium until the last sampling day (Table 3). The amount of soluble P released was found to vary significantly among isolates and sampling days. After 12 days of incubation the highest amount of Preleased from TCP was 674 µg ml^{-1}. This indicates that *Enterobacter kobei* (PSBL5) is the most efficient in TCP solubilization in culture medium. On the other hand

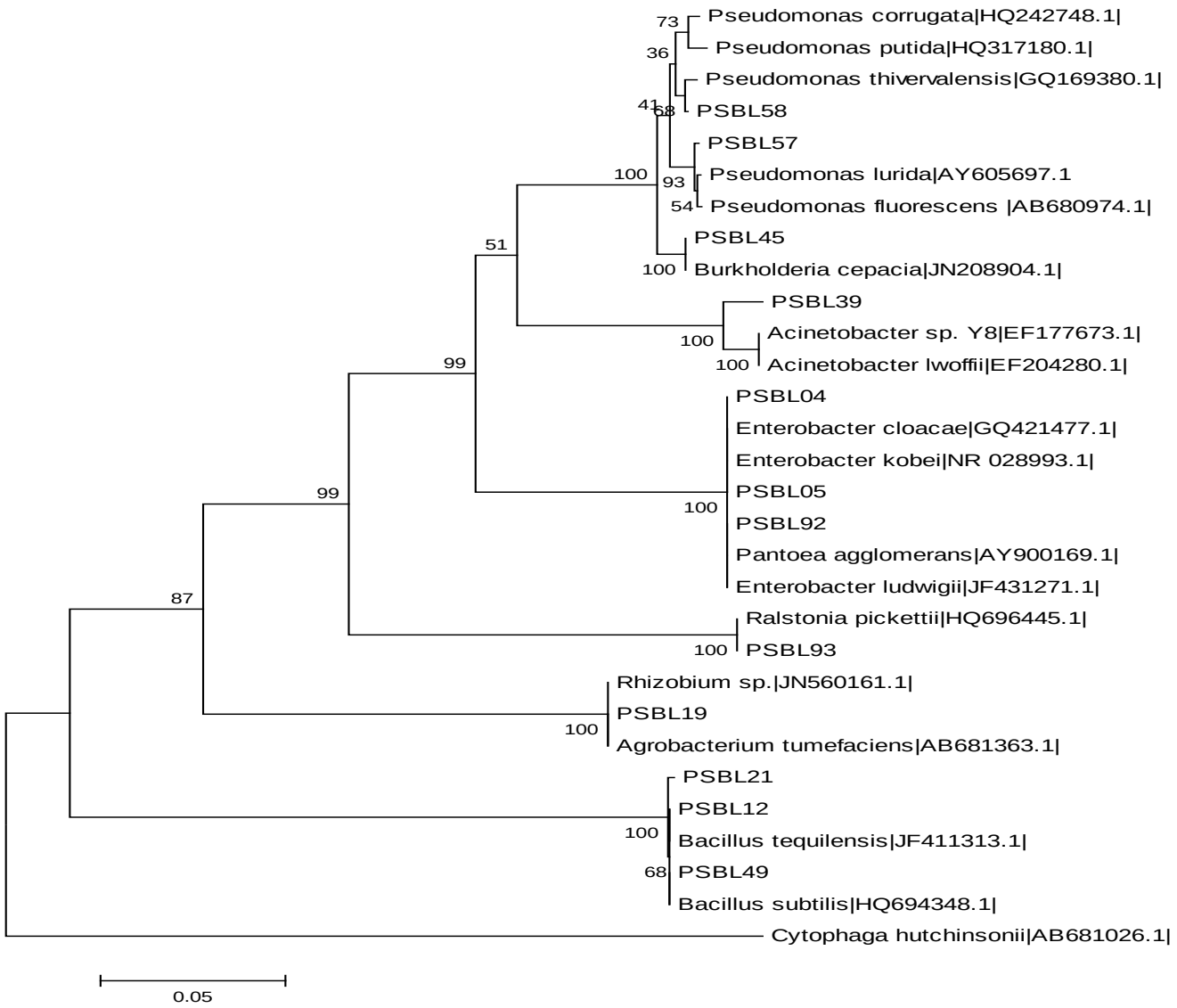

Figure 1. Neighbour-Joining phylogenetic dendrogram of 16S rRNA gene sequence showing the positions of different PSBL strains. Bootstrap values are shown at branching point. Cytophaga sequence is used as out group.

Pseudomonas fluorescens (PSBL33) was found to be poor in solubilization of TCP as it released only 52 µg ml^{-1} of P after 12 days of incubation. Generally, the amount of released P was found to increase progressively until the last sampling day for most isolates. However, it was also found to show a slight decline for *Enterobacter* species (PSBL28) and *P. fluorescens* (PSBL33) after the 8^{th} day of incubation while it was stabilized for *Pseudomonas koreensis* (PSBL45).

During the process of TCP solubilization all isolates showed a sharp drop in pH of the culture medium from 7.0 to 5.4-5.8 (Table 3). The lowest pH was recorded for isolate *P. koreensis* (PSBL45) that is, 5.4 while the highest was recorded for isolate *Enterobacter* species (PSBL26) which was 5.8 on 4^{th} day of incubation. After a sharp decline on day 4, a further decrease in pH was noted only for isolate *Bacillus* sp. (PSBL31) while all the others showed a slight increment. The correlation analysis showed that there is a statistically significant ($p \leq 0.05$) inverse relation between the amount of P released and pH of the broth ($r = -0.82$) indicating that acid production might be the main mechanism of TCP solubilization.

Similarly, the rock phosphate solubilization capacity of the isolates was investigated in PK broth and the isolates showed good solubilization ability of rock phosphate. The amount of soluble phosphorus released and the corresponding change in pH of the medium were varied among isolates and sampling dates (Table 3). The highest amount of soluble P was 78 µg ml^{-1} which was

Table 3. Different insoluble phosphate sources solubilization efficiencies PSB isolates obtained from lentil rhizosphere.

Isolates	Tricalcium phosphate						Rock phosphate						Bone phosphate					
	4th day		8th day		12th day		4th day		8th day		12thday		4th day		8th day		12thday	
	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})
PSBL04	5.6	378^{c}	5.6	483^{cd}	5.7	575^{c}	3.4	9^{f}	3.7	10^{g}	3.7	57^{b}	4.6	16^{g}	4.3	20^{f}	4.4	20^{e}
PSBL05	5.6	378^{c}	5.6	609^{a}	5.5	674^{a}	4.4	46^{d}	5.4	75^{a}	5.7	78^{a}	6.0	239^{b}	6.1	245^{b}	6.1	286^{a}
PSBL21	5.5	375^{c}	5.5	415^{e}	5.5	505^{d}	3.6	49^{c}	3.9	52^{d}	4.7	47^{e}	6.2	187^{d}	6.4	184^{c}	6.4	194^{b}
PSBL26	5.8	62^{d}	5.8	64^{f}	6.0	616^{b}	3.8	7^{f}	4.0	44^{e}	4.0	47^{e}	4.5	30^{f}	4.3	64^{e}	4.3	63^{d}
PSBL28	5.4	410^{ab}	5.4	529^{b}	5.4	492^{ef}	3.5	63^{a}	3.7	63^{b}	3.7	63^{b}	6.4	273^{a}	6.0	276^{a}	5.9	280^{a}
PSBL31	5.4	408^{ab}	5.3	474^{d}	5.2	670^{a}	5.2	44^{d}	5.6	61^{c}	5.0	65^{b}	5.3	201^{c}	5.2	248^{b}	5.3	297^{a}
PSBL33	5.4	52^{d}	5.5	49^{f}	5.8	52^{g}	3.7	60^{b}	3.8	60^{c}	3.9	62^{c}	6.4	204^{c}	6.3	180^{c}	6.4	187^{b}
PSBL37	5.5	380^{c}	5.5	401^{e}	5.5	478^{f}	5.5	41^{e}	5.6	39^{f}	5.6	38^{f}	6.2	191^{d}	6.1	180^{c}	6.1	187^{b}
PSBL45	5.4	398^{b}	5.5	491^{c}	5.6	495d^{e}	4.3	62^{a}	4.9	61^{c}	4.7	63^{bc}	5.9	126^{e}	5.9	143^{d}	5.9	150^{c}

Numbers in the same column followed by the same superscript letter do not differ significantly (p>0.05) by DMRT test.

released by *E. kobei* (PSBL5) while the least was 38 µg ml^{-1} which was recorded for *Pseudomonas fluorescens* (PSBL37) on the 12th days of incubation. Generally, the amount of soluble P in culture medium increased gradually up to the 12th days of incubation for most isolates except *Bacillus subtilis* (PSBL21) and *P. fluorescens* (PSBL37) which showed a slight decrease. Compared to other insoluble P sources the lowest amount of soluble P was obtained from rock phosphate dissolution by all tested isolates.

During the course of rock phosphate solubilization a sharp drop in pH of the growth medium was observed. The pH was varied from 3.4-5.2 which was noted for *Enterobacter ludwigii* (PSBL4) and *Bacillus* sp. (PSBL31), respectively. Compared to TCP and BM the lowest pH was recorded during the solubilization of rock phosphate. However, this low pH was not accompanied by a higher amount of soluble P as the lowest amount of it was obtained from rock phosphate compared to other insoluble P sources. Although statistically not significant the correlation analysis showed that there is an inverse relationship (r= -0.34) between the amount P released and the pH of the growth medium suggesting acid production as the mechanism of rock phosphate solubilization.

The amount of soluble phosphorus released from old bone meal and corresponding drop in pH of the culture medium by the PSB isolates is shown in (Table 3). All tested isolates showed BM solubilization ability with different level of efficiencies. There was a significant variation among isolates in terms of P solubilization and associated change in pH of the culture medium. Isolate *Bacillus* sp. (PSBL31) was comparatively more efficient in solubilizing BM than all other PSBL isolates, followed by *E. kobei* (PSBL5) and *Enterobacter* species (PSBL28) with 297, 286 and 280 µg ml^{-1} soluble P in culture medium, respectively. Isolate *E. ludwigii* (PSBL4) and *Enterobacter* species (PSBL26) were found to be the least efficient in solubilizing BM with 20 and 63 µg ml^{-1}soluble P, respectively. The amount of soluble P was found to increase progressively for all isolates except, PSBL37 and *P. fluorescens* (PSBL33) which showed a slight decrease after the fourth day.

Similar to other insoluble P sources a sharp drop in pH was observed during the course of BM solubilization by all isolates. On the fourth day of incubation drops in pH of the culture medium range from 4.5 to 6.4 which were recorded for isolates *Enterobacter* species (PSBL26) and *Enterobacter* species (PSBL28), respectively. The pH of the medium was found to decrease steadily until 12th day except for some isolates such as *B. subtilis* (PSBL21), *Enterobacter kobei* (PSBL5), *P. fluorescens* (PSBL33) and *P. koreensis* (PSBL45), which showed a slight increment or stability. Though it is not statistically significant there was positive correlation (r= 0.27) between soluble P and pH of the culture medium. This suggests that mechanisms other than acid production were involved in the dissolution of BM and hence further study on the solubilization mechanisms of BM is recommended. All PSB isolates were able to release significantly higher

amounts of soluble P from different insoluble phosphate sources into the culture medium. In general, the highest amount of soluble P was released from TCP followed by BM after 12 days of incubation. The lowest dissolution and sharpest drop in pH was obtained during solubilization of RP. Likewise, previous studies (Rodriguez and Fraga, 1999; Keneni et al., 2010) showed that phosphate solubilizing microorganisms release more soluble P from insoluble inorganic salts than naturally occurring phosphate sources such as rocks and bone meal that consists apatite.

All isolates displayed a significant drop in pH of the culture medium during the solubilization of all tested insoluble phosphate sources. In all cases phosphate solubilization was found to increase with the decreasing pH of the growth medium. Several studies have indicated that the drop in pH is due to the production of organic acids during bacterial growth (Chen et al., 2006; Ivanova et al., 2006; Muleta et al., 2013; Vyas and Gulati, 2009). The decrease in pH of the culture medium associated with the production of organic acids resulted in a considerable amount of P solubilization (Chen et al., 2006). An inverse relationship was established between the drop in pH of the culture medium and the amount of soluble P during solubilization of rock phosphate and TCP. These results are consistent with the report of earlier studies (Rodriguez Fraga, 1999; Naik et al., 2008; Vyas and Gulati, 2009) which showed that solubilization of insoluble phosphate sources were mediated particularly by the decreasing pH of the medium. Several studies showed that mineral phosphate solubilization by PSB isolates is through the release of low molecular weight organic acids (Goldstein, 1995; Kim et al., 1997; Vyas and Gulati, 2009). These organic acids chelate the cations bound to the insoluble phosphate through their hydroxyl or carboxyl groups, thereby converting it into soluble forms (Kim et al., 1997).

The fluctuations and differences in insoluble P sources solubilization efficiencies observed among isolates could be attributed to differences among test isolates in the amount of immobilized P (Muleta et al., 2013). When cells in the culture immobilize phosphate for microbial biomass production, the corresponding values decrease, creating such fluctuations (Muleta et al., 2013). Our observation, a decrease in soluble P and concomitant increase in the pH values of the growth medium indicates re-utilization of available P (Rashid et al., 2004; Tripura et al., 2007). In such cases the organic acids excreted to the growth medium could be reused by the isolates for their own metabolism (Tripura et al., 2007). This could be due to an exhaustion of the original carbon source (Rashid et al., 2004).

Our results indicate that PSB isolates native to Ethiopian soils have good rock phosphate solubilization ability. The dissolution of rock phosphate to a significant extent by these indigenous PSB isolates would give the opportunity to utilize rock phosphate deposits found in different parts of the country (Assefa 1991). In Ethiopia, studies (Bekele and Hofner, 1993) have indicated successful use of rock phosphate under greenhouse conditions. A study conducted elsewhere indicated that 50% of the costly superphosphate could be replaced by rock phosphate; a cheap source of P, when applied in conjunction PSB inoculants (Sundara et al., 2002). Thus, inoculation of native PSB isolates with superior rock phosphate solubilization ability could improve the productivity of lentil and other crops. Our results demonstrated that some PSB isolates released a considerable amount of P from insoluble animal bone; a rich source of P. Previous study (Postma et al., 2010) indicated that PSB strains showed excellent solubilization abilities of animal bone, a source of P that can be recycled. Furthermore, the superior BM solubilization of these isolates would also help to use the problematic waste accumulated around the abattoirs found in different urban areas of the country as P-fertilizer along with PSB inoculants. These could reduce our dependence on the very expensive chemical fertilizers.

Multiple PGP characteristics of PSB isolates from lentil rhizosphere

All PSB isolates obtained from lentil rhizosphere were screened for multiple PGP characteristics such as IAA production and inhibition of *F. oxysporum* growth under *in vitro* conditions. In addition to their superior phosphate solubilization activity, PSB isolates exhibited different PGP properties (Table 4). Dual culture assay indicated that PSBL31 and PSBL53 inhibited the growth of *F. oxysporum* with 41 and 32% of mycelia growth inhibition (Table 4). These isolates were found to be member of the genera *Bacillus* and *Pseudomonas*. Similarly, studies showed that PSB isolates obtained from different agriculturally important crops rhizosphere were found to be antagonistic to different fungal pathogens such as *Fusarium* species, *Phytium* species, *Rhizoctonia* species and others under *in vitro* conditions (Peix et al., 2001b; Naik et al., 2008). In Ethiopia lentil production has been constrained by wilt and root rot caused by different fungal plant pathogens (Ahmed and Ayalew, 2006). Of these wilt disease caused by *Fusarium oxysporum f*. sp. *lentis* has been responsible for major yield loss (Ahmed and Ayalew, 2006) and thus PSB isolates which inhibited the growth of this pathogen would give dual advantage as biofertilizer and biocontrol agents. Consequently, such isolates are highly recommended for greenhouse and field experiments.

In addition, 27.5% of the PSB isolates were found to be capable of producing IAA (Table 4). Quantitative analysis indicated that the highest amount of IAA produced was 48 µg ml^{-1} while the lowest was 14 µg ml^{-1} which was recorded for isolates PSBL19 and PSBL96, respectively (Table 4). The greatest number of IAA producer were

Table 4. Multiple PGP characteristics of PSB isolated from lentil rhizosphere.

Isolate	IAA (µg ml^{-1} ± SD)	*In vitro* inhibition of FoL growth (%)± SD
PSBL04	18.2±2.0^{cd}	-
PSBL19	47.5±1.6^{a}	-
PSBL21	16.7±2.5^{de}	-
PSBL28	14.7±1.5^{e}	-
PSBL31	-	41±2.5^{a}
PSBL33	-	-
PSBL37	17.9±1.9^{cd}	-
PSBL52	19.6±1.5^{c}	-
PSBL53	15.5±1.2^{e}	32±1.7^{b}
PSBL57	-	
PSBL92	20.7±1.2^{bc}	-
PSBL93	21.6 ±2.1^{bc}	-
PSBL96	14.3±0.7^{e}	-
PSBL97	14.7±0.3^{e}	-

Numbers in the same column followed by the same superscript letter do not differ significantly (p> 0.05) by DMRT; **+**: the character present/ -: the character absent, SD: standard deviation; FOL: *Fusarium oxysporum f.sp. lentis*

from the Gram negative isolates (90%) belonging to genera *Enterobacter, Pseudomonas, Agrobacterium, Ralstonia* and *Burkholderia*. The most capable strains belonged to the genera *Agrobacterium* and *Ralstonia.* Similarly, several previous studies demonstrated that PSB isolates obtained from the rhizosphere of different crops were able to exhibit different levels of IAA production (Naik et al., 2008; Shahab et al., 2009). These studies indicated that most of the studied isolates produced considerable amounts of IAA (Shahab et al., 2009). Such isolates were found to be excellent in promoting plant growth under greenhouse conditions (Shahab et al., 2009). The findings of the present investigation showed that IAA producing PSB isolates could be easily isolated from local soil and might be exploited as multipurpose inoculants after greenhouse and field tests.

Response of Lentil to inoculation PSB with different P sources

The effects of inoculation of selected PSB isolates on lentil growth, nodulation, and N and P content of shoot were evaluated under greenhouse conditions. During this experiment *Enterobacter kobei* (PSBL5), *Bacillus subtilis* (PSBL21), *Enterobacter* species (PSBL26), *Bacillus sp.* (PSBL31) and *Pseudomonas fluorescens* (PSBL37) were used as inoculants. The results showed that shoot dry matter yield of lentil was improved by inoculation of PSB isolates over the uninoculated plants (NC) control (Table 5). Their effect was comparable to P-fertilizer application. In addition to their phosphate solubilization two isolates (PSBL21 and PSBL37) were found to be IAA producers. However, further studies using IAA mutant strains of these isolates are needed to determine the exact contribution of IAA production in the promotion of plant growth when isolates of multiple PGP attributes are used as inoculants.

All PSB isolates except *Enterobacter* species (PSBL26) increased the number of nodules at a statistically significant level (*p≤0.05*) over the NC. This improvement was found to be comparable to the P-fertilized plant. The numbers of nodules were also found to vary considerably among the isolates. The highest number of nodules was induced by *E. kobei* (PSBL5) while the least was by *Enterobacter* species (PSBL26). These isolates increased nodule number by 43.9 and 38.2% over the NC, respectively. It is interesting to note that *E. kobei* (PSBL5) was superior to P-fertilization in terms nodulation induction. The result of our study indicated that the increase in nodule number is positively correlated with the increase in shoots dry weight (r=0.72).

All PSB isolates used in this experiment increased the concentration of N in the shoot of lentil significantly (*p≤0.05*) over the NC (Table 5). The highest N concentration was observed in *Enterobacter kobei* (PSBL5) and *Bacillus* sp. (PSBL31) inoculated plants and a 36.4% increase was obtained over the NC. The variations in N concentration obtained among isolates were not statistically significant. Their effect on N concentration was found to be comparable to P-fertilization. The results of our study indicated that the N content of shoot is positively correlated with the nodule number (r=0.67) and

Table 5. Effects of inoculation of PSB different on growth of lentil under glasshouse conditions.

Treatments	Nodule number/plant ± SD	Shoot dry weight(g)/Plant ± SD	Total Nitrogen(%) of shoot ± SD	Phosphorous concentration in shoot(mg/g SDW) ± SD
PC	34.3±2.5^{ab}	1.03±0.06^{a}	3.27±0.16^{a}	12.1±0.1^{ab}
NC	28.0±3^{c}	0.87±0.05^{b}	2.50±0.11^{b}	8.2±0.1^{c}
PSBL5	40.3±3.5^{a}	0.98±0.02^{ab}	3.41±0.15^{a}	12.8±0.1^{a}
PSBL21	36.0±4.0^{ab}	0.99±0.03^{ab}	3.31±0.16^{a}	11.3±0.15^{ab}
PSBL26	33±4^{bc}	0.96±0.14^{ab}	3.32±0.13^{a}	10.7±0.25^{abc}
PSBL31	38.7±3^{ab}	0.95±0.04^{ab}	3.41±0.17^{a}	12.0±0.1^{ab}
PSBL37	34.7±2.1^{ab}	0.97±0.08^{ab}	3.14±0.14^{a}	10.0±0.1^{bc}

Numbers in the same column followed by the same letter do not differ significantly (p> 0.05) by DMRT; PC: positive control; NC: negative control; SD: standard deviation

shoot dry weight (r=0.53) of the plants. Peix et al. (2001b) have also observed similar results in plants inoculated with PSB strain of *Burkholdera cepacia.* It has been documented that phosphorus deficiency in soils has a deleterious effect on the symbiotic interaction between rhizobia and legumes such as nodulation, N_2-fixation, and hence growth and productivity (Gyaneshwar et al., 2002). Several studies have demonstrated that inoculation of seedlings with PSB isolates increased the level of available phosphorus in the rhizosphere soil (Sundara et al., 2002; Vyas and Gulati, 2009). In this way, inoculation of lentil with efficient PSB could improve growth, nodulation and thus the nitrogen-fixation of this crop.

Similarly, all PSB isolates improved the concentration of P in the shoot of lentil at statistically significant level over the NC. The highest concentration of P was obtained by *Enterobacter kobei* (PSBL5) isolate inoculation followed by *Bacillus sp.* (PSBL31). These isolates increased P concentration by 56.1 and 46.3%, over the NC control, respectively. The P content of the plant was positively correlated with the N content (r=0.61) and shoot dry weight (r=0.72) of the plant. Previous studies have demonstrated that inoculation of PSB isolates increased the available P in soil (Sundara et al., 2002; Vyas and Gulati, 2009) and concomitantly improved P uptake of the plant (Peix et al., 2001a; Peix et al., 2001b; Vyas and Gulati, 2009).

Conclusion

In general, from the results of the present study it is possible to conclude that rhizosphere soils of lentil from different producing areas of Ethiopia harbor a considerably high number of PSB isolates. They belong to different bacterial genera: *Pseudomonas, Bacillus, Agrobacterium, Enterobacter, Burkholderia, Ralstonia, Chryseomonas* and *Acinetobacter.* The PSB isolates were able to release a considerable amount of P from TCP, RP and BM. The present investigation revealed the diversity of PSB with superior phosphate solubilizing capacity, multiple plant growth promoting traits and biocontrol properties. The greenhouse experiment showed that inoculation of lentil with PSB has improved the nodule number, dry matter yield, N and P content of the plant significantly. Some of the isolates such as *Enterobacter kobei* (PSBL5) and *Bacillus* (PSBL31) are of particular interest for further research and need to be tested in the field in different agroecological zones, as they could be used as efficient biofertilizer in the lentil production system. The knowledge generated on diversity and PGP characteristics of phosphate solubilizing bacteria from rhizosphere soil would be useful to design strategies to use phosphate solubilizing bacterial inoculants technology in the crop production systems of the country.

Conflict of interest

Author did not declare any conflict of interest.

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Full Length Research Paper

Diversity and distribution of endophytic bacterial community in the Noni (*Morinda citrifolia* L.) plant

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Noni (*Morinda citrifolia* L.) is a plant used by traditional cultures and also in modern health care products. Various chemical substances are derived from the plant and include, but are not limited to anthraquinone flavonol glycosides, iridoid glycosides, lipids glycosides and triterpenoids. Also commonly found on the plant are endophytic bacteria however, there are no reports on endophytic bacterial community of Noni. We collected samples from five sites of Noni plant (roots, branches, leaves, fruits and seeds) and performed 16S rDNA analysis. Results show that these five parts harbor a highly similar bacterial composition with the top four being *Sphingomonas*, *Pseudomonas*, *Halomonas* and *Geobacillus*. *Sphingomonas* and *Pseudomonas* were found to be widely distributed in plant endophytic biotope; while there are little reports on plant-associated *Halomonas* and *Geobacillus,* indicating distribution in the plant hosts. Unknown genus also is abundant in five sites of Noni, ranging from 26.70 to 33.66%, implicating necessity to reveal them. This study provides information on endophytic bacteria in the Noni for future analysis based on a metagenome strategy.

Key words: Noni, endophytic bacteria, diversity, metagenome.

INTRODUCTION

Plants host an abundant microbial community in rhizosphere, phyllosphere and endosphere areas as previous research has reported and the noni plant microbiome has received significant attention in recent years (Lebeis et al., 2012; Turner and James, 2013; Bulgarelli et al., 2013; Berg et al., 2014). Microbes colonizing plant surfaces and interior areas are vital for plant health and productivity (Bonfante, 2010; Berendsen et al., 2012; Ferrara et al., 2012; Monteiro et al., 2012), but some of them could lead to disease development of plants (James and Olivares, 1998; Monteiro et al., 2012; Van Overbeek et al., 2014). Prior reports indicate that

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plant microbiome could be beneficial for human health through consumption of raw plants (Blaser et al., 2013; Van Overbeek et al., 2014). Therefore, understanding the microbial composition of plants may assist in the development of sustainable agriculture (Berg, 2009; Lugtenberg and Kamilova, 2009).

Several scientific teams documented the feasibility of promoting environment-friendly agriculture through manipulation of plant microbiome (Bloemberg and Lugtenberg, 2001; Philippot et al., 2009; Adesemoye et al., 2009; Singh et al., 2010; Bakker et al., 2012). Bloemberg et al. (2001) revealed that plant microbiome could reduce incidence of plant disease, and research conducted by Bakker et al. (2012) showed contribution of plant microbiome to agricultural production.

Plant microbiome also holds the potential to keep plant productivity with decreased chemical inputs (Adesemoye et al., 2009) and function as a key player in biogeochemical cycles (Philippot et al., 2009; Singh et al., 2010). Although endophytic microbes were ever considered contaminants in some of prior reports (Ryan et al., 2008; Reinhold-Hurek and Hurek, 2011; Mitter et al., 2013), they should be the most stable microbial partners of plants.

Various researchers have identified endophytic bacteria in plants (Hallmann et al., 1997; Compant et al., 2010; Monteiro et al., 2012; Sessitsch et al., 2012) and implicated their significance in promoting plant growth and the ability to control phytopathogens (James, 2000; James et al., 2002; Compant et al., 2010; Reinhold-Hurek and Hurek, 2011; Sessitsch et al., 2012; Suarez-Moreno et al., 2012). However, it is challenging to isolate and inoculate these inner bacteria, making it difficult to get a whole-picture of interaction network among various bacteria and between their hosts. A culture-independent strategy is increasingly used to uncover the endophytic bacterial community such as those in rice and sugarcane (Sessitsch et al., 2012; Fischer et al., 2012).

To duplicate the method performed on rice and sugarcane to evaluate bacterial types and concentrations, we performed 16S rRNA analysis on five different plant parts of the medicinal plant named Noni (*Morinda citrifolia* L.) (Chan-Blanco et al., 2006). These endophytic bacteria could produce various bioactive compounds (Su et al., 2005), that may improve immunity and anti-tumor activity (Furusawa et al., 2003; Brown, 2012). Others have reported that lignin is associated with antioxidant activity (Kamiya et al., 2004). This work will provide an informative reference on this “accessory organ” of Noni, and the first summary of endophytic bacterial community of Noni.

MATERIALS AND METHODS

Sample collection and sterilization

Roots, branches, leaves, seeds and fruits of Noni (*Morinda citrifolia* L.) (Supplementary Figure 1) were randomly collected from mature Noni trees, which were growing in cultivation field of Hainan Noni Biological Engineering Development Co., Ltd. in Sanya, Hainan (18°18′01″N, 109°31′36″E, South China), and stored at the temperature of 4°C.

The samples were washed with sterile water, immersed in 70% alcohol for 3 min, washed with fresh sodium hypochlorite solution (2.5% available Cl^-) for 5 min, rinsed with 70% alcohol for 30s, and finally washed five to seven times with sterile water. Aliquots of the final rinsing water were spread on Luria-Bertani (LB) solid medium plates and cultured for 3 days at 28°C for detection of bacterial colonies (Liu et al., 2013). The samples without bacteria on the surface were used for subsequent analysis.

DNA extraction, amplification, and sequencing

All selected roots were pooled as a single sample to average the deviations in the endophytic bacterial community, which was also done for branches, leaves, fruits and seeds. Then about 5.0g of surface-sterilized samples of each site were frozen with liquid nitrogen and quickly ground into a fine powder with a precooled sterile mortar. Then, the CTAB procedure was used to extract bacterial DNA (Liu et al., 2012), which was used as template to amplify V6 region of the 16S rDNA by primers (967F5’-CAACGCGAAGAACCTTACC-3’) and 1046R (5’-CGACAGCCATGCANCACCT-3’). The purified PCR products were mixed in equal concentration, and sequenced by HiSeq 2000 (Illumina, USA.) following the manipulation instructions at BGI Shenzhen (China).

Acquisition of unique tags and OTUs

The reads with more than 2 bases (quality value lower than 20) were filtered. Then the reads with more than 3 mismatches within amplification primers region were removed, and the low quality bases which is located at the 3’ end were trimmed. Besides these, the reads which contained more than 15 bases of adapter sequences (3 mismatches allowed), 9 N bases or 10 consecutive same bases were removed.

The processed paired-reads were overlapped with each other to form V6 tags under following standard: minimum overlapping length was 30 bp without mismatch or N base. Non-redundant tags were produced by Mothur (version 1.27.0), and the unique tags were the typical tags representing all the similar tags. Unique tags were listed based on abundance and pre-clustered by single-linkage pre-clustering (SLP) following 98% similarity. Then the unique tags were annotated and clustered into operational taxonomy units (OTUs) following 97% identity.

Taxonomy assignment and abundance analysis

Unique tags were classified by alignment to Silva RefSSU database using BLAST (version 2.2.23, and the key parameters were ‘-p blastn -m 8 -F F -a 2 -e 1e-5 -b 50’), and the best alignments were selected. If more than 66% of the unique tags in OTUs were aligned to the same species, the OTUs was assigned to the species and then the analysis went into the next taxonomic rank. The abundance of tags in different classification levels was calculated according to the alignment results.

Analysis of sample complexity and similarity

Alpha diversity was measured by indexes including chao1, ACE, Shannon and Simpson. Values of rarefaction was calculated by

Table 1. Data statistics on different tissue regions of Noni.

Sample name	Data production (M)	Tag number	Unique tag number	OTU Number
Root	497.99	340,000	22,290	3,697
Branch	443.19	350,092	18,482	2,608
Leaf	538.07	339,441	19,753	2,853
Fruits	488.82	346,141	19,611	2,871
Seed	488.47	345,173	19,724	2,951

Table 2. Sample complexity indexes for different samples.

Sample name	Chao1	ACE	Shannon	Simpson
Root	14,451.321	33,017.373	4.348	0.039
Branch	10,357.145	23,206.406	3.965	0.051
Leaf	11,374.413	27,411.963	4.009	0.045
Fruits	10,964.413	22,452.468	3.907	0.052
Seed	11,973.855	24,230.996	3.944	0.051

The bigger chao1, ACE, and Shannon are the more complex the sample was; the smaller Simpson is the more complex the sample was.

Mothur (version 1.27.0) and the rarefraction curve was drawn by R (version 2.11.1) in which the extracted tags were used as X-axis and the OTUs number was used as Y-axis.

Phylogenetic analysis was executed based on species abundancy at the genus level, and the samples were clustered by their phylogenetic distance. The representative sequence for each OTUs was selected at random, and the neighbor-joining phylogenetic tree (1,000 bootstraps) was constructed by combining all OTUs using MEGA4.

RESULTS

Species classification and complexity

V6 region of 16S rDNA was amplified and sequenced to produce 339,441 to 350,092 tags for root, branch, leaf, fruit and seed samples (Table 1). Using the protocol summarized in the methods, the unique tags were clustered into Operational Taxonomic Units (OTUs) (Table 1) and aligned with Silva108 database to identify the bacterial community of each tissue region in Noni. Arranged from 66.34 to 73.30% in different tissues, tags could be classified at the genus level (less than 5.63% at the species level) as Supplementary Figure 2A showed, making it feasible to analyze bacterial composition at this taxonomic level.

Various indexes including chao1, ACE, Shannon and Simpson were employed to evaluate the complexity of samples, indicating that root harbored the most abundant species in comparison to other four sites (Table 2). This was also documented in rarefaction analysis summarized in Supplementary Figure 2B.

Discrepancy of species composition in different sites of Noni

As Figure 1 showed, five sites are colonized by the same most abundant phyla: Proteobacteria (67.90 to 72.67% relative abundance), Firmicutes (19.39 to 22.26%) and Actinobacteria (5.95 to 6.87%) accounting for more than 95% of the species components for each site.

To understand the specific compositions, the bacterial community was profiled according to their relative abundance at the genus level (Figure 2). Among those taxa examined, *Sphingomonas* was the most abundant genus in all samples, ranging from 19.88 to 25.97%. The second abundant genus was *Halomonas* in the endophytic communities of fruits, leaves and seeds, while it *Pseudomonas* and *Geobacillus* were predominant in the branches and roots respectively. Genus *Pseudomonas and Halomonas* were widely distributed over all five parts with high relative abundance. Tags which could not be classified hold 26.70 to 33.66% abundance suggesting necessity to disclose them in the follow-up study.

Similarity analysis on bacterial community of five tissues

The dendrogram (Supplementary Figure 3) indicated that Noni fruits hold more similar bacterial types than the leaves or root. In fact, the leaves and root held the endophytic bacterial community under the similarity higher than 97%. Five sites shared 46 genus with high relative abundance (Supplementary Figure 4), including

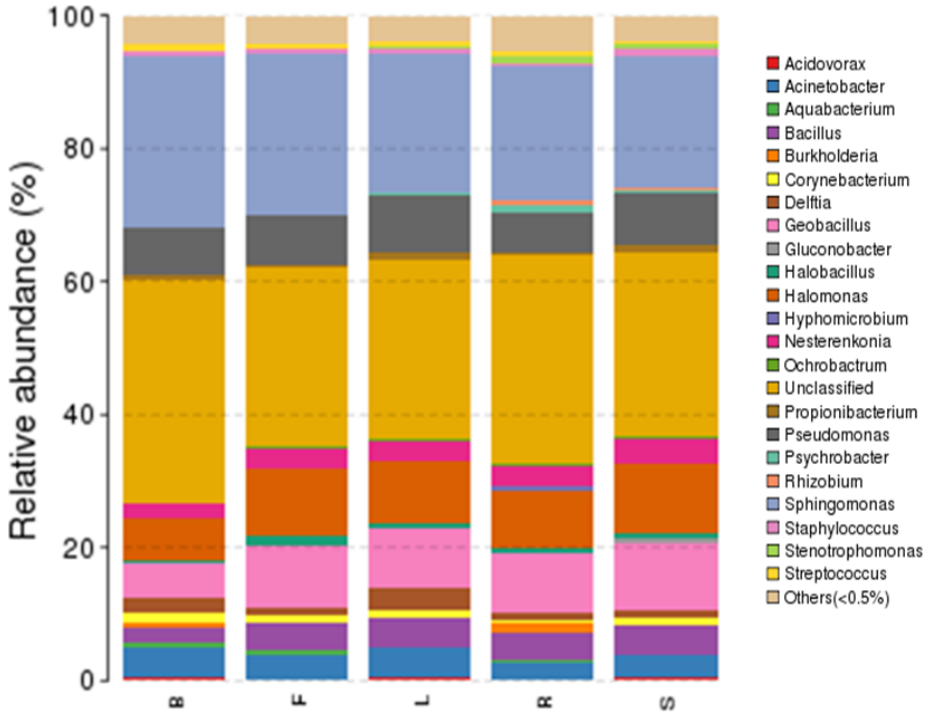

Figure 2. Distribution of bacteria in different parts of Noni at the genus level. F: fruit; L: leaf; B: branch; R: root; S: seed.

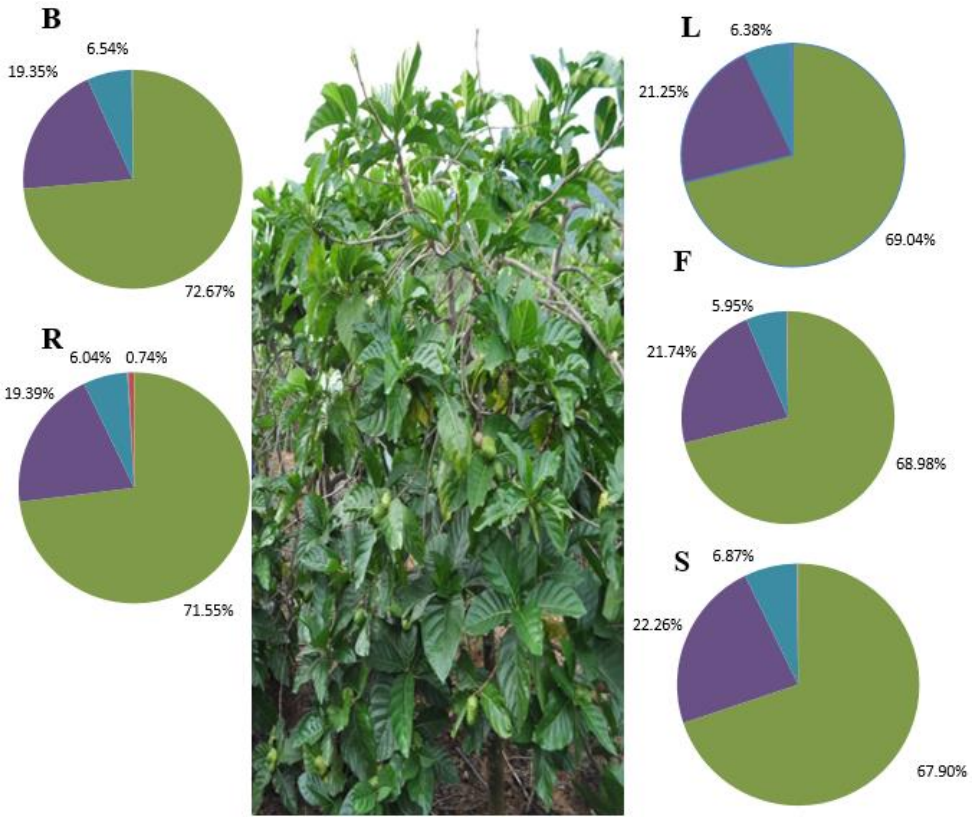

Figure 1. Distribution of bacteria in different parts of Noni at the phylum level. Green color represents Proteobacteria, purple stands for Firmicutes, Actinobacteria and Acidobacteria are highlighted in blue and red respectively. F: fruit; L: leaf; B: branch; R: root; S: seed.

the top four abundant genus *Sphingomonas*, *Pseudomonas*, *Halomonas* and *Geobacillus.* Roots harbored 13 special genus and this number were 6, 2, 4, 4 for branches, leaves, fruits and seeds respectively, but the highest relative abundance of these special genus was just 0.12% suggestive of an insignificant role they play.

DISCUSSION

Noni is an important plant providing various -derived health food raw material but the research about Noni mainly concentrated on its efficacy component detection and functional test (Liu et al., 2014a; Xu et al., 2014). Little research exists on the endophytic bacteria which may affect Noni's healthy growth, production of bioactive ingredients and the plant products' quality (Cheng et al., 2013; Cao et al., 2014; Liu et al., 2014b).

In this study, we found that bacterial community in different sites of Noni tend to be stable and most abundant genus for each of the five plant parts were *Sphingomonas*, *Pseudomonas*, *Halomonas*, and *Geobacillus*. Endophyte *Sphingomonas* is widely distributed in various plants including tomato, *Sedum alfredii*, *Dendrobium officinale* (Chen et al., 2014a; Khan et al., 2014; Chen et al., 2014b; Yang et al., 2014). Several reports have proved significant role of *Sphingomonas* and *Pseudomonas* for plant hosts by increasing plant biomass, improving cadmium uptake, fixing nitrogen and producing phytohormone including gibberellins and indole-3-acetic acid (IAA) (Chen et al., 2014a; Khan et al., 2014; Chen et al., 2014b; Yang et al., 2014). Genomic analysis of some *Pseudomonas* strains isolated from plants indicated that they uphold the potential to be involved in plant growth promotion, environmental adaptation and antagonism to fungal pathogens (Duan et al., 2013; Illakkiam et al., 2014). Based on our knowledge, there were no report about function analysis on plant-derived endophytic *Halomonas* and *Geobacillus*, requiring more efforts to elucidate how they contribute to the host. This is also needed for unclassified genus with high abundance.

Culture-independent methods applied in this work will provide a significant reference to reveal micro-ecosystem in Noni and isolate endophytic bacteria with potential value for plant growth, bioactive compounds or pathogen resistance. Findings in this research will be also referable to identify and assemble bacterial genomes from metagenomic samples (Nielsen et al., 2014) of Noni. This will be a significant alternative to understand functional networks for endophytic bacteria in Noni due to difficulties in removing host contamination in metagenome analysis and culturing some endophytes and laid the foundation for better development and use of noni plant resources.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

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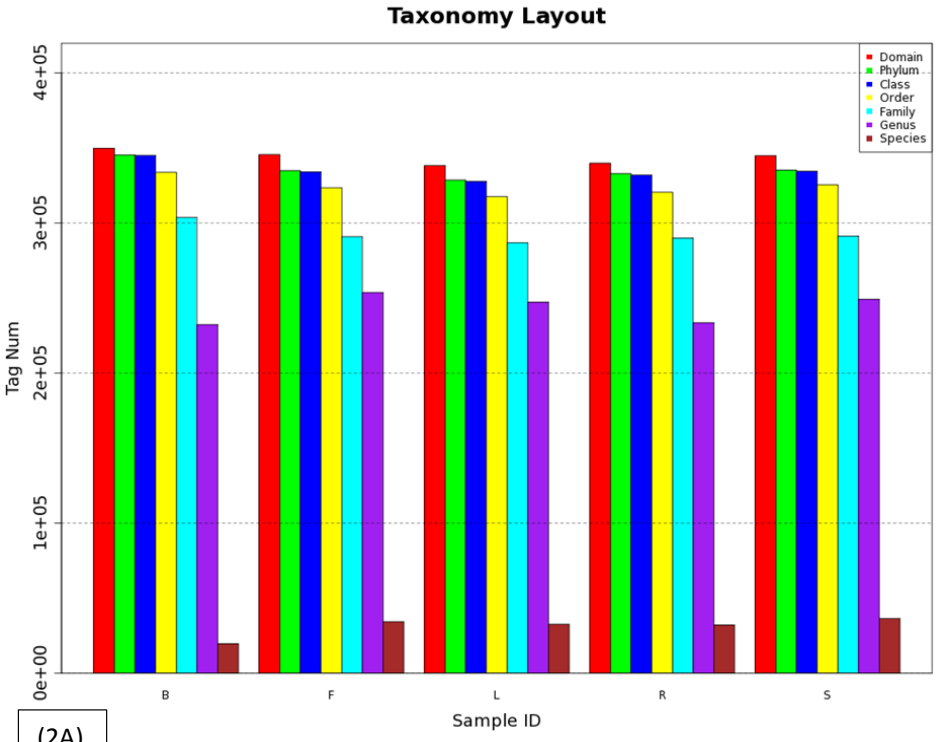
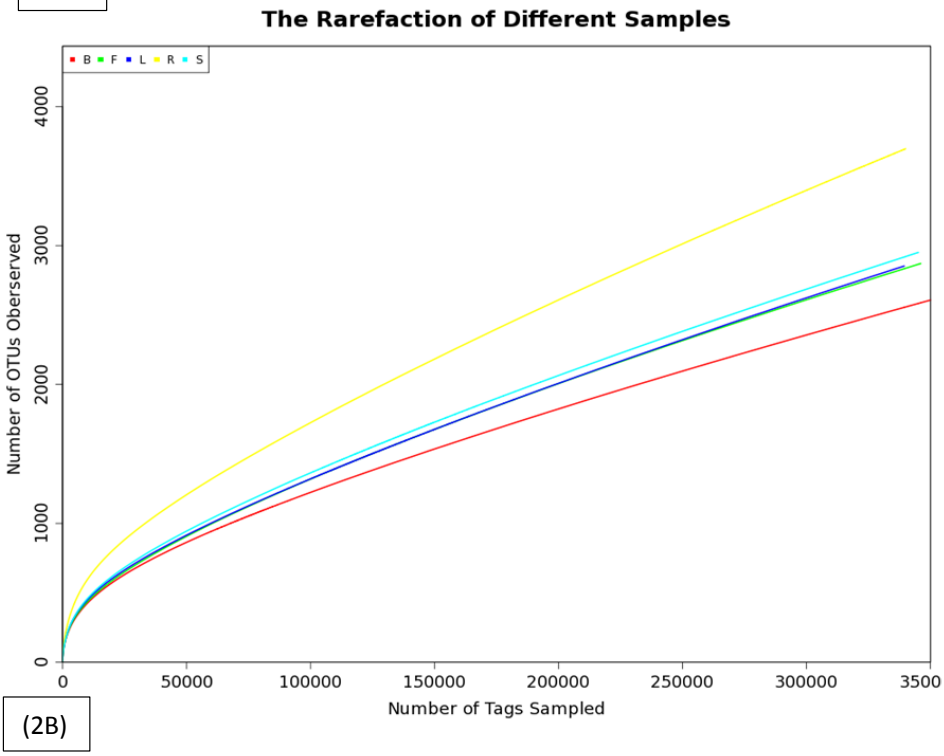
Supplementary Figure 1. Base of Noni cultivation and samples collected from five sites. F: fruit; L: leaf; B: branch; R: root; S: seed.

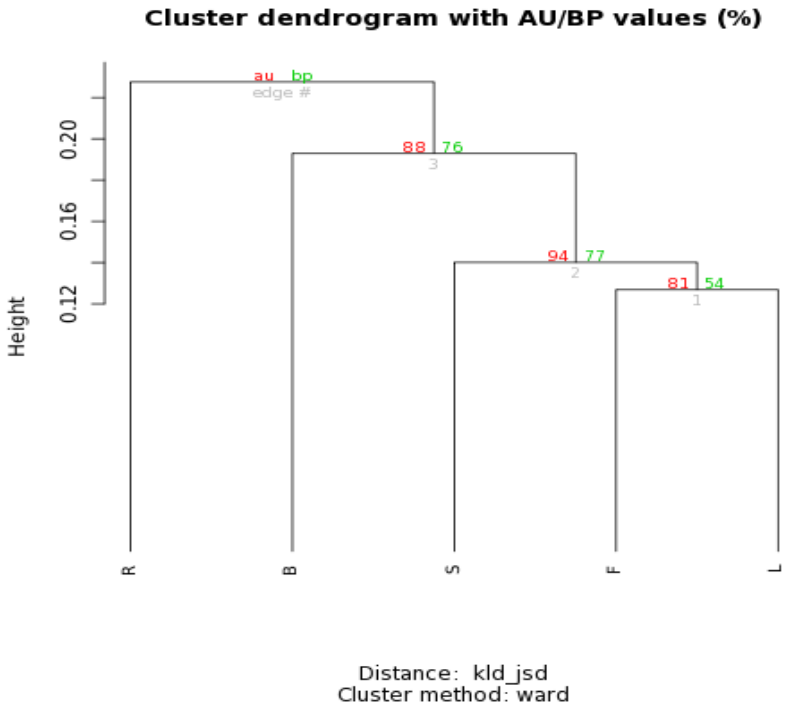


(2A)

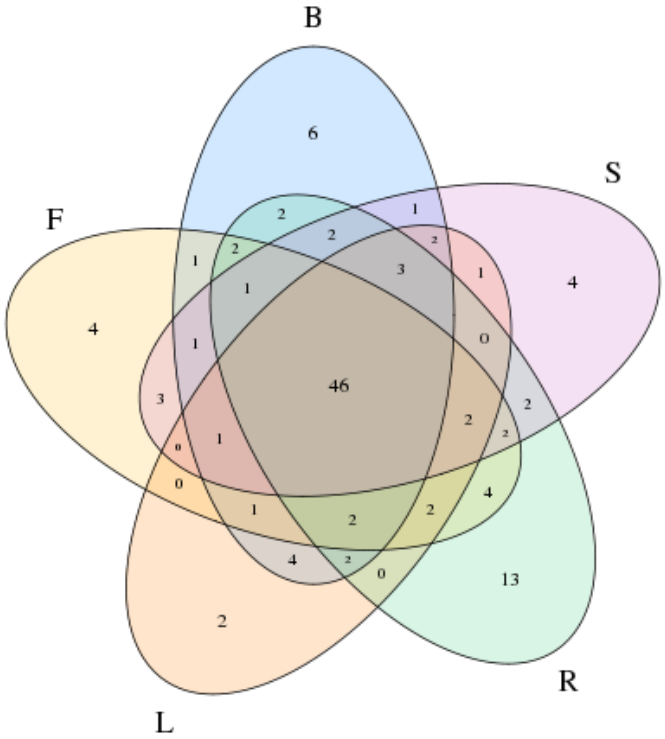


(2B)

Supplementary Figure. (2A) Number of tags that could be assigned to specific taxonomic level. F: fruit; L: leaf; B: branch; R: root; S: seed. (2B) Rarefaction of different sites of Noni. F: fruit; L: leaf; B: branch; R: root; S: seed.



Supplementary Figure 3. Community clustering results of different parts of Noni. In the picture, the communities of fruit and leaf exhibited a close relationship, and the community components of root was far from other samples. Five samples were compared together and the general relationship was exhibited.



Supplementary Figure 4. Venn diagram describing discrepancy of species similarity of five parts at the genus level five samples were compared together and the general relationship was exhibited.

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Full Length Research Paper

Effects of practices of maize farmers and traders in Ghana on contamination of maize by aflatoxins: Case study of Ejura-Sekyeredumase Municipality

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Contamination of maize by aflatoxins is of major concern to governments and the international community because high degrees of aflatoxin in food render the food unsafe for human consumption. The disposal of such foods also constitutes an economic loss in food production. This paper reports the findings of a study conducted during the 2013 minor maize farming season in Ejura-Sekyeredumase Municipality in the Ashanti region, and in Agbobloshie market in the Greater Accra Region of Ghana. The study was to investigate management practices employed at the market level and on farms by maize traders and smallholder farmers, respectively, and their impact on aflatoxin contamination. Purposive sampling was used to select 150 farmers from maize farming communities across 10 cluster zones based on geographical location of farms within the municipality. Maize traders were also selected from a market close to maize farms and a market close to consumers for the study. In all, 30 traders were randomly selected from each market. Maize samples were collected from both markets and selected farms to determine the presence and level of aflatoxins using the Vicam Aflatest immunoaffinity column method. The study revealed that, farmers and traders adopt practices that expose maize grains to aflatoxin contamination. These include: use of farmer-saved seed stock as planting material; delayed harvesting, heaping harvested maize cobs on the field; planting by broadcasting method, use of hand dipping and teeth cracking method to determine dryness of maize, use of wooden stalls with no proper ventilation for maize storage at market centres and temporal storage in the open using tarpaulin resulting in heat build-up and moisture re-absorption. Types of aflatoxin determined from sampled maize grains were G2, G1, B2 and B1. Grains from the farms showed below detection limit at 1 ppb of aflatoxins. However total values of 50.234, 70.102 and 30.943 ng/g were, respectively obtained from three composite samples taken from Ejura market. A similar occurrence was observed at Agbobloshie market, where higher levels of 677.480, 101.748 and 4831.942 ng/g were detected in composite samples analysed. All respondents had no knowledge of aflatoxin contamination and it causes. Moreover, 63% of traders from both markets believed that, consuming contaminated maize have no health implications for consumers as food products from maize are normally cooked before consumption. In conclusion, the study reveals that, practices of farmers and traders has direct effect on maize quality. It was noted that, aflatoxin contamination of maize is likely to increase from the farm through markets and ultimately compromise the health of consumers. Farmers and traders need to be encouraged to adopt best practices in maize production and marketing to ensure food safety of the final consumer. Education on aflatoxin and its health implications must also be given the necessary attention.

Key words: Maize, traders and farmers practices, aflatoxins contamination, Ghana.

INTRODUCTION

Maize is one of the most important staple food crops in Ghana. Maize is a very important food crop for both humans and livestock. It is a source of energy, vitamins and negligible amount of protein. According to Coulter et al. (1993), maize is a staple crop grown in almost all parts of the country, and is the most important source of carbohydrate in most Ghanaian meals. The economic importance of maize and its role in ensuring food security in Ghana cannot be over emphasized. Annual production has been more than 1,000,000 MT since 2000, averaging 1,772,300MT over the period 2009 to 2012 (MoFA, 2013). The maize market in Ghana is dominated by several small-scale traders, with a greater proportion being women. Five main participants in the maize trade may be identified: the farmer/seller, the local assembler, the commission agent, the long distance wholesaler and the market-based wholesaler/the market-based retailer (Obeng et al., 1990).

Post-harvest activities such as drying and storage are among the key areas along the maize value chain that is of critical importance to small-holder farmers/traders in Africa. On-farm storage and storage of produce by small-scale traders in Africa, represents 75-85% of national storage (Pother and Hotchkisss, 1995). However, due to ineffective storage practices adopted by small-holder farmers and traders, some level of grain losses due to insect infestation, mould growth and discolouration, contamination by aflatoxins, re-wetting and germination of grains is usually encountered. According to Addo et al. (2002), major storage techniques utilised by small-holder producers in Western Africa vary greatly, but include on-field, open storage, jute bags, polyethylene or polypropylene bags, raised platforms, conical structures with thatched roofs and giant woven baskets. Moreover, drying of maize by small-holder farmers is normally done either in storage or while the crop is on the field. According to Kaaya et al. (2006), delay field drying of maize could result in serious grain losses during storage. Platform drying, which raises the maize off the ground for longer-term drying, was however, reported to be associated with losses of up to 3.5% in Zambia (Rembold et al., 2011).

Maize, just as any other crop can be contaminated with storage fungi, some of which may develop as by-products of mycotoxins that can be harmful to animal and human health. Mycotoxins that develop from *Aspergillus flavus* and common post-harvest fungi in maize are called aflatoxins. These toxins are hazardous to animals and human health, and constitute a factor in economic loss in food production in the world (Lubulwa and Davis, 1994). Aflatoxin, which commonly affects maize, causes illness and even death when consumed in large quantities. According to WHO (2006), acute aflatoxicosis is an under-recognized and under-reported cause of liver damage; aflatoxin is a Group 1 human liver carcinogen. Low-level, chronic exposure is carcinogenic, and has been linked to growth retardation, underweight, neurological impairment, immunosuppression and mortality in children (Strosnider et al., 2006). High levels of aflatoxins have been found in groundnuts and cereal grains in countries such as Gambia, Ghana, Guinea, Nigeria, Senegal, South Africa and Uganda.

In 1991, World Health Organisation (WHO) explained that, food-borne diseases created an enormous burden on the economies of developing countries, and consumer costs included; medical, legal, and other expenses, as well as absenteeism at work and school. Economic consequences as a result of rejection of exports and loss of credibility as trading partners have also been reported. In Nigeria, the Food and Drug Administration destroyed aflatoxin-contaminated food worth more than US$ 200 000. The quantity of safe food required to replace contaminated food during the outbreak of acute aflatoxicosis in Kenya in 2004 was 166,000 tonnes for 1.8 million people over a six months period (WHO, 2006).

Contamination by aflatoxins can occur both at pre-harvest and post-harvest. Aflatoxins infestation in maize starts in the field or during storage of the grains (Kumar et al., 2000), thus making the grains unwholesome for consumption. According to Wilson and Payne (1994); Hell et al. (2008), the predisposing factors of infection include; improper drying, high relative humidity and temperature, farmers' production practices, intercropping with aflatoxin infected grains, early and delayed harvesting and poorly constructed storage structures and storage practices as well as stress induced while crops are growing.

While aflatoxin itself is invisible and tasteless, its presence may be correlated with other attributes that facilitate or result from fungal growth, including physical damage to the protective outer layer of the kernel, discoloration, and compromised taste quality (Hoffmann et al., 2013). Based on maize consumption patterns and possible aflatoxin contamination levels of 20 ppb, the population in countries with high hepatitis B virus (HBV) infection rates could be at risk of liver cancer at 11 per 100 000 population per year (WHO, 2006). Given that maize is the primary staple grain for Ghanaians, accounting for 36% of total food caloric intake (Kirimi et al., 2011); even relatively low levels of exposure may have significant negative health effects (Shephard, 2008).

In light of the discussion above, this study was

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undertaken to assess management practices adopted by farmers, and traders in handling and storage of maize and its impact on aflatoxin contamination.

MATERIALS AND METHODS

Selection of study sites

The survey on farming practices was undertaken in Ejura Sekyere-Dumase Municipality in the Ashanti Region. Maize samples for the aflatoxin test were collected from two major markets in Ghana Ejura market (Maize market close to maize producing areas or farms) in the Ashanti Region and Agbobloshie market in the Greater Accra region of Ghana (Maize market close to consumers. It is normally located in city centers).

The rationale for selecting Ejura Sekyeredumase Municipality was because it is one of the leading maize producing areas that accounts for more than 60% of maize produced in Ghana. About 60% of the labour force in the Municipality also engaged in agriculture, and maize is the main crop cultivated. Strategically, the Municipality is located in the transition zone between the Northern and Southern zones of the country and has one of the largest maize markets in the sub-region. The Ejura market is a major maize 'producing' market. Maize sold in this market mainly comes from farming communities such as Kasei, Nokaresa, Nyamebekyere, Ashakoko, Yaabraso, Bemi in the Municipality. Wholesale traders (middlewomen/men) and retailers from Kumasi, Takoradi, Obuasi, Accra and other parts of the country, as well as, from neighbouring countries like Burkina Faso, Niger, Mali, Togo and Ivory Coast all buy maize from this market.

The Agbobloshie market is also amongst the largest maize markets located close to consumers in Ghana. It is located in the capital, Accra, where the traditional food of the indigenes known as *kenkey* is prepared from maize. About 80% of maize sold in the market has its roots from the Ejura and Techiman market.

Data collection and research instrument

The study was undertaken during the 2013 minor maize season. Two methods for data collection were employed: a survey to identify practices of farmers and traders used in maize production and marketing respectively. Maize samples collected from selected farms and markets were later tested for the incidence or presence of aflatoxins.

After reviewing literature on recommended best practices in maize production and marketing, 2 sets of semi-structured questionnaires were developed to investigate empirically the practices used by farmers and traders in maize production and marketing respectively in the study areas. The questionnaire for the farmers sought information on farmer's household demographics and agronomic activities (type of land preparation, type of seeds planted, time of planting, planting method used, weeding practices, fertiliser application etc.); harvesting activities (time of harvest, criteria used to assess maize maturity, harvesting method and yield) as well as post-harvest management practices by farmers on drying, shelling, transportation, and storage. Traders' management practices on maize handling at the market centres, as well as, their storage practices were also investigated. The traders' and farmers' perceptions or knowledge of contaminated maize and aflatoxins were also investigated.

Face-to-face interview script was used to solicit responses for the survey questions. The questions were standardised to increase interviewer consistency (Fowler, 2002). Sampling spear, and sampling bags, weighing scales, tally counter to count grains for analysis, Mini GAC plus grain moisture analyser, stereo micro-scope to identify weevils and other insects, forceps, and High-Performance Liquid Chromatography (HPLC) system were used to collate data on aflatoxins levels in sampled maize grains from the selected markets and farms.

Survey sample of respondents

The target respondents were maize farmers and traders in the selected study sites. The maize farming areas in the Municipality were put into 3 cluster zones based on geographical or ecological location of farms (forest, thicket and grassland, and guinea savannah). One hundred and fifty (150) maize farmers were purposively selected across 134 communities in the three cluster zones for the survey based on their farming activities and production output.

Maize traders in both markets were also put into clusters depending on quantity of bags handled and storage method practiced. Three clusters were formed in each market, Cluster 1-retailers who buy maize (<10 bags) within the market's and sell to individuals who buy for their personal consumption. Such traders usually do not have storage facilities at the market and may store the few bags in the open, covering at night with tarpaulin. Cluster 2 involved retailers who buy maize (10– 50 bags) from wholesale traders and only sell to food processors, and millers. These were classified as contract traders. Contract traders have storage facilities at the markets, and will only store at the request of their customers from a few days to a maximum of 4 weeks. Cluster 3 was made up of wholesale traders or middlewomen/men who buy maize (>50bags) directly from farmers or bring maize from producing markets to sell at the consuming markets. They sometimes own storage facilities or store their maize at warehouses close to the market. In all, 10 traders were randomly selected from each cluster, bringing the total number of traders selected for the survey to 30.

Collection of maize smples and aflatoxin analysis

Collection of maize samples was done in accordance with the process recommended by the FAO for maize collection for aflatoxin analysis as reported by Njapau (2008). Specifically, multiple samples from randomly selected parts of each farm or market cluster zone were combined to produce a representative 3-kg composite sample. A maximum weight of 1-kg grains from the composite was sampled from each farm in the three locations, and market cluster zones for the analysis. Maize samples from the farms were collected before harvesting. The samples were kept and transported for analysis in paper bags to control moisture content. The analysis for aflatoxin levels was done at the Aflatoxin Laboratory of the Department of Food Science and Technology, KNUST, Kumasi. Moisture content of maize samples was determined using a grain moisture analyser, while the presence of insect infestation and mould was determined using forceps, a stereo microscope and visual inspection.

Extraction and clean-up

A mixture of ground maize (25 g) with 5 g of sodium chloride and 125 ml of methanol/ deionized water (70:30) was blended at high speed for 2 min and filtered through a fluted filter paper. The extract (15 ml) was diluted with 30 ml of deionized water and filtered through a1.0 µm microfiber filter. In reference to Reiter et al. (2010), the diluted extract (15 ml) was passed through Vicam Aflatest immunoaffinity column (IAC), which was washed twice with10 ml deionized water. Aflatoxin was eluted from the IAC with 1 ml HPLC grade methanol and 100ul of the eluent was injected into the HPLC.

HPLC determination

A Cecil-Adept Binary Pump HPLC coupled with Shimadzu 10AxL fluorescence detector (Ex: 360 nm, Em: 435) with Phenomenex HyperClone BDS C18 Column (150 x 4.60 mm, 5 um). The mobile phase used was methanol: water (40:60, v/v) at a flow rate of 1 ml/min with column temperature maintained at 40°C. To 1 L of mobile phase were added 119 mg of potassium bromide and 350 ul of 4 M nitric acid (required for postcolumn electrochemical derivatisation with Kobra Cell, R-Biopharm Rhone. Aflatoxin Mix (G_1, G_2, B_1, B_2) standards were prepared from Supelco® aflatoxin standard of 2.6 ng/µL in methanol. Calibration standards were prepared by spiking 25 g blank maize matrix with 2.6, 13, 52, 78 and 104 ppb. The correlation coefficient (R^2) of the standard curve using spiked samples for each toxin was greater than 0.974. Recovery was greater than 77% at 26 ppb of total aflatoxin of spiked sample. LOD (Limit of Detection) was established at 1 ppb.

Data analysis and presentation

The data was analysed using Statistical Package for Social Science (SPSS) version 16 and Microsoft excel. The data were subjected to simple descriptive and inferential statistics.

RESULTS AND DISCUSSION

Personal characteristics of farmers

Majority (62%) of the respondents interviewed were smallholder farmers with an average farm size of 2 ha. Majority of the farmers (71%) interviewed were aged between 30-50 years with a dominance male population of about (65%). Average households had family sizes from 5 to 9 members. They usually assist in farming activities such as planting, weeding, harvesting etc, implying reliable and constant access to labour for farm work. Out of the 150 farmers interviewed, 45% had no formal education and 36% had their education up to the primary school level. This has the potential of impeding farmers understanding of aflatoxins infestations, its causes, implications and measures to minimize its infestation since education facilitates farmers' adoption of innovations (Onemolease et al., 2005).

Practices of maize farmers in the study area

Majority (62%) of the farmers were smallholder farmers. From the survey, it was realised that farmer-saved seeds was the common planting material used every season. This reflects an over reliance on the use of farmers own maize seed varieties. These are more susceptible to insect and disease attacks, and have a lower yield compared to improved varieties (Tengan et al., 2011). Among the key factors that influence farmer's choice of seed material was cost. Cost of seeds per kg for improved varieties such as *Obatanpa, Akomasa, Abelehe etc.* was GHC 9 ($1 = GHS 2.36 as at January 2014 when the study was conducted). This made majority of farmers (82%) rely on their own seed stock for planting material. Among farmers who cultivated improved varieties, 85% preferred *Obatanpa* to other varieties due to its drought tolerant characteristics and yield potential of 5.5 ton/ha. Though it is recommended to be planted in the major season, most of the farmers planted it in the minor season, disregarding the effect time of planting could have on the yield potential. This was confirmed by an estimated average yield of 1.5 ton/ha (Estimated yield obtained during field investigation of maize food losses in the study area) as opposed to the 5.5 ton/ha that could potentially be realised. Harvesting maize in the study area is usually done by women and children through manual means. The study reveals that, majority of farmers (69%) did not harvest based on the physiological maturity period of the planted maize. Consequently, they employ traditional practices like observing the dried tassels of cobs and drooping of cobs as a sign of maturity before harvesting. Other traditional practices used by the farmers to determine the dryness of their maize was by cracking/biting with their teeth. These techniques are not accurate, and therefore, harvested maize may still have high moisture content, thereby making the grains highly susceptible to aflatoxin contamination (Hell et al., 2008).

Late harvesting of maize during the minor season was identified as a common practice in the study area. Though, late harvesting may expose maize grain to diseases and pest attack, thereby increasing quantitative and qualitative loss of maize, approximately 93% of farmer's interviewed practice this method. With mechanical drying perceived to be very expensive, farmers resort to this practice as the best method in drying their maize grains in the minor season since most often there are no rains during this period. Extended field drying of maize could result in serious grain losses during storage (Borgemeister et al., 1998; Kaaya et al., 2006) and as such, harvesting immediately after physiological maturity is recommended to mitigate the likelihood of aflatoxin infestation (Hell and Mutegi, 2011). Kaaya and Kyamuhangire (2006), observed that, aflatoxin levels increased by about 4 times by the third week, and more than 7 times when maize harvest was delayed for 4 weeks in Uganda.

The study also reveal that, farmers sometimes leave the harvested maize on the field for a couple of days or weeks before shelling and transporting home or to the market. According to Hell and Mutegi (2011), leaving the harvested crop in the field prior to storage promotes fungal infection and insect infestation. Udoh et al. (2000) reported that, this is a common practice in Africa, and is often due to labour constraints, and the need to let the crop dry completely prior to harvest.

Management practices of maize traders at Ejura and Agbogbloshie markets

Approximately 97% of traders interviewed from both

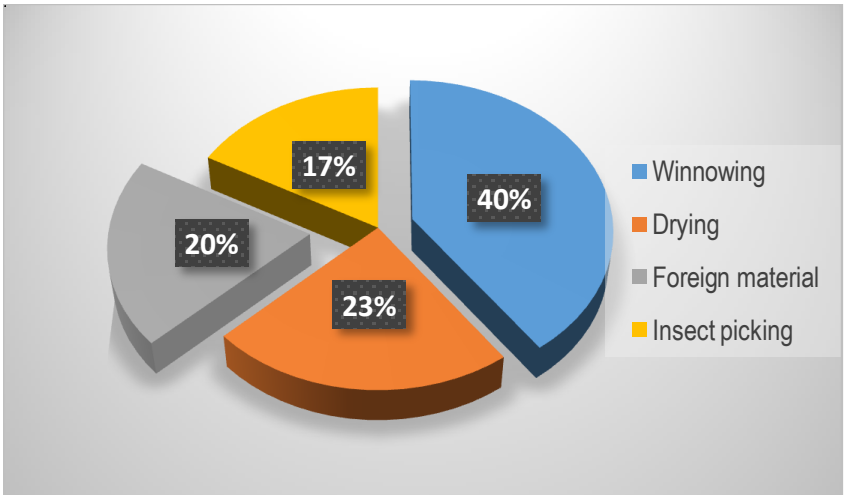

Figure 1. Post-harvest activities at the market center.

markets had their education not exceeding primary school level, with 57% trading in maize for 10 years and above. The analysis also revealed that only traders in Agbogbloshie market performed some basic post-harvest management activities such as winnowing or cleaning, pest control and intermittent exposure of grains to the sun to control weevil infestation (Figure 1). This is a common phenomenon in consumer markets as traders may likely have their stock kept for long before they are sold out. However, approximately 83% of respondents at both market centers did not practice any management practices to control storage pest.

Inspection of storage facilities of traders at both market centers revealed that, 50% of the traders use temporal wooden stalls to store their maize. The structures were identified to be poorly constructed, with no openings for ventilation, thereby, making the stored maize susceptible to insects and fungi infestations. Approximately, 23% resort to leaving their produce in the open after a day's trade, and covered with tarpaulin at night and rainy days. This practice exposes maize grains to humid conditions, thereby, increasing the likelihood of fungi infestation or mould growth. This is confirmed by Christensen and Mirocha, (1976), who reported that, the growth of *A. flavus* increases dramatically when relative humidity increases above 85%. They further stated that, in this range, even a small increase in moisture can be very influential in terms of increasing the risk of aflatoxin contamination. It was, however, discovered that 27% of traders store their maize in ordinary rooms or warehouses. Bagged maize in these stores is put on wooden platform to prevent contact with the floor. This has the potential of reducing or preventing contamination from insect and fungi infestation. Hell (1997) reported that maize stored in baskets and platform stores showed low mean aflatoxin levels.

Assessment of grains from both markets revealed some level of mould and weevil infestation. While the level of infestation was low at Ejura market with only 10% of the traders whose samples were assessed having signs of weevil and mould infestation, grains from Agbogbloshie market were heavily infested with weevils. Evidence of high weevil infestation was identified among 83% of maize traders whose samples were assessed at the Agbobloshie market. As rightly noted by Bekele *et al*., (1997), high level of insect infestation of stored maize are due to poor storage facilities, improper storage methods, poor food distribution, poor transportation facilities and insects pest resistibility to chemicals used to store the maize. The other reasons are climatic conditions (high relative humidity) which are conducive for insect activity. All these state factors were clearly identified at the two markets. The infestation of maize grains by insects, makes it more susceptible to aflatoxin contamination. This is confirmed by a study conducted by Lamboni and Hell (2009), who reported that, storage pests, in particular *Cathartus quadricollis* and *Sitophilus zeamais*, have been shown to play an important role in the contamination of foods with fungi, especially those that produce toxins. Edusei et al. (2004), also reported that, damage done by insects encourages infection by bacterial and fungal diseases through transmission of their spores.

Moisture content of sampled maize

The moisture contents of maize samples from the Ejura market were found to be in the range of 12.5 to 13.4%. This is close to the recommended moisture content (13%) for effective maize storage proposed by Christensen and Kaufmann (1974) cited in Garuba et al. (2011). The lower moisture content observed can be attributed to late harvesting. The harvested maize

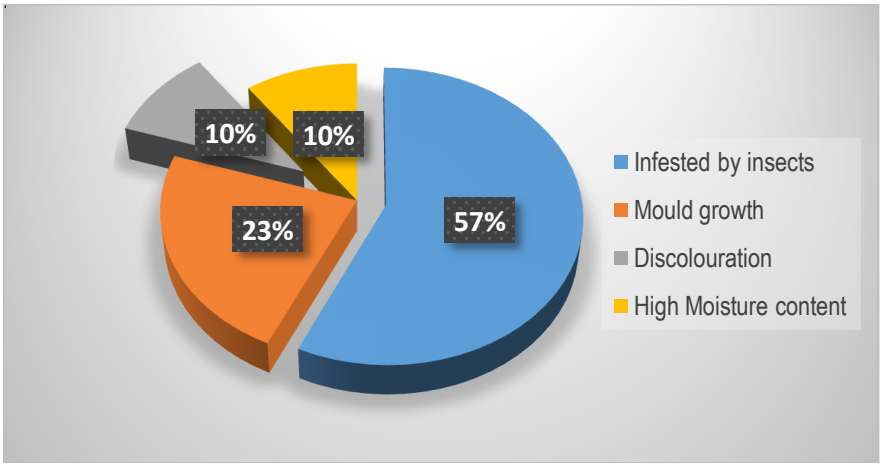

Figure 2. Traders perception of contaminated maize.

normally ends up in the Ejura market. Recorded moisture content of maize samples collected from the Agbogbloshie market was between 13.1 to 16.6%. The high moisture content recorded can be attributed to reabsorption of moisture by such grains due to the humid conditions created by the use of tarpaulin at night and when it rains. The recorded high moisture content of maize samples at Agbogbloshie market correlates with the high insect infestation observed. This is corroborated by Shejbal (1997), who reported that, grains of moisture content above 13% are likely to be attacked by pest and moulds.

Trader's knowledge or perception of contaminated maize

All the respondents (farmers and traders) indicated they have no knowledge of aflatoxin contamination. However, 57% perceived contaminated maize as grains infested by insects such as weevils. Approximately 23% also perceived contaminated maize as one with mould growth,10% perceived contaminated maize as one with discolouration, and 10% believe maize with high moisture content above the recommended storage moisture of 13% is contaminated (Figure 2).

Poor management practices are principally the cause of contamination, and contribute to the vulnerability of maize to fungi infections, which can further lead to aflatoxin contamination. But interestingly, majority (63%) of the respondents believed that, consumption of contaminated maize will have no health effect on humans, mainly due to the rigorous cooking maize food products are subjected to before eating.

Aflatoxin contamination

Aflatoxins are produced as metabolites by the *Aspergillus Flavus* and *Aspergillus Parasiticus,* and exist in nature world widely. The common aflatoxins are B1, B2, G1 and G2. Among these mycotoxins, the aflatoxin B1 is of most toxicity followed by G1; the toxicities of B2 and G2 are relative weak (Yang and Rong, 2011). According to the European Food Safety Authority (EFSA), (2007), aflatoxins are genotoxic and carcinogenic, and can cause both acute and chronic toxicity in humans. They reported that, they are most commonly found in cereals. Types of aflatoxin determined from sampled maize grains from both farms and markets were G2, G1, B2 and B1 (Table 1). When estimating aflatoxin levels, the values that were less than the limit of detection (LOD) were substituted with LOD.

Aflatoxin levels determined on maize grains sampled from the three farms were recorded as below detection (Limit of detection of G2, G1=1.5 ng/g and B2, B1=0.8 ng/g) limit of 1 ppb (<LOD @1ppb). However, as presented in Table 1, total values of 50.234, 70.102 and 30.943 ng/g were respectively obtained from maize samples collected from the Ejura market. Moreover, higher levels of aflatoxin, 677.480, 101.748 and 4831.942 ng/g were obtained from samples taken from the Agbobloshie market.

The high occurrence of aflatoxins detected in sampled maize from both markets could be attributed to, but not limited to the following reasons: delayed shelling after harvesting which occurs due to inadequate and unreliable services of mobile sheller operators. Farmers thereby, resort to heaping harvested cobs (Figure 3) on the farm sometimes for one to two weeks before shelling is done.

Many of the sheller operators tend to be in a hurry to move to other farms during the period and, therefore, do not take time or allow farmers to separate the moulded or infested cobs (see Figure 4) from the good ones before they are fed into the shellers. Thus, shelled grains after the shelling process becomes a mixture of both good and aflatoxin infested grains. Other potential reasons are; delay in transporting grains home or to the market due to

Table 1. Levels of aflatoxins in sampled maize from markets.

Sample ID	G2	G1	B2	B1	Total
EJ_1	19.132	<LOD	10.844	20.258	50.234
EJ_2	10.698	18.863	15.196	25.345	70.102
EJ_3	<LOD	<LOD	12.178	18.765	30.943
AG_1	<LOD	Absent	23.564	653.916	677.480
AG_2	<LOD	<LOD	10.498	91.250	101.748
AG_3	26.302	1670.888	133.856	3000.896	4831.942

LOD= limit of detection (G2, G1= 1.5ng/g; B2, B1= 0.8ng/g). R^2 = 0.974. Maximum limit for safe consumption of aflatoxin contaminated maize is 20ng/g (FDA, 2011). $AG_{1,2,3}$ and $EJ_{1,2,3}$ represents composite maize samples from the three clusters zones at Agbobloshie and Ejura markets respectively.

Figure 3. Heaped maize cobs on the field waiting for shelling (Source; Field photograph).

Figure 4. An infested cob among heaped cobs on the field. (Source: Field photograph).

Table 2. Summarised results of parameters determined.

Parameter	Farm	Ejura market	Agbobloshie market
Moisture content (%)	<13	12.5 – 13.4	13.1 – 16.6
Weevil infestation	Absent	Low	High
Presence of mould	No	Yes	Yes
Aflatoxins present	Yes	Yes	Yes
Level of aflatoxins	<LOD	>LOD	>LOD

LOD= limit of detection (G2, G1= 1.5 ng/g; B2, B1= 0.8 ng/g).

poor road networks, and use of rickety vehicles which often break down; prolong storage under unhygienic and unfavourable storage environments at the market places and poor storage practices (storage in the open) which leads to high infestation by weevils, and urine of rodents. Re-wetting of the grains is likely to occur under such storage conditions.

Aflatoxin contamination cannot be completely eradicated from foods; however, exposure through food should be kept as low as possible. According to Food and Drugs Authority (2011), level for aflatoxin in milk-stage of maize acceptable for human consumption is 0.5 ng/g, when dried is 20 ng/g and 100ng/g for feeds for cattle, swine and poultry. The results of this study, however, showed that, aflatoxin contamination levels recorded on sampled grains was very high and beyond the recommended level. This study suggests that consumers of maize from the study areas are at a risk of exposure to dire health implications such as aflatoxicosis. Pier (1991) reported that, aflatoxins have been implicated in sub-acute and chronic effects in humans. These effects include primary liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis through repeated ingestion of low levels of aflatoxin. It is also considered that aflatoxins may play a role in a number of diseases, including Reye's syndrome, *kwashiorkor* and hepatitis, as well as, affecting the immune system. There is a high risk of Hepatitis B and hepatitis C carriers developing liver cancer when they are exposed to aflatoxin (Williams et al., 2004). Aflatoxin contamination has also been linked to micronutrient deficiencies in animals (*ibid*).

The results as summarised in Table 2 show that, aflatoxin contamination is likely to increase along the value chain of maize, from the farm to the market. Therefore, farmers and traders lack of knowledge on the subject and non-existing monitoring protocols by the appropriate authorities is a major concern.

Conclusions

The study sought to use a case study approach to investigate the effect of practices of farmers and traders in maize production and handling at the farm level and market centers, respectively, on contamination of maize by aflatoxins in selected farming communities and markets. The findings suggest that pre-harvest practices adopted by farmers in the study areas among the sample pool, as well as, post-harvest handling methods were inappropriate and inefficient. These practices exposed maize grains to insect infestation, fungi infection and increased levels of aflatoxin contamination, which could have significant economic implications for the farmers/traders and health implications for the final consumer.

It is clear that, aflatoxin contamination in maize is likely to increase through the channels of distribution from the farm up to the market centers before the cereal reaches the final consumer. In the interest of food security and safety of consumers, who have a right to safe food as declared by the Universal Declaration of Human Rights, 1948,farmers and traders need to be educated and encouraged to adopt management practices that reduce the incidence of aflatoxin contamination in the field and during handling at market centers. This will make maize grains less susceptible to aflatoxin infestation thereby ensuring the safety of the final consumer.

Conflict of interests

The author(s) did not declare any conflict of interest.

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