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

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Prevalence of *mec*A, PVL and *ica* genes in *Staphylococcus aureus* strains isolated from urinary tract infections patients

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The prevalence of *Staphylococcus aureus* among urinary tract infections (UTIs) patients has been increasing worldwide. The aim of this study was to determine the occurrence of the mecA, PVL, ica genes in a collection of MRSA urinary isolates by PCR. Methicillin resistance *S. aureus* (MRSA) is considered to have emerged from *S. aureus* through the acquisition of staphylococcal cassette chromosome (SCCmec), which carries the *mec*A gene for methicillin resistance. Panton-Valentine leukocidin (PVL)-producing strains of MRSA appear to be associated with increased risk of transmission, complications and hospitalization. IcaA and icaD genes have been reported to play a significant role in biofilm formation in *S. aureus*. Out of 50 isolates of *S. aureus* from UTI patients, 36 (72%) were found to be MRSA by oxacillin screen agar. All these MRSA strains were found to be positive for *mec*A genes, 9 (25%) were found to be positive for PVL and 23 (64%) were positive for both *icaA* and *icaD* genes. MRSA isolated from UTI patients show the presence of *mec*A, PVL, *ica* genes, which may have consequences for the treatment of UTIs especially in catheter-associated and nosocomial infections.

Key words: Urinary tract infections (UTIs), *mec*A gene, *PV*L gene, *ica* gene, MRSA.

INTRODUCTION

Urinary tract infections (UTIs) are the third common infections after respiratory and gastro-intestinal infections, and most common cause of both community-acquired and nosocomial infections (Najar et al., 2009; Hryniewicz et al., 2001). Although most UTIs are caused by Gram negative bacteria, other species such as *Staphylococcus* spp. are emerging (Bonadio et al., 2001; Farajnia et al., 2009). Several studies have reported the increasing prevalence of *Staphylococcus aureus* among UTI patients (Nwanze et al., 2007: Akortha and Ibadin, 2008). *S. aureus* is an important human pathogen whose pathogenicity largely depends on producing a broad spectrum of extracellular and cell wall-associated virulence determinants. Among them, a wide variety of surface adhesins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) has been described (Sauer et al., 2008). MRSA is considered to have emerged from *S. aureus* through the acquisition of SCCmec, which carries the *mec*A gene for methicillin resistance (Takano et al., 2008). Methicillin resistance is

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clinically very important because a single genetic element confers resistance to the beta-lactam antibiotics, which include penicillins, cephalosporins and carbapenems (Grundmann et al., 2006). MRSA isolation frequency has increased in association with UTIs in Japan, USA and France (Shigemura et al., 2005; Baba-Moussa et al., 2008; Johnson et al., 2006). PVL is a cytoxin, and a member of the bi-component family of staphylococcal leukocidins and one of the β-pore-forming toxins. The toxins subunits bind to leukocyte cell membrane inducing trans-membrane pore formation and subsequent cell lysis (Khosravi et al., 2012). PVL is a synergohymenotropic toxin (Lina et al.,1999) that can damage the membranes of human polymer-phonuclear cells and macrophages by forming pores in the membranes of leukocytes, resulting in an increase in membrane permeability and cell lysis (Prévost et al., 2001). PVL is described as a key virulence factor because it can be found virtually in all community acquired (CA)-MRSA strains that cause soft-tissue infections (Vandenesch et al., 2003). PVL is carried by <5 % of isolates of *S. aureus*, both methicillin-sensitive *S. aureus* and MRSA (Dyer, 2007; Holmes et al., 2005). PVL-producing strains of CA-MRSA appear to be associated with increased risk of transmission, complications and hospitalization. Biofilm formation, especially on medical implants such as catheters, is another important virulence mechanism for *S. aureus*. Bacterial cells in a biofilm show much greater resistance to antibiotics than free living cells; biofilms also help micro-organisms evade host immune responses. The inter-cellular adhesion (ica) locus consists of the genes icaADBC, and among the ica genes, icaA and icaD have been reported to play a significant role in biofilm formation in *S. aureus* and *Staphylococcus epidermidis* (Cramton et al., 1999). Co-expression of icaA and icaD genes leads to the full phenotypic expression of the capsular poly-saccharide (Vasudevan et al., 2003). This study investigated the presence of *mec*A gene and various virulence factors (*pv*L and *ica* genes) of MRSA strains isolated from UTI patients in Khartoum State, Sudan.

MATERIALS AND METHODS

The bacteria investigated comprised of *S. aureus* isolates collected from UTI patients from four main tertiary care hospitals in Khartoum (Khartoum Teaching Hospital, Sahiroon Hospital, Soba University Hospital, Ibrahim Malik Hospital) from 2011 to 2014 which were stored at –70°C in Tryptic Soy broth with 20% glycerol at the microbiology laboratory (research lab) of Sudan University for Sciences and Technology (SUST). Identification of *S. aureus* was based on the colony morphology, Gram staining, catalase (Sigma), coagulase tests and latex slide agglutination Staphytect Plus test (Oxoid). *S. aureus* isolates were detected as MRSA (oxacillin-resistant) by inoculating the organism onto Oxacillin Screen Agar (Mueller-Hinton agar plates supplemented with 4% NaCl and oxacillin 6 µg/ml) according to NCCL (2001) guidelines, and confirmed by cefoxitin disc test (Oxoid). Any growth after incubation for 24 h at 35°C was interpreted as a positive MRSA (Louie et al., 2001). Reference strains MSSA ATCC 25923 and MRSA ATCC43300 were used as negative and positive controls, respectively.

The *mec*A, PVL, *ica*A and *ica*D genes of MRSA isolates were detected by PCR. Chromosomal DNA was isolated from overnight cultures grown on blood agar at 37°C. Genomic DNA was extracted by using microwave method (Ahmed et al., 2014) with some modification. Briefly, cell pellets were incubated for 30 min at 65°C, after washing with TE and addition of 50 µl of 10% SDS (Sigma). The lysates were centrifuged and supernatants were removed. The micro-tubes were then placed in a microwave oven and heated three times for 1 min at 750 W. The pellets were dissolved in TE (Tris-EDTA, Sigma) bufferand were extracted with an equal volume of chloroform/isoamyl alcohol (24:1) for 15 min. The aqueous phase was recovered by centrifugation for 20 min and precipitated with ethanol. The primers used in this study are shown in Table 1. They were synthesized by IDT (Integrated DNA technologies, Interleucvenlaan, 12A,B 3001, Belgium) . A 50 µl PCR mixture containing 3 µl of DNA template, 1 µl (100 pmol) of each primer and a 25 µl of Taq PCR Master Mix polymerase containing 100 mM Tris-HCl, 500 mM KCl at pH 8.3 at 20°C, 1.5 mM $MgCl_2$, 200 M of each of deoxyribonucleoside triphosphate and 0.025U Taq polymerase (Qiagen, USA) was prepared. Amplification of DNA was performed using Mastercycler PCR machine (Eppendorf Co, Germany). PCR thermocyling conditions for each reaction are shown in Table 2. During the PCR reaction for *ica*A and *ica*D, a further 1U of Taq DNA polymerase was added after the first 30 cycles. Ten microlitres of each PCR product was mixed with 2 µl loading buffer and separated on a 2% agarosegel (Sigma) in TBE buffer. Amplified products were visualized under UVP BioDoct-It digital imaging system (UVP, Inc., Cambridge, UK) after staining with ethidium bromide (Sigma).

RESULTS AND DISCUSSION

Out of 50 urinary *S. aureus* isolates, 36 (72%) were found to be positive MRSA by Oxacillin screening agar. All MRSA strains were positive for the *mec*A genes (Figure 1), 9 (25%) were positive for PVL (Figure 1) and 23 (64%) were positive for both *ica*A and *ica*D genes (Table 3). It was also found that 23 trains that were positive for *ica*A were also positive for *ica*D (Figures 2 and 3).

All MRSA strains isolated by oxacillinscreening agar were found to be *mec*A-positive by PCR. Many studies have evaluated the sensitivity of different culture media and methods for the primary phenotypic detection of MRSA (Monsen et al., 2003; Davies and Zadik, 1997; NCCLS, 2001). In addition, the low-cost of such methods in comparison with PCR based kits make them likely to be used in low resource settings. The results of this study showed that 36 (72%) of *S. aureus* were *mec*A-positive. MRSA UTIs are increasing throughout the world. Similar studies from various countries (Onanuga and Awhowho, 2012; Araki et al., 2002; Martineau et al., 2000) reported different rates of MRSA among urinary isolates. CA-MRSA is more likely to produce PVL than HA-MRSA (Aires-de-Sousa et al., 2006). The results in this study demonstrated that 9 (25%) of MRSA were PVL-positive. All PVL- positive strains were community acquired strain. Although the PVL from *S. aureus* have rarely been described in cases of UTI (Park et al., 2008; Baba-Moussa et al., 2008), PVL carriage appears to be a possible virulent factor particularly among community-acquired strains. The ability of *S. aureus* to form biofilms

Table 1. Primers of genes used in the study.

Primer	Primer Sequence (5'_3')	Product size (bp)	Reference
*mec*A-P4 *mec*A-P7	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162	Milheiriç et al. (2007)
Luk-pvl- F *Luk-pvl*- R	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	433	Lina et al . (1999)
*ica*A-F *ica*A-R	TCTCTTGCAGGAGCAATCAA TCAGGCACTAACATCCAGCA	188	Cramton et al. (1999)
*ica*D-F *ica*D-R	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	198	Cramton et al. (1999)

Table 2. PCR thermocycling conditions.

PCR	Temperature (°C)/Time					Cycle no.
	Initial denaturation	Cycling condition				
		Denaturation	Annealing	Extension	Final extension	
Pvl	94/5 min	94/40 sec	53/40 sec	72/1 min	72/10 min	35
*mec*A	94/5 min	94/40 sec	53/40 sec	72/1 min	72/10 min	35
*ica*A	94/5 min	94/30 sec	55.5/30 sec	72/30 sec	72/1 min	50
*ica*D	94/5 min	94/30 sec	55.5/30 sec	72/30 sec	72/1 min	50

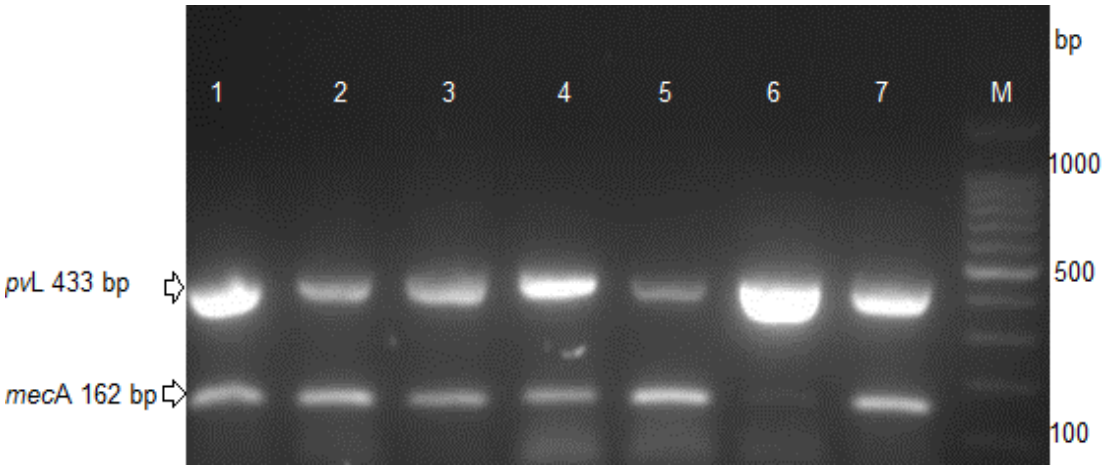

Figure 1. Multiplex PCR assay for *mecA* and PVL gene detection. Lane1; positive control, Lane 2, 3, 4, 5 and 7; 162-bp *mecA* and 433-pb PVL genes fragment, Lane 6; a 433-pb PVL with negative *mecA* gene, Lane M; 100-bp DNA ladder.

Table 3. Frequency of *mec*A, *pv*L, *ica* genes among MRSA isolates.

Gene	Positive no. (%)	Negative no. (%)	Total (%)
*mec*A	36(100%)	0(0%)	36(100%)
*pv*L	9(25%)	27(75%)	
*ica*A	23(64%)	13(36%)	
*ica*D	23(64%)	13(36%)	

helps the bacterium to survive in hostile environments within the host and is considered to be responsible for persistent infections (Christensen et al., 1985; Bernardi et al., 2007). Synthesis of the capsular polysaccharide is, at least in parts, mediated by the *ica* operon. Upon the activation of this operon, a polysaccharide intracellular adhesion (PIA) is synthesized. In the present study, it was observed that 23 (64%) of the MRSA isolates

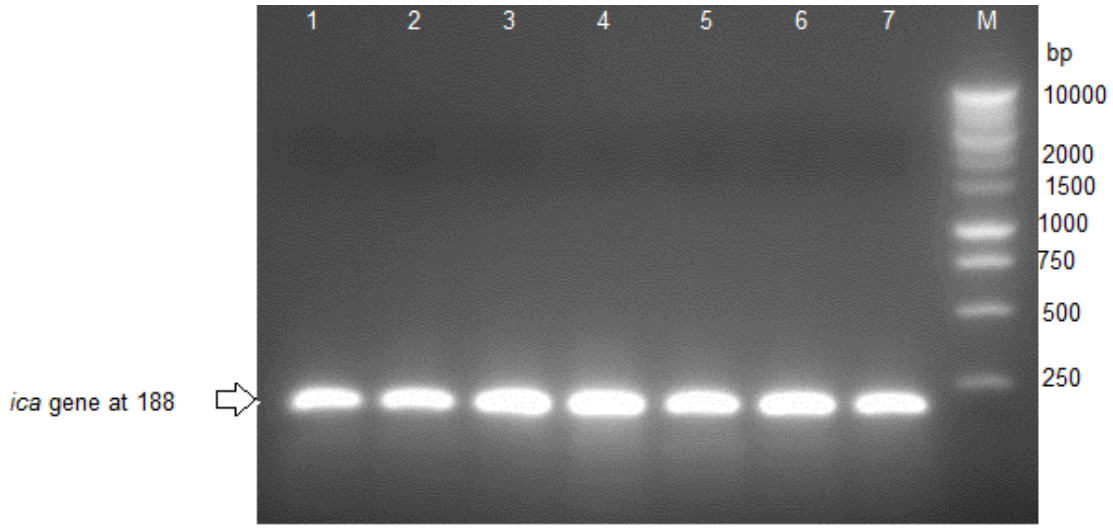

Figure 2. PCR assay for *ica*A gene detection. Lane 1; positive control, Lane 2, 3, 4, 5, 6 and 7; 188-bp *ica*A, Lane M; 1kb DNA ladder.

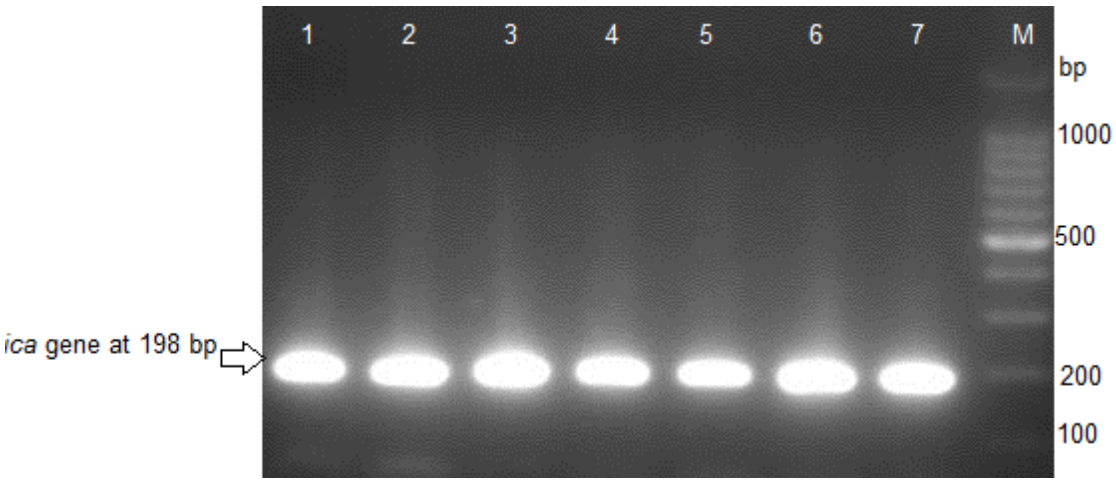

Figure 3. PCR assay for *ica*D gene detection. Lane 1; positive control, Lane 2, 3, 4, 5, 6 and 7; 198-bp *ica*D, Lane M; 100-bp DNA ladder.

harbored loci, *ica* and 9 (25%) harbored *pvL*. These results were higher than that of Park et al. (2008) who believed that the *ica*A genes may enhance the adherence of *S. aureus* to host cells of the urinary tract, and may play a pathogenic role in UTI patients. Several studies described the roles of regulatory elements associated with biofilm formation on the regulation of virulence (Yarwood et al., 2004; Caiazza and O'Toole, 2003). The formation of slime and biofilms by *S. aureus* strains causing catheter-associated and nosocomial infections have been shown to be associated with the presence of the *ica*A and *ica*D genes (Ziebuhr et el., 1997; Arciola et al., 2001). In conclusion, the results of this study suggest that MRSA isolated from UTI patients showed the presence of *mec*A, PVL, *ica* genes, which may have consequences for the treatment of UTI especially in catheter-associated infections. Close surveillance of these strains is essential to monitor their spread among UTIs particularly among hospital inpatients.

Conflict of Interest

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

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Full Length Research Paper

Evaluation of antimicrobial activities of *Rosa damascena* cv. Taifi extract

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Antimicrobial activities were evaluated from the known varied weights of rose petals (50 to 400 mg) and pollens extracts of *Rosa damascena* cv. Taifi in different aqueous solutions and solvents such as sterilized *zamzam* water, distilled water, ethanol, acetone, isoamyl alcohol and 100 millimolar (mM) Tris-HCl at pH 8.7 by using liquid nitrogen. The highest activity was recorded against ethyl alcohol and acetone extracts of *R. damascena* cv. Taifi. The roses were obtained from Al-Kamal factory from *Al-Hada* and Mohammed Bin Othman Farm in *Abha*. The research was conducted at the Department of Biotechnology, Taif University from February to May 2013. The bacterial strains from ATCC and the nutrient agar were utilized for the study. Experiment results shows that the ethyl alcohol and acetone extract of rose's petals and pollen have shown an activity against *Pseudomonas aeruginosa* (ATCC 27853). The acetone extract of rose's petals showed activity against *Candida albicans* (ATCC 14053) and *Pseudomanas aeruginosa* (ATCC 27853). Also, the ethyl alcohol extract of rose's petals had shown activity against *Escherichia coli* (ATCC 25922). The water extract of rose petals showed an activity against *C. albicans (*ATCC 14053) 200 mg/ml. The acetone extract of rose's petals appeared to have a moderate activity against *C. albicans* (ATCC 14053) and showed the highest antimicrobial activities against *P. aeruginosa* (ATCC 27853). Encouraging results were due to the fact that the plants are rich antibacterial sources and further study should be carried out to confirm the purification of the antibacterial compounds.

Key words: Agar well diffusion method, antimicrobial activities, medicinal plant extract.

INTRODUCTION

Rosa damascena cv. Taifi belongs to Rosaceae family, mainly known for its perfuming characteristic and its major products are rose water and essential oil (Lavid et al., 2002). The chemical constituents such as terpenes, glycosides, flavonoids and anthocyanin of this plant have beneficial effects on human health. Its pharmacological

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effects are widespread and most of the central nervous system (CNS) effects are hypnotic, analgesic and anticonvulsant. The respiratory, cardio-vascular, laxative, anti-diabetic, antimicrobial, anti-HIV, anti-inflammatory and antioxidant and other effects of this plant has been proven (Boskabady et al., 2011; Ginova et al., 2013).

R. damascena originated from the Lyzangan Valley near Faris in Iran (Kiani et al., 2008). Farmers shifted it from Iran to Bulgaria in the 17th century and to Turkey in the 1880s. The largest producers of rose oil today are Bulgaria and Turkey (Baydar et al., 2004).

More than 200 roses species and over 18000 cultivar forms of the rose plant have been well known (Gudin, 2000) and used in perfume, medicine and food industry (Jabbarzadeh and Khosh-Khui, 2005). It is mainly known for its perfuming effects (Widrlechner, 1981).

The medicinal functions of Rosaceae are partly due to the presence of phenolics compounds which possess a wide range of pharmacological activities such as free-radical scavengers, antioxidants, anti-inflammatory, anticancer, antimutagenic and antidepressant (Ng et al., 2000; Ren et al., 2003; Hongratanaworakit, 2009). Fresh flower (FF) and spent flower (SF) extracts of *R. damascena* flower were studied against 15 species of bacteria such as *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus feacalis, Escherichia coli, Klebsiella pneumoniae, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus* and *Yersinia enterocolitica* for antibacterial activity and both extracts were effective against all the bacteria except *E. coli.* The FF extract was more effective than the SF extract (Özkan et al., 2004).

The essential oils of *R. damascena* and several plants at their low concentrations exhibited inhibitory and bactericidal activities against Gram-positive *S. aureus* (ATCC 25923); Gram-negative *E. coli* (ATCC 25922); *P. aeruginosa* (ATCC 27853) and yeast *C. albicans* (ATCC 14053) as reported by Lisin et al. (1999).

In this work, the rose petals and pollen from *R. damascene* cv. Taifi were extracted with six different solvents against five micro-organisms.

MATERIALS AND METHODS

The delicate and intense fragrance *R. damascena cv. Taifi was* collected from Al Hada region in Taif and Mohamed Bin Othaman Farm in Abha in early morning hours. The flowers were kept in fridge initially for 2 h. The rose petals and pollen were weighed individually and cleaned initially with sterilized distilled water and then ethyl alcohol.

Liquid nitrogen was poured into the cleaned rose petals and pollen which were taken in already cleaned mortar and pestle, homogenized into a fine powder in order to increase the surface area and facilitate the extraction procedure. The microbial strains *B. subtilis* (ATCC 6633); *C. albicans (*ATCC 14053); *E. col*i (ATCC 25922); *M. luteus* (ATCC 9341); *P. aeruginosa (*ATCC 27853) were obtained from American Type Culture Collection (ATCC). The different solvents used were *zamzam* water; sterilized distilled water; ethyl alcohol; isoamyl alcohol; acetone and 100 millimolar Tris-HCl at pH 8.7. The solvents, sterilized water and z*amzam* water were prepared by autoclaving at 121°C at 15 psig for 20 min. The ethyl alcohol, isoamyl alcohol and acetone were purchased from Loba Chemie Pvt. Ltd, Mumbai, India and Tris-HCl at pH 8.7 was prepared in the laboratory. The chemicals used were liquid nitrogen; NaOH; nutrient agar and nutrient broth were obtained from Himedia Lab Pvt. Ltd., Mumbai, India. NaOH was also purchased from Loba Chemie Pvt. Ltd, Mumbai, India. The *zamzam* water was obtained from the bottling factory under the Saudi Ministry of "King Abdullah Bin Abdul-Aziz *zamzam* project" distribution station in Kuday, Mekkah. Oxytetracycline was obtained from Sigma and stored at 4°C.

The rose petals and pollens with bioactive components were subjected to extraction procedures. Varying amounts (50 to 400 mg) of the powdered material was dissolved in 1.5 ml of each solvent and the extract collected was kept in a shaking incubator at 30°C for 2 h. The organic solvents and water extract was filtered and evaporated until dryness. The extract was stored at 4°C until further use. The individual fractions were then centrifuged at 10000 rpm for 10 min. After centrifugation, the supernatant was kept at -20°C. Rose petal from 50 to 400 mg/ml and pollen of equal weight (100 mg) were used for the study of "Evaluation of Antimicrobial Activities of *R. damascena* cv. Taifi Extract". After extraction, the tubes were kept in the shaker at 150 rpm at 30°C for 2 h and then kept in freezer at -80°C. The bacterial strains were maintained on nutrient agar and freshly prepared sub-cultures in nutrient broth were used during this project. The standard agar-well diffusion was employed to determine the antimicrobial activities for both rose petals and pollen extracts method (Collins et al., 1995). Agar was cooled to 50-60°C before adding any thermo-labile substance (Sambrook and Russell, 2001). Suspension of the bacterial cultures were covered wholly on the agar plates and allowed to dry. Then, in the nutrient agar, 30 wells (6 mm in diameter) were made on each plate using sterile yellow tip. Following this, 50 µl of the test solution, that is, the supernatant of rose extract and pollen were added inside the laminar flow cabinet for 15-20 min to allow the solutions in the wells to diffuse. The agar plates were then inverted and incubated for 24 h at 37°C. After incubation, clear areas in the region of the wells containing antibacterial compounds appeared. This diameter of the clear area (called the inhibition zones) around the wells were measured and recorded. Antibacterial activities of each solvent extract were expressed in terms of average diameter of the inhibition zone (evaluated in milliliter). Each rose extract was tested in the same manner. The concentration and solvents that give the optimum result were identified.

The inhibition zone values of the rose petal and pollen extracts was compared with known standard antibacterial agent oxytetracycline. An accurately weighed 10 mg of oxytetracycline base was put in 96% ethanol in 100 ml standard volumetric flask and the flask was swirled to dissolve oxytetracycline base. Accurately measured portion was diluted with sterilized distilled water to get a known concentration of 0.01 mg of oxytetracycline per millilitre. The values obtained for the five tested microorganisms with oxytetracycline at 1.0 µl/ml are *B. subtilis* (ATCC 6633) 3.5 mm/ml; *E. coli* (ATCC 25922) 6 mm/ml; *P. aeruginosa* (ATCC 27853) 2.9 mm/ml; *M. luteus* (ATCC 9341) 4 mm/ml and *C. albicans* (ATCC 14053) 6.5 mm/ml.

RESULTS

The antimicrobial activity of rose petal extracts of *R. damascena* with the six solvents against different types of microbial strains showed diverse inhibition zones. When compared with all the solvent extractions, the *zamzam*

Table 1. Antimicrobial activities (inhibition zones mm/ml) of Taif rose petals extracted with 6 different solvents against 5 microorganisms.

Solvents of extraction	Bacteria	Rose petals weights in milligrams/ml (50 - 400) Inhibition zones (mm)							
		50	**100**	**150**	**200**	**250**	**300**	**350**	**400**
zamzam	*Bacillus subtilis*	6±1	6±1	8±1	6±1	-	-	10±2	10±2
Distilled water		6±1	8±1	-	-	-	4±2	-	6±1
Ethyl alcohol		4±2	4±2	4±2	8±1	4±2	4±2	-	-
Acetone		-	-	-	-	-	-	-	12±1
Tris-HCl		-	-	4±2	12±1	-	-	6±1	-
zamzam	*Escherichia coli*	4±2	-	-	-	-	4±2	-	4±2
Distilled water		-	4±2	4±2	6±1	-	-	4±2	4±2
Ethyl alcohol		-	-	-	8±1	6±1	10±2	10±2	10±2
Acetone		-	-	-	-	-	4±2	-	-
Tris-HCl		4±2	4±2	-	4±2	4±2	-	4±2	6±1
zamzam	*Candida albicans*	-	4±2	-	-	-	-	-	4±2
Distilled water		-	-	-	10±2	-	-	12±1	-
Ethyl alcohol		8±1	-	-	8±1	4±2	4±2	8±1	6±1
Isoamyl alcohol		-	-	-	4±2	4±2	4±2	-	-
Acetone		4±2	6±1	6±1	-	10±2	12±1	10±2	12±1
Tris-HCl		-	-	-	-	4±2	-	-	-
zamzam	*Micrococcus leutus*	4±2	-	-	-	6±1	-	-	4±2
Distilled water		4±2	4±2	6±1	4±2	4±2	4±2	4±2	4±2
Ethyl alcohol		4±2	4±2	4±2	4±2	4±2	8±1	6±1	4±2
Tris-HCl		6±1	4±2		6±1	-	10±2	6±1	-
zamzam	*Psedomonas aeroginosa*	4±2	6±1	4±2	4±2	-	-	4±2	-
Distilled water		-	-	4±2	6±1	4±2	4±2	4±2	4±2
Ethyl alcohol		8±1	6±1	10±2	12±1	4±2	6±1	6±1	6±1
Acetone		14±2	10±2	14±2	10±2	12±1	14±2	14±2	8±1
Tris-HCl		10±2	-	-	4±2	-	-	-	-

water extracts of rose's petals showed antibacterial activity against *B. subtilis (*ATCC6633) and water extracts of rose petals showed antibacterial activity in *C. albicans* (ATCC 10231), *M. luteus* (ATCC 9341) and *P. aeruginosa* (ATCC 27853). The ethyl alcohol extracts of rose petals showed highest antibacterial activity against *P. aeruginosa* (ATCC 27853) and moderate antibacterial activity against *E. coli* (ATCC8739).

The most effective inhibition value was measured as 13 mm as diameter for *P. aeruginosa* (ATCC 27853) against ethyl alcohol extracts of rose petals. The acetone extracts of rose petals showed clearing zone having a diameter of 6 to 16 mm against *C. albicans* (ATCC 10231) and *P. aeruginosa* (ATCC 27853). The isoamyl alcohol extracts of rose petals showed very little antibacterial activity against *C. albicans (*ATCC 10231). The Tris-HCl at pH 8.9 (100 mM) extracts of rose petals showed less inhibition zone (6 mm) in four microorganisms except, *C. albicans* (ATCC 10231) (Table 1).

Comparing the concentration of the extracts with different solvents in which the inhibition values differ against the tested microorganisms, a concentration of 350 and 400 mg/ml z*amzam* water and 200 and 350 mg/ml water extracts of rose's petals showed anti-bacterial activity against *B. subtilis (*ATCC 6633), 150 and 200 mg/ml ethyl alcohol extracts of rose petals showed highest antibacterial activity against *P. aeruginosa* (ATCC 27853) whereas 200 and 400 mg/ml ethyl alcohol extracts of rose petals showed moderate antibacterial activity against *E. coli* (ATCC8739) and *C. albicans* (ATCC 10231) (Table 1).

The antimicrobial activity of rose pollen extracts of *R. damascena* with the six solvents against different types of microbial strains also showed diverse inhibition zones. When compared with all the solvent extractions, the ethyl alcohol extracts showed highest antibacterial activity

Table 2. Antimicrobial activities (inhibition zones mm/ml) of Taif rose pollen grains extracted with 6 different solvents against 5 different microorganisms.

Pollen/solvent (mg/ml)	*Bacillus subtilis* (mm)	*Escherichia coli* (mm)	*Candida albicans* (mm)	*Micrococcus leutus* ((mm)	*Pseudomonas aeroginosa* (mm)
Pollen/zamzam	-	4±2	10±2	6±1	-
Pollen/distilled water	-	-	8±1	4±2	4±2
Pollen/ethyl alcohol	-	14±2	-	4±2	12±1
Pollen/isoamyl alcohol	-	4±2	4±2	-	-
Pollen/acetone	-	4±2	-	-	8±1
Pollen/Tris-HCl	-	4±2	-	4±2	4±2
Pollen/Tris-HCl	-	4±2	-	4±2	4±2
Oxytetracycline at 10 µl/ml	3.5±0.2	6±0.1	6.5±0.2	4±0.2	2.9±0.1

(among all microorganisms) in *E. coli* ATCC 25922 and *P. aeruginosa* (ATCC 27853). The *zamzam* water extracts of rose pollen extracts showed antibacterial activity against *C. albicans* (ATCC 10231) and *M. luteus (*ATCC 9341*)*. The acetone extracts of pollen showed lesser antibacterial activity in *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) as compared to ethyl alcohol extracts of rose pollen. Nil antibacterial activity was found in *B. subtilis* (ATCC 6633) irrespective of the solvents (Table 2).

The most effective inhibition value was measured as 14 mm as diameter for *E. coli* (ATCC 25922) and 13 mm as diameter for *P. aeruginosa* (ATCC 27853) against ethyl alcohol extracts of rose pollen. The *zamzam* extracts of rose pollen showed clearing zone having a diameter of 12 and 7 mm against *C. albicans* (ATCC 10231) and *M. luteus* (ATCC 9341) respectively. The acetone extracts of rose pollen showed inhibition value of 6 and 9 mm as diameter against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853), respectively. The Tris-HCl at pH 8.9 extracts of pollen showed 6 mm, a less inhibition value against *E. coli* (ATCC 25922), *M. luteus (*ATCC 9341*)* and *P. aeruginosa* (ATCC 27853) (Table 2).

DISCUSSION

The results obtained by the standard agar well diffusion clearing zone method showed that concentrated ethyl alcohol and acetonic extracts had inhibitory effects (upto 16 mm) on most of the tested microorganisms as represented in Table 1. While the concentrated aqueous extract showed variable inhibitory effects on the tested microorganisms (Tables 1 and 2). The ethyl alcohol extracts of rose petals showed highest antibacterial activity against *P. aeruginosa* (ATCC 27853). The earlier antimicrobial study with native isolated bacteria from sea cucumber recorded moderate antimicrobial activity against *P. aeruginosa* as reported by Farouk et al. (2007). The bacteria isolated from flowers of *R. damascena* cv. Taifi also showed antibacterial activity and enzymatic activity was reported by Farouk et al. (2014). At a concentration of 150 and 200 mg/ml ethyl alcohol extracts of rose petals showed highest antibacterial activity against *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 8739). This result complies with other reported research outcomes that the low dilution of alcoholic extract showed a higher antimicrobial activity as compared to high dilution (Hirulkar and Mona, 2010; Farouk and Benafri, 2007). Halawani (2014) also reported that the ethanolic extract of *R. damascena* showed a positive significant bacterial activity. *R. damascena* cv. Taifi should be investigated to better understand its antimicrobial properties, safety and efficiency. In the last two decades, various studies were conducted in many countries to verify such efficiency (Ikram and Inamul, 1984; Izzo et al., 1995; Kubo et al., 1995; Shapoval et al., 1994). Lesser research reports are available for the antimicrobial activity of *R. damascena* pollen. The ethanol extracts of pollen showed higher antimicrobial activity against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) than the acetone extracts of pollen.

Conclusion and recommendations

However, in this study, the ethyl alcohol and acetone extracts of rose petals and pollen of *R. damascena cv. Taifi* had showed moderate result against *P. aeruginosa* and the acetone extract (200 mg/ml) of rose petals showed good antimicrobial activity against *C. albicans* and highest antimicrobial activity against *P. aeruginosa*. In addition, the ethyl alcohol extract (200 to 400 mg/ml) of rose petals showed good result against *E. coli.* The water extract of rose petals showed good result against *C. albicans*. These results are encouraging and promising and further study should be carried out to confirm the purification of the antimicrobial compounds. In this study, with different concentrations of rose petals and pollen extracts, it is shown that *R. damascena cv. Taifi* has medicinal values.

Conflict of Interests

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

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Full Length Research Paper

Chrome agar *Candida* for species level identification of isolates of *Candida* sp. from oral cavity

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Recently, *Candida* has become an important nosocomial pathogen. They are normal flora of skin, mouth, gut and vagina of healthy humans. They become opportunistic with immunocompromised and immunosuppresed individual. Since it is not possible to identify the species directly on Sabouraud's dextrose agar (SDA), CHROM agar for candida is used for easy recognition of species by colour of the colonies. This study was conducted in 38 patients with symptoms of oral candidiasis aged 25 to 75 years. Oral swabs were taken from oral cavity and were cultured on Sabouraud's dextrose agar and CHROM agar for candida. Gram staining and germ tube test were done with the samples. Four different species of *Candida* were isolated from the samples using CHROM agar, that is, *Candida albicans, Candida tropicalis, Candida glabrata* and *Candida krusei*. It was observed that, of the 38 isolates, *C. albicans* was obtained in higher rate (58%) followed by *C. tropicalis* (24%), *C. krusei* (16%) and *C. glabrata* (2%). *C. albicans* produced light green colonies, *C. tropicalis* produced dark blue with purple diffusion colonies, *C. glabrata* showed pink with a darker mauve center colonies, *C. krusei* produced pink with pale borders colonies. Thus, CHROM agar candida medium was found to be helpful in direct and easy identification of multiple yeast species simultaneously.

Key words: *Candida albicans*, oral swabs, CHROM agar, differential medium.

INTRODUCTION

Yeasts, especially *Candida albicans* is a member of the native born microbial flora of the skin, mucous membranes of the gut, mouth and vagina in healthy human. Although, *C. albicans* rarely causes infections in healthy human without predisposing factors, immune-suppressed patients can suffer from mucosal, cutaneous or systemic candidiasis. Oropharyngeal candidiasis is the most common opportunistic infection. Oral thrush is a common form of the oropharyngeal candidiasis and its clinical features include white patches appearing as discrete lesions on the buccal mucosa, throat, tongue and gum linings that develop into confluent pseudo-membranes resembling milk curds (Marsh and Martin, 2009). The incidence of candidiasis has increased markedly with the advent of diseases like AIDS and the development of immune suppressive therapy (Smitha and Shashanka, 2011). Among the various species of *Candida*, *C. albicans* was the most frequently isolated species (72.7%)

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(Back-Brito et al., 2009). Although *C. albicans* remains the major species isolated, non-albicans such as *C. glabrata, C. krusei* and *C. tropicalis* also involved in the incidence of candidiasis. In a study conducted by Vijaya et al. (2011), non *C. albicans* was isolated at a higher rate (55.8%) than *C. albicans*. Isolation and prompt identification of infecting microorganism from the mixed yeast population are required for early antifungal therapy.

Traditional method of identification of *Candida* species is germ tube formation by the fungi in serum (Mackenzie, 1962). In most clinical investigations, fungal pathogens are routinely cultured on Sabouraud's Dextrose Agar (SDA) (Baveja, 2010). The drawback with these media is that, the colonies on these media are very similar in appearance and their subsequent identification requires considerable investigative time (Zarei Mahmoudabadi et al., 2000; Beighton et al., 1995).

CHROM agar for *Candida* is a differential culture medium which facilitates the species level identification of *Candida* isolates of various clinical specimens. These chromogenic media provide different colours of colonies secondary to chromogenic substances that react with enzymes secreted by the organisms (Murray et al., 2005; Yucesoy et al., 2001). A major advantage of these media is that identifications of species can be done in shorter duration within 48 h with great accuracy (Pfaller et al., 1996). In addition, mixed yeast infections are seen in the oral cavity frequently in immunocompromised patients, CHROM agar is useful because differences in the colour of the colony make the identification simple and selective (Odds and Bernaerts, 1994; Pfaller et al., 1996). Therefore, the present study was conducted to evaluate the performance of CHROM agar for the isolation, direct presumptive identification and species differentiation of *Candida* from oral specimens.

MATERIALS AND METHODS

Preparation of CHROM agar *Candida*

CHROM agar *Candida* (Himedia India) was prepared according to the manufacturer's instructions. CHROM agar *Candida* is composed of (per litre): peptone (10 g), glucose (20 g), agar (15 g), chloramphenicol (0.5 g) and Chromogenic mix (2 g). Twelve grams of CHROM agar *Candida* powder (one vial) was added to 250 ml of sterile distilled water in a sterile Erlenmeyer. The suspension was completely dissolved by boiling (<100°C) and mixing. The medium does not require sterilization by autoclave, therefore after cooling in a water bath to 45°C, the agar was poured into sterile Petri dishes (Odds and Bernaerts, 1994). After being allowed to cool, the plates were stored at 4°C prior to use. CHROM agar was prepared as per the instruction manual. *Candida* species isolate were inoculated on CHROM agar and incubated at 37°C for 48 h.

Collection of samples

A total of 38 clinical samples were obtained from patients attending tertiary care Hospital, Coimbatore, TamilNadu, India, with symptoms of oral candidiasis. Oral swabs were collected with all aseptic precautions using sterile swabs from tongue and buccal mucosa by gently rubbing a sterile cotton swab over the lesional tissue (18) (Ax'ell et al., 1985). The swabs were then dispensed in a test tube containing sterile SDA broth.

Processing of samples

The samples were inoculated on HiCHROM *Candida* differential agar and Sabouraud's Dextrose agar and incubated at 37°C for 48 h. From that Gram staining was done. Germ tube test was done which is the standard laboratory method to differentiate the *C. albicans* from other *Candida* species. The test involved the induction of hyphal outgrowths (germ tubes) when sub cultured in serum at 37°C for 2 - 4 h (Williams and Lewis, 2000). Growth on the CHROM agar was observed with in 24 h in most of the cases. For few isolates, the plate had to be incubated for up 48 h to appreciate the growth. Colour of the colonies was noted and the species was identified.

RESULTS AND DISCUSSION

A total of 38 species isolated from oral specimen were studied for morphological characteristics by Gram staining and cultural characteristics by growth on SDA. Gram positive budding yeast cells were observed in Gram staining. On SDA (Figure 1a) creamy white colored, smooth, pasty convex colonies were observed. After 48 h incubation at 37°C, positive cultures produced colonies of 1 to 5 mm in diameter. On CHROM agar appearance of *Candida* species were as follows: *C. albicans* - Green (Figure 1b), *C. tropicalis* - metallic blue (Figure 1c), *C. krusei* - pink (Figure 1d) and *C. glabrata* - Mauve (Table 2).

The germ tube test (Figure 1e) was used for the confirmation of *C. albicans*. *C. albicans* alone gave positive result for germ tube test (Table 2). A distribution of *Candida* species isolated is shown in Table 1. Of the 38 isolates obtained, predominantly isolated *Candida* species was *C. albicans* (58%) and then *C. tropicalis* (24%), *C. krusei* (16%) and *C. glabrata* (2%). Distribution of specimen between different age group is shown in Table 3.

Among the 38 *Candida* species isolated from the oral cavity, *C. albicans* was found to be predominant with 58%. This observation correlated with the previous studies. Manjunath et al. (2012) found that, *C. albicans* was the most common isolate from both HIV and non-HIV infected patients. This observation was also reported (Odds and Bernaerts, 1994; Al-Dwairi et al., 2014). According to Back-Brito et al. (2009) and Williams and Lewis (2000), the majority of yeast isolates from oral swabs were *C. albicans*, but it was often recovered in association with other yeasts. This was followed by *C. tropicalis* 24%, *C. krusei* 16% and *C. glabrata 2*% (Table 1). In our study, the isolation rates of *Candida* species is high in ages ranging from 36-70 years old. This observation is more or less similar with the results shown by Zaremba et al. (2006) and Pinho Resende et al. (2002).

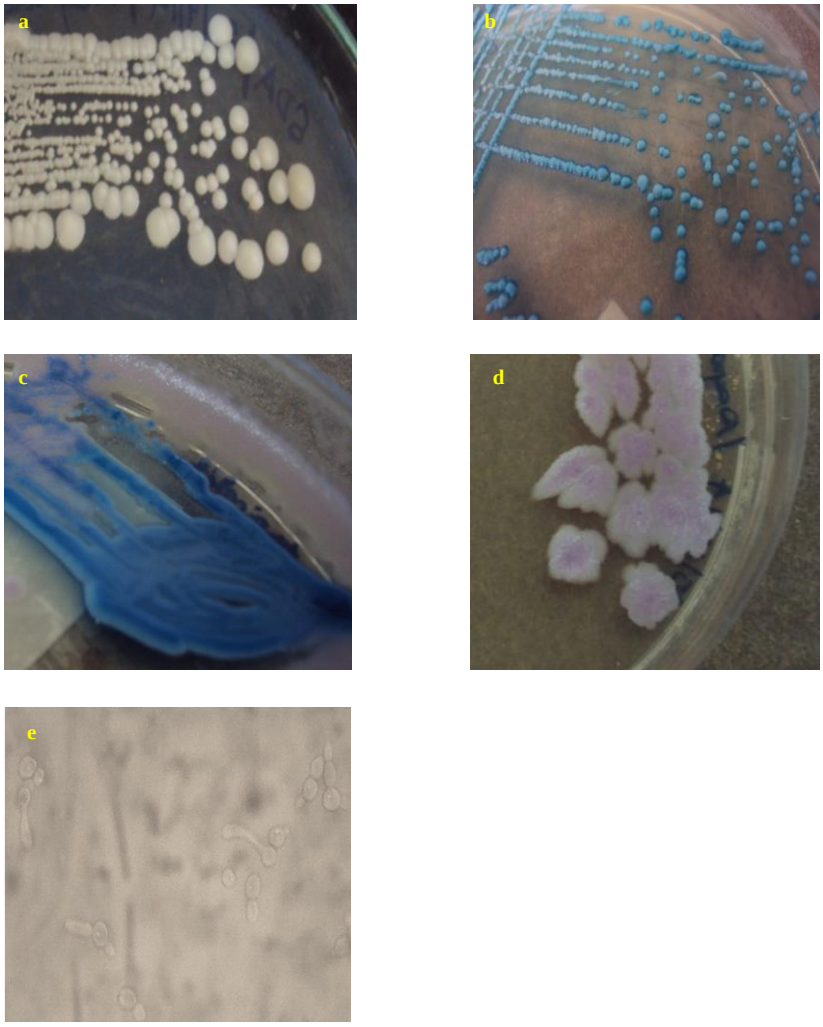

Figure 1. Growth of *Candida* sp. on SDA (a), *Candida albicans* on CHROM agar (b), *Candida tropicalis* on CHROM agar (c), *Candida krusei* on CHROM agar (d) and Germ tube formation by *Candida albicans* (e).

Table 1. Distribution of different species of *Candida* isolated from oral cavity

Species	No. of isolates	Percentage of isolates
C. albicans	22	58%
C. tropicalis	9	24%
C. krusei	6	16%
C. glabrata	1	2%

Table 2. Growth characteristics of *Candida* species isolated from oral cavity.

Species	Growth on CHROM agar	Germ tube test
C. albicans	Green	Positive
C. tropicalis	Metallic blue	Negative
C, krusei	Pink	Negative
C. glabrata	Mauve	Negative

Table 3. Age distribution between the collected isolates.

Age (years)	No. of isolates	Percentage of isolates
21-35	9	24%
36-50	17	45%
51-70	12	31%
Total	38	100%

It has generally been assumed that old age represent a predisposing condition for increased candidal colonization. Lockhart et al. (1999) found that frequency and intensity of carriage of candidal colonization increased as a function of age. According to Sumitra and Megha (2014), sensitivity and specificity of CHROM agar for *C. albicans* were 100 and 96%, *C. tropicalis* were 100% and 100%, *C. krusei* were 100% and 100% and *C. glabrata* 75% and 100%, respectively. Germ tube test has been the gold stranded method for species differentiation of *Candida* yeast. But it may lead to false positive and false negative results. Though SDA has been used for routine culturing of yeast cultures, precise identification by colony appearance is not possible with mixed cultures (Jean-Philippe et al., 1996). In our study, with in 48 h, candida species were differentiated based on colony colour and morphology.

Hence, the identification of *Candida* species is technically simple, rapid and cost effective as compared to technically demanding time consuming and expensive conventional method.

In recent years, other differential media have been developed that allow identification of certain *Candida* species based on colony appearance and colour following primary culture (Houang et al., 1997). The advantage of such media is that the presence of multiple *Candida* species in a single infection can be determined which can be important in selecting subsequent treatment options (Odds and Bernaerts, 1994). CHROM agar *Candida* is a new Chromogenic differential culture medium that is used for the isolation and identification of some of the most clinically important yeast pathogens on the basis of colony colour. CHROM agar *Candida* has previously been shown to be an effective and selective medium for the direct identification of *Candida* species from clinical materials (Odds and Bernaerts, 1994; Pfaller et al., 1996). This medium has previously also been used for the isolation and identification of yeasts from dental samples (Beighton et al., 1995) and from swabs of soft tissues in oral cavity (Odds and Bernaerts, 1994). A major advantage of CHROM agar is the ability to detect mixed cultures of yeasts in clinical specimens.

Conclusion

CHROM agar *Candida* medium was found to be helpful, allowing direct and presumptive identification of *C. albicans* and the easy recognition of association of multiple yeast species. Thus, CHROM agar for *Candida* was proved to be easy to use, time saving and appears to be well suited for routine use in the clinical mycology laboratories.

Conflict of Interests

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

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Full Length Research Paper

Antimicrobial assay and phyto-chemical analysis of *Solanum nigrum* complex growing in Kenya

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Solanum nigrum **complex is a group of plants used as indigenous vegetables and also a source of traditional medicine in Kenya and other parts of the world. The main objective of this study was to determine the antimicrobial property and phytochemical composition of *S. nigrum* complex. Samples of *S. nigrum* complex were collected from Kenya. The plants were dried under the shade and then ground to a fine powder. Crude extracts were prepared from the plants using methanol and evaluated for antifungal and antibacterial activities against the fungi *Fusarium culmorum, Fusarium avenaceum, Fusarium moniliforme* and bacteria *Staphylococcus aureus, Salmonella typhi, Pseudomonas aureginosa, Proteus mirabilis, Shigella* spp., *Pseudomonas syringae, Escherichia coli* and *Basillus subtilis*. Minimum inhibition concentration (MIC) of the samples against active microbes was determined. Phytochemical composition was analysed. T-test was used to analyse the significance of the activity indexes of the extracts against the different groups of the microbes. The samples collected were found to belong to the species *S. nigrum, Solanum scabrum* and *Solanum villosum* which all belong to the *S. nigrum* complex. The samples showed a considerable antibacterial and antifungal activity against the tested microbes. The highest antibacterial activity was 29.00 mm which was shown by *S. villosum* species against *S. aureus*. The antibacterial and antifungal activities were dose dependent with low MIC values as such 0.09 g/ml given by *S. nigrum* Mill species against all eight tested bacteria. The antimicrobial activity was associated with the wide array of phytochemical compounds observed in the samples and they include tannins, saponins, flavonoids, steroids, terpenes, phenolic compounds, alkaloids and cardiac glycosides. It was concluded that the *S. nigrum* complex is rich in many phytochemical compounds like saponins, flavanoids, steroids, glycosides, terpenoids among others which are responsible for the antimicrobial activities of the plants. It can be used not only in treatment of plant fungal infections but also in the management of bacterial human diseases and further research is recommended.**

Key words: *Solanum nigrum* complex, phytochemical, antimicrobial.

INTRODUCTION

Solanum nigrum complex comprise of both native and bred *Solanum* species used as vegetables and source of fruits in Kenya and other parts of the world (Schippers, 2000). Both indigenous (wild types) and modified (hybrid)

varieties have been cultivated (Peter et al., 2009). These plants are believed to have a high nutritional value. The leaves are eaten as vegetable in most parts of the world while the ripe seeds are also edible (Edmonds and Chweya, 1997). There are several species which belong to the *S. nigrum* complex such as *S. nigrum, Solanum villosum, Solanum scabrum, Solanum americana, Solanum burkankii,* and *Solanum schenopodioides* among others.

All these species highly resemble each other and these pose a problem in the taxonomy of the species (Edmonds and Chweya, 1997). Species in the *S. nigrum* complex exhibit considerable genetic variation, both florally and vegetatively. These variations occur in different populations of the same species. Sometimes, the character may be genetically controlled in one variant, but phenoltypically plastic in another (Edmonds and Chweya, 1997).

Other than being used as vegetables, *S. nigrum* complex also forms an important part of traditional medicine in Africa. In Kenya, unripe fruits are used to soothe toothache. They are also squeezed on babies' gums to ease pain during teething. Leaves are used to treat stomach-ache and extracts from leaves and fruits are used to treat tonsillitis (Edmonds and Chweya, 1997).

In Africa, bacteria and fungi are a major challenge in both medical and agricultural fields; they cause fatal infections to humans, animals and plants. People have been using the conventional medicine made of artificial chemicals to manage and treat these infections. Some of these treatments are supposed to be used for a very long time for example treatment of tuberculosis and opportunistic infections due to human immunodeficiency virus (HIV).

Most of these synthetic drugs are also too expensive and unaffordable to most people. Given that plants in the *S. nigrum* complex are edible and universally acceptable as vegetables, there is need to establish their antimicrobial potential with a view of using them as herbal medicine for management of various diseases.

Research shows that herbal remedies have been used successfully and they pose fewer side effects as compared to the synthetic medicines. Species in the *S. nigrum* complex have been used for treatment of microbial and non-microbial diseases successfully in the traditional medicine but no research has been done in Kenya to establish its efficacy. There are very many different species and variants of *Solanum* that are grown in the country and are used traditionally both as a vegetable and as traditional medicine.

Evidently, there are no sufficient scientific studies that confirm the antimicrobial properties of the *S. nigrum* complex in Kenya. This study shows the *in vitro* antimicrobial activity of these plants against pathogenic microorganisms that cause the most common cases of infectious diseases in Kenya.

METHODOLOGY

Samples were collected from various farmers in Western Kenya. Voucher specimens of the plants were stored at the herbarium at the University of Eldoret under reference number SN23/12.

Preparation of plant sample

The samples were dried in a dark room up to a constant weight for a period of one month, turning the plants up and down daily to ensure they dried evenly without fungal growth or spoilage. The samples were then pulverized using a super mixer grinder and the powdered plants were parked in well labelled paper bags and stored at room temperature.

Preparation of crude extracts

Powdered samples (500 g) were soaked overnight in 1400 ml of distilled methanol and filtered. The residue was re-extracted three times with 500 ml of methanol and the filtrates combined. The combined filtrates were concentrated using a rotary evaporator with the water bath temperature maintained at 70°C to prevent thermal decomposition of labile compounds. The samples were placed in clean dry bottles to await further analysis.

Screening for antifungal and antibacterial activity

Preparation of the sample for antimicrobial screening

Ten (10) samples were screened. The first five samples (*S. nigrum* from Kisumu, *S. nigrum* from Bungoma, *S. cabrum* from Kakamega, *S. villosum* from Bungoma, and *S. scabrum* from Bungoma) were weighed to an approximate mass of 50 mg and dissolved in 2 ml of purified methanol to make an approximate concentration of 25 mg/ml. The last five samples (*S. villosum* from Eldoret (Ziwa), *S. scabrum* from Kisumu, *S. villosum* from Eldoret (Ziwa1) *S. nigrum* from Kakamega and *S. scabrum* from Eldoret AMP) were prepared at approximate concentration of 100 mg/ml.

Test microorganisms

The following eleven test microorganisms were used in antimicrobial sensitivity tests; Gram positive bacteria: *S. aureus, Basillus subtilis* and *Proteus* ssp.*;* Gram negative bacteria: *Escherichia coli*, *Shigella* ssp., *Salmonella typhi, Pseudomonas* ssp. and *Pseudomonas syringae*; Fungi: *Fusarium culmorum, Fusarium avenaceum* and *Fusarium moniliforme.* The strains were all obtained from Kenya Medical Research Institute in Nairobi, Kenya.

Preparation of McFarland solution

The McFarland solution was prepared by mixing water solutions of 1% (w/v) anhydrous barium chloride and 1% (v/v) sulphuric acid (H_2SO_4) in the ratio of 0.05:9.95 respectively (McFarland, 1907).

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Preparation of the inoculums

Nutrient broth and sterile distilled water were used to prepare broth cultures of bacterial and fungal test organisms respectively. The nutrient broth media was prepared according to the manufacturers' instructions. The autoclaved media and distilled water were aseptically transferred to sterile capped test tubes of about 6 ml each. Pure isolates of sub-cultured bacteria and fungi colonies were aseptically transferred to the respective broth media and the concentration of the inoculum adjusted to make an approximate cell concentration of 1.0×10^8 cells /ml (Baris et al., 2006) with turbidity approximates that of McFarland 0.5 standard.

Antimicrobial sensitivity tests

Disc diffusion method

Antimicrobial sensitivity tests of the methanol extracts was determined by disc diffusion assay method as described by Rojas et al. (2006) and Moshi et al. (2006). Muller Hilton agar and Sabouraud dextrose agar (SDA) were prepared for bacteria and fungi respectively. The media was autoclaved and allowed to cool in a 45 to 50°C water bath. The freshly prepared and cooled media was transferred to Petri dishes (90 mm in diameter) in a Laminar flow to give a uniform depth of approximately 4 mm. The agar media was allowed to cool and solidify at room temperature. About 0.2 ml of the test inoculum was evenly spread on the surface of the solidified agar media using a sterile cotton swabs. 10 µl of each sample solution was dispensed on sensitivity blank discs using a micro-dispenser and the discs allowed drying in a laminar flow hood for about 4 h after which they were placed at the centre of the labelled inoculated plates.

The treated plates were stored in a refrigerator at 4°C for 24 h to allow sufficient diffusion of the samples into the media without microbial growth and then transferred to incubator at 37°C for 24 h for bacterial and seven days for fungal. The test was carried out in triplicates. Antimicrobial activities were determined by measuring the diameters of zones of inhibition (activity index) in millimetres. Negative control was prepared by dispensing 10 µl methanol on blank discs and allowed to dry. Chloramphenical discs (30 µg/disc) and nystatin discs (25 µg/disc) were used as a positive control for bacterial cultures and fungal cultures, respectively. Some inoculated plates were prepared without any disc as a proof for viability of the inoculums. The plates were put in the same conditions as the plates with test samples and then their activity indexes were determined and recorded.

Minimum inhibition concentration (MIC) assay for antimicrobial tests

Sample preparation

A stock solution with a concentration of 25 mg/ml was prepared for each extract using methanol as the solvent. A series of dilutions with concentrations of 90, 80, 70, 60, 50 and 40% were prepared using distilled methanol. The antimicrobial activity tests were performed using these solutions as per method described earlier.

Determination of the phyto-chemical composition

Phyto-chemical tests were carried out on the methanol crude extracts of *S. nigrum* complex leaves using standard procedures to identify the constituents. The presence of alkaloids and flavonoids was determined as described by Sofowora (1993), while the methods described by Edeoga et al. (2005) was used for terpenoids, tannins, phlobatannins, saponins and cardiac glycosides. Steroids were detected using the method of Shanmugavalli et al. (2009). Presence of the compounds was identified by specific colour changes.

Statistical analysis

T-test was used to determine the level of significance between the antimicrobial results of the extracts at different sample concentrations. The efficacy of the samples against Gram negative bacteria and Gram positive bacteria was also compared, Statistical Package for Social Sciences version 16.0 (SPSS) was used for this analysis. The means and standard deviations (±SD) of the diameters of zones of growth inhibitions for the treatments were determined using Microsoft Excel software.

RESULTS AND DISCUSSION

The samples had different morphological characteristics. These morphological characteristics included the colour of flowers which was either yellow (*S. nigrum*, *S. villosum*) or purplish yellow (*S. scabrum*); colour of berries which was either black (*S. nigrum* and *S. scabrum*) or orange (*S. villosum*) when ripe; leaf size also differed among the species. Some leaves were wider (*S. cabrum*) while others were narrow (*S. nigrum* and *S. villosum*). There were also differences in the size of berries; some were smaller (about 5 mm diameter) (*S. nigrum* and *S. villosum*) while others were larger (20 mm in diameter) (*S. scabrum*). Some of the leaves had smooth edges (*S. scabrum* and *S. nigrum*) while others had serrated edges (*S. villosum*).

The samples showed variations in the percentage yields (Table 1). *S. villosum* of Bungoma had the highest yield of 15.01% while *S. scabrum* of Eldoret had the least (6.14%). The variation was attributed to the difference in the amount and type of the soluble compounds in the samples (Edeoga et al., 2005). The rest samples had the percentage yields ranging between these two values.

The different species of the *S. nigrum* complex showed a considerable amount of antibacterial activity. *S. villosum* of Bungoma was active on all the tested microbes. It gave the best activity of 29 mm against *S. aureus*. *S. scabrum* from Eldoret AMP was also active on all tested microbes followed by *S. nigrum* of Kakamega. The results showed that the three species of the *S. nigrum* complex studied had very good activity on both Gram negative and Gram positive bacteria tested. However, the results showed that differences in activity vary with concentration of the sample just like observed by other scientists in the previous studies on the same plants (Sheen, 2009; Aliero and Afolayan, 2005). The results showed that there was more antibacterial activity seen for the samples at higher concentration than for the samples at lower concentration. This was statistically proven by the T-test results where the T value of the paired means which was 24.52 was out of the expected range of 5.925 - 6.959 at 95% confidence interval

Table 1. Percentage yields of extracts from *S. nigrum* complex

Samples	K01	K02	KO3	K04	K05	K06	K07	K08	K09	K10
Plant dry mass used (g)	174.37	500	500	500	500	500	200	350	350	500
Mass of liquid phase (g)	9.76	7.12	9.27	8.26	11.13	15.76	12.05	10.08	9.43	15.24
Mass of solid phase (g)	12.40	25.30	34.40	66.80	59.50	23.66	17.39	11.79	19.38	15.45
Total mass of crude extract (g)	22.16	32.42	43.67	75.06	70.63	39.42	29.44	21.87	28.81	30.69
Percentage yield	12.70%	6.48%	8.73%	15.01%	14.12%	7.88%	14.72%	6.24%	8.23%	6.14%

K01 = *S. nigrum*; Kisumu, K02 = *S. nigrum*; Bungoma, K03 = *S. cabrum*; Kakamega, K04 = *S. villosum*; Bungoma, K05 = *S. scabrum*; Bungoma, K06 = *S. villosum*; Eldoret (Ziwa), K07 = *S. scabrum*; Kisumu, K08 = *S. villosum*; Eldoret Ziwa (A1), K09 = *S. nigrum*; Kakamega, K10 = *S. scabrum*; Eldoret AMP.

Table 2. MIC values of the liquid samples against bacteria.

Bacterial	MIC values (g/ml)									
	Samples									
	K01L	K02L	K03L	K04L	K05L	K06L	K07L	K08L	K09L	K10L
E. coli	0.49	0.60	0.40	0.70	0.42	0.09	1.20	0.22	0.30	0.60
S. aureus	0.35	0.20	0.16	0.10	0.14	0.09	0.70	0.22	0.16	0.60
P. syringae	0.21	0.70	0.56	0.60	0.35	0.09	1.20	0.22	0.14	0.30
B. subtilis	0.49	0.70	0.64	0.60	0.35	0.09	0.20	0.22	0.04	0.20
P. mirabilis	0.14	0.80	0.48	0.20	0.14	0.09	1.20	0.16	0.30	0.20
S. typhi	0.49	0.50	0.16	0.70	0.35	0.09	1.20	0.22	0.12	0.80
Shigella spp	0.75	0.70	0.56	0.60	0.49	0.09	0.60	0.22	0.14	0.30
P. aureginosa	0.75	1.01	0.82	1.02	0.73	0.09	1.20	0.22	0.12	1.32
Mean	0.46	0.65	0.47	0.57	0.37	0.09	0.94	0.21	0.17	0.54

K01 = *S. nigrum*; Kisumu, K02 = *S. nigrum*; Bungoma, K03 = *S. cabrum*; Kakamega, K04 = *S. villosum*; Bungoma, K05 = *S. scabrum*; Bungoma, K06 = *S. villosum*; Eldoret (Ziwa), K07 = *S. scabrum*; Kisumu, K08 = *S. villosum*; Eldoret Ziwa (A1), K09 = *S. nigrum*; Kakamega, K10 = *S. scabrum*; Eldoret AMP.

(Appendix 1). The antibacterial activity of the tested samples was concentration dependent (Sheeba and Thambidurai, 2009). *S. nigrum* from Kisumu, *S. nigrum* from Bungoma, *S. cabrum* from Kakamega, *S. villosum* from Bungoma and *S. scabrum* from Bungoma, were all active against *S. typhi*, *P. aureginosa* and *E. coli.* These bacteria are notorious in causing gastro-intestinal and urinary tract infections, dermatitis, bacteremia (Kenneth, 2012), traveller's diarrhea, typhoid fever, paratyphoid fever, salmonellosis and other nosocomial infections. *S. nigrum* and *S. scabrum* are capable of managing these infections. This explains the successful use of the extracts from *Solanum* species in traditional treatment of stomach infections (Edmonds and Chweya, 1997). The *S. villosum* from Eldoret (Ziwa) had no significant effect on any tested microbe. However, *S. scabrum* from Kisumu had significant effect on *B. subtilis* only. *S. nigrum* from Kakamega, and *S. scabrum* from Eldoret AMP had significant effect on all tested microbes except on *S. aureus.* Variation in activity was also associated with variation in the presence of many phytochemical compounds in the samples (Table 4) (Gugulothu et al., 2011).

The samples showed a variation in the MIC values against the different bacteria (Table 2). For instance, the lowest MIC from *S. nigrum* Mill from Kisumu, was 0.14 g/ml on *P. mirabilis*. For *S. nigrum* from Bungoma, the lowest MIC was 0.20 g/ml on *S. aureus*. For *S. scabrum* from Kakamega, the lowest MIC was 0.16 g/ml observed on *S. aureus* and *S, typhi*. For *S. villosum* from Bungoma, the lowest MIC was 0.10 g/ml on *S. aureus*. *S. scabrum* from Bungoma had the lowest MIC of 0.14 g/ml on *S. aureus* and *P. mirabilis*. *S. villosum* from Eldoret had the lowest MIC of 0.09 g/ml on all microbes tested both Gram negative and Gram positive bacteria. *S. scabrum* from Kisumu had the lowest MIC of 0.20 g/ml on *B. subtilis* and *S. villosum* from Eldoret, Ziwa had the lowest MIC of 0.16 g/ml on *P. mirabilis* and on the rest of the test microbes an MIC of 0.22 g/ml. *S. nigrum* from Kakamega had the lowest MIC of 0.04 g/ml on *B. subtilis* and *S. scabrum* from Eldoret, AMPATH had the lowest MIC of 0.20 g/ml on *B. subtilis* and *P. mirabilis*. This variation was associated with the variation in type and amount of phytochemical compounds like saponins and cardiac glycosides, which were found to be present in all the samples and have been proven to have antibacterial activity (Harbone, 1973; Foerster, 2006; Al-Bayati and Al-mola, 2008). Other compounds like tannins, sterols and

Table 3. MIC values of the liquid samples against fungi.

Fungi	MIC values (g/ml)				
	Samples				
	K01L	K02L	K03L	K04L	K05L
F. culmorum	0.35	0.20	0.16	0.70	0.28
F. moniliform	-	0.60	-	0.90	-
F. avenaceum	0.35	0.70	0.24	0.30	0.14
Mean	0.35	0.50	0.20	0.63	0.21

K01 = *S. nigrum*;Kisumu, K02 = *S. nigrum*; Bungoma, K03 = *S. cabrum*; Kakamega, K04 = *S. villosum*; Bungoma, K05 = *S. scabrum*; Bungoma.

Table 4. Phytochemical composition of liquid samples of *S. nigrum* complex.

Compound	K01L	K02L	K03L	K04L	K05L	K06L	K07L	K08L	K09L	K10L
Saponins	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	-	-	-	-	-
Sterols	+	+	+	+	+	+	-	-	-	-
Cardiac glycosides	-	+	+	+	+	+	+	+	+	+
Flavanoids	+	+	+	+	+	+	+	+	+	+
Phlabotanins	-	-	-	+	-	+	+	+	+	+
Terpenoides	+	+	+	+	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+	+	+	+	+
Phenolic compounds	+	+	+	+	+	+	+	+	+	+

(+) Represent presence of the tested compound in the sample, (-) represent absence of the tested compound in the sample, L - liquid samples, K01 = *S. nigrum*; Kisumu, K02 = *S. nigrum*; Bungoma, K03 = *S. cabrum*; Kakamega, K04 = *S. villosum*; Bungoma, K05 = *S. scabrum*; Bungoma, K06 = *S. villosum*; Eldoret (Ziwa), K07 = *S. scabrum*; Kisumu, K08 = *S. villosum*; Eldoret Ziwa (A1), K09 = *S. nigrum*; Kakamega, K10 = *S. scabrum*; Eldoret AMP.

phlabotanins were present in some samples and absent on others in no particular order (Table 4). This qualitative variation and the possible quantitative variation in the presence of the phytochemical compounds were responsible for the variation in MIC values of the samples against the different bacterial species. Higher MIC values show that the bacteria are resistant to the test sample (Sheen, 2009).

S. nigrum from Kisumu was tested against *F. culmorum* and *F. avenaceum* giving activity indexes of 0.35 g/ml for both fungi. *S. nigrum* from Bungoma was analyzed for MIC against *F. culmorum, F. moniliform* and *F. avenaceum* with the MIC values of 0.2, 0.6 and 0.7 g/ml for the three fungi respectively. *S. scabrum* was analyzed for MIC against *F. culmorum* and *F. avenaceum.* With the two fungi having MIC values of 0.16 and 0.24 g/ml, respectively. *S. villosum* from Bungoma was tested for MIC value against the three fungal species. The MIC values were 0.7, 0.9 and 0.3 g/ml for the fungi *F. culmorum, F. moniliform* and *F. avenaceum*, respectively. *S. scabrum* from Bungoma was analyzed for MIC against the fungi *F. culmorum* and *F. avenaceum* with MIC values of 0.28 g/ml and 0.14 g/ml, respectively (Table 3).

F. culmorum and *F. moniliforme* were inhibited by *S. nigrum, S. scabrum* and *S. villosum* although the later had very low inhibition zones as compared to the standard antifungal, nystatin. *F. avenaceum* was inhibited by *S. nigrum* and *S. villosum*. These are *Fusarium* species of fungi known to cause plant infections, food poisoning and various mycoses. Therefore these samples were considered as potential treatments for the three fungi. *S. nigrum* complex species which lacked tannins had no effect on the fungi tested. According to Victor et al. (2005), tannins have considerable antifungal activity. *F. culmorum* is plant pathogenic and causes seedling blight, foot rot, ear blight, common root rot and other diseases of cereals, grasses and a wide variety of monocots and dicots (Rodriguez and Regina, 2008). It is also capable of producing mycotoxins in plants especially cereals. The most common mycotoxins of *F. culmorum* in wheat are deoxynivalenol (DON) and 3-acetyldeoxynivalelon (3-ADON) which are harmful to human and animal health (Marasas et al., 1984). This shows a potential application of these plant samples in control and treatment of plant diseases as well as preventing food poisoning by mycotoxins in cereals and grains. *F. avenaceum* is a globally distributed fungus commonly isolated from soil and a wide range of plants. Severe outbreaks of crown and stem rot of the flowering ornamental, lisianthus (*Eustoma grandiflorum*), have been

attributed to *F. avenaceum*. This fungus has been reported to be an occasional pathogen of stored cabbage (Geeson, 1983).

Extracts of the *S. nigrum* complex were found to poses a wide range of different phytochemical compounds. These compounds are tannins, saponins, terpenes, terpenoids, cardiac glycosides, phenolic compounds, anthraquinones, flavanoids and phlobatanins. All these compounds are known to be biologically active and hence the observed antimicrobial activity (Kessler et al., 2003). All the plant extracts that contained tannin can therefore be recommended for treatment of diarrhoea (Yu et al., 2000; Gertrudes, 2006), inhibit multiplication of retroviruses because of their ability to inhibit reverse transcriptase (Nonaka et al., 1990), treatment of asthma and other respiratory diseases (Burkil, 1994). These tannins can be extracted and used to prepare insecticide for spraying on plants (Buttler, 1998). Tannins have been reported to be toxic to filamentous fungi, yeasts and bacteria (Scalbert, 1991). Their antimicrobial action is made possible by their capacity for protein complexation through hydrogen and covalent bonding and inactivation of microbial adhesions, enzymes and cell envelope transport proteins (Haslam, 1996). The consumption of tannins as green teas (Gertrudes, 2006) and wines prevents different illnesses (Serafini et al., 1994) and inhibits viral reverse transcriptase. Flavonoids were present in the extracts. Their health promoting effects include anti-inflammatory, anti-viral, anti-cancer, anti-oxidant and anti-allergic effects (Balch and Balch, 2000) which make *S. nigrum* complex samples useful in all these medical applications. Saponins found in the samples are known to reduce the level of low density cholesterol and are therefore useful in human diet for controlling cholesterol levels (Assiak et al., 2001). They also control cancer by interfering with cholesterol rich membranes of cancer cells (Dong et al., 2005). They also have antioedema and immunoregulatory effects (Victor et al., 2005), antibacterial, antifungal, anti-inflammatory, antiviral (Al-bayati and Al-mola, 2008) and anti-protozoan (Cheeke, 1998) activities which add to the beneficial application of *S. nigrum* complex.

Another class of compounds which was found in all the extracts was steroids which have important application in the pharmaceutical companies in the production of sex hormones used to bring hormonal balance in expectant and lactating women. It brings libido to men and solves other fertility problems (Victor et al., 2005). This may be the reason why the leaves of *S. nigrum* are recommended as vegetable for expectant mothers or breast feeding mothers to ensure their hormonal balance. This is because steroidal structure could serve as potent starting material in synthesis of these hormones (Okwu, 2001). It has been proven that steroids also have antimicrobial effects against bacteria causing stomach infections. It has also been shown to have analgesic and anti-inflammatory effects, and also used in decreasing serum cholesterol levels (Cyberlipid, 2008). Cardiac glycosides were present in all the samples except *S. nigrum* from Kisumu. Therefore, all the studied species of *S. nigrum* complex can be used in treatment of cardiac disorders (Clifford et al., 1973; Leverin and McMatron, 1999). Phlobatanins and alkaloids were not common in most samples, but they were found to be present in *S. villosum* of Bungoma. Research shows that these compounds have antimicrobial (Ogunwenmo et al., 2007), anticancer (Sneden, 2005), anti-inflammatory (Sofowora, 1993) effects and also act as immune boosters (Jeffery and Harborne, 2000). Alkaloids have their antibacterial effect based on the fact that they help the white blood cells to dispose harmful micro-organisms, (Jeffery and Harbone, 2000).

Glycosides are responsible for the characteristic bitter taste of the black night shade. They are in high concentration in unripe berries and they prevent insects and birds from feeding on the immature fruits and seeds. They also prevent decay of damaged plant tissues (Ogunwenmo et al., 2007). Phenolic compounds, especially the hydroxylated phenols have been found to be toxic to microorganisms with their relative activity increasing with increasing level of oxidation (Scalbert, 1991), number of hydroxyl groups attached to the main structure and their specific sites (Pashin et al., 1986). Phenols and flavonoids have antibacterial activity associated with their ability to complex with nucleophilic amino acids in proteins and the bacterial cell wall leading to destruction of the protein structure and subsequent enzyme inactivation and loss of function (Mason and Wasserman, 1987).

Conclusion and recommendation

The *S. nigrum* complex of Western Kenya comprises of the *S. nigrum, S. scabrum* and *S. villosum*. These different species of the genus *Solanum* contain both antibacterial and antifungal activities against both plants and animal/human pathogens. The study also showed that the antimicrobial activity in these species is due to the presence of a wide array of phytochemical compounds which include saponins, terpenes, sterols, flavonoids, phenolic compounds, cardiac glycosides, tannins, phlabotannins and alkaloids. More work can be done in order to find out the mechanism of inhibition by these plant species which could prove to be of much importance in controlling some of the pathogens which have resistance to the existing antibiotics available in the market.

Conflict of Interests

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

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APPENDIX 1: T- test for comparing the antimicrobial activity of samples at concentrations of 100 mg/ml and 25 mg/ml

******* Two-sample T-test (paired) *******
Calculated using one-sample t-test with the null hypothesis that the mean of C1 - C2 is equal to 0

One-sample t-test

Variate: 100 mg (C1), 25mg (C2) [1].

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
C1-C2	328	6.442	22.64	4.759	0.2628

95% confidence interval for mean: (5.925, 6.959)

Test of null hypothesis that mean of C1-C2 is equal to 0

Test statistic t = 24.52 on 327 d.f.
Probability < 0.001

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Relative plant growth promoting potential of Himalayan Psychrotolerant *Pseudomonas jesenii* strain MP1 against native *Cicer arietinum* (L.)., *Vigna mungo* (L.) Hepper; *Vigna radiata* (L.) Wilczek., *Cajanus cajan* (L.) Millsp. and *Eleusine coracana* (L.)Gaertn.

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Plant growth promoting properties of *Pseudomonas jesenii* strain MP1 were tested against five native crops *viz. Cicer arietinum* (L.) (Chickpea), *Vigna mungo* (L.) Hepper. (Blackgram), *Vignaradiata* (L.) Wilczek. (Greengram), *Cajanus cajan* (L.) Millsp. (Pigeonpea) and *Eleusine coracana* (L.) Gaertn. (Finger millet). The strain significantly ($p<0.05$) stimulated the growth of shoot length, root length, plant fresh weight and plant dry weight of each crop, over their respective untreated controls. Moreover, MP1 treated plant leaves typically showed significant increase in their chlorophyll content, nitrate reductase activity and P content. Chickpea and black gram responded better to MP1 inoculation relatively to other crops. Further, total bacterial and diazotrophic count of MP1 treated soils along with their available phosphorus (P) and nitrogen (N) content were found to increase significantly, in comparison to their respective untreated controls. Microbial community analysis using denaturant gradient gel electrophoresis (DGGE) revealed that the soil bacterial communities were minimally affected by MP1 inoculation. Conclusively, the sustainable agriculture plan in Himalaya may be developed on a strategy of exploring psychrotolerant *P. jesenii* MP1 strain as representative candidate of indigenous biodiversity for individual and/or mixed cropping.

Key words: Psychrotolerant, plant growth promotion, Himalayan agriculture, microbial community analysis.

INTRODUCTION

The need of sustainable agriculture development is revitalizing the interest in plant growth promoting rhizobacteria (PGPR), particularly those involving economically important crops in terms of food and forage. PGPR are able to promote plant growth directly by either assisting in nutrients acquisition (N, P and minerals) or

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modulating plant hormone levels, or indirectly in the forms of biocontrol agents (Glick, 2012). These potential PGPR can fertilize several important agronomic plants such as sugarcane (Mirza et al., 2001), rice (Isawa et al., 2010), maize (Couillerot et al., 2012) or wheat (Upadhyay and Singh, 2014).

Agriculture plays an important role in sustaining livelihood of local people in marginal land of Uttarakhand Himalayas. Being an organic state use of chemical fertilizers for crop production is not recommended. Hence, the application of PGPR as an alternative to chemical fertilizers has emerged as a promising approach. Given the existence of novel and unique gene pool (Soni and Goel, 2010; Suyal et al., 2014), Himalayan soils may be a rich reservoir of novel and diverse microorganisms, as also confirmed by their tremendous potential of biodegradation (Soni et al., 2008) and PGP traits (Selvakumar et al., 2011; Singh et al., 2012). However, despite being potent most of them are not under practice; probably due to their restricted environmental adaptations. In this context, PGP traits of indigenous psychrotolerant *Pseudomonas jesenii* MP1 strain that was able to well adapt to fluctuating temperatures, could be used effectively as a low cost bioinoculant in Himalayan agricultural lands.

Numerous studies conducted over the past three decades have clearly shown that the plant genotype and the soil type are two main drivers that shape the rhizosphere microbiome (Bakker et al., 2012). Moreover, microbial application in an ecosystem may cause tremendous changes in the number and composition of the taxonomic groups (Mendes et al., 2013). These changes may be undesirable if important native species are lost, thus affecting subsequent crops. Therefore, to exert beneficial effects in the root environment, it has to be rhizosphere competent. Various culture independent methods has been used to investigate the effect of bacterial inoculation in soil microbial communities *viz.* denaturant gradient gel electrophoresis (DGGE) for *Rizobium* (Herrmann et al., 2012), quantitative real time PCR (qPCR) for *Rizobium* (Babic et al., 2008), ribosomal intergenic spacers analysis (RISA) for *Azospirillum* (Schumpp and Deakin, 2010), fatty acid methyl ester analysis (FAME) (Kozdroj et al., 2004), etc.

The primary objectives of this study were to evaluate PGP traits of psychrotolerant MP1 strains against four native pulses *viz*. chick pea, black gram, green gram and pigeon pea and one cereal *viz.* finger millet; to investigate native bacterial diversity response to PGPR inoculation and assessment of the effect of MP1 strain inoculation on soil health. Here, it is pertinent to mention that the demand of these pluses and millet produced in Himalayan hills is on an increase, not only for taste and nutritive value but because they are a bio-food. The positive growth response of all the crops signifies the use of *P. jesenii* MP1 strain to the agriculture practices in Himalayan and/or similar agro-ecosystems for improved crop production and sustainability.

MATERIALS AND METHODS

Strain and culture conditions

Strain MP1, originally isolated from agricultural soil sample from Munsyari (2200 m, 30.60°N/80.20°E) from Western Indian Himalayas, was obtained from departmental culture collection. Strain was maintained aerobically in Burk medium at 28°C.

In vitro assessment for plant growth promoting attributes

MP1 was assessed for the presence of important plant growth promoting traits *viz.* N_2 fixation, P solubilization and indole acetic acid (IAA) production. The procedures were taken from respective standard protocols (Supplementary material).

In vitro seed germination assay

In vitro comparative seed germination assay was conducted to determine the effect of MP1 strain on seed germination of local varieties of five crops *viz.* chickpea, blackgram, greengram, pigeonpea and finger millet. In the experiment, 60 seeds of each crop were imbibed separately in 5 ml of a 1×10^8 ml^{-1} bacterial suspension. Controls were imbibed with Burk medium broth only. After 15 min excess suspension was decanted off and the seeds were plated out on to filter paper laid over 0.5% water agar in Petri dishes (20 seeds per Petri dish) at 28°C under a diurnal cycle of white light. The number of seeds germinated was recorded as seedlings with coleoptile lengths >5 mm on day 3 and 6 post-inoculation. The number of germinating seeds was taken as the mean of three Petri dishes so that each value was the mean of 60 imbibed seeds (three Petri dishes × 20 seeds), expressed as a percentage of the controls.

Chemical analysis of the soils

Soil samples were analyzed using “K054 Soil Testing Kit” Himedia Laboratories Pvt Ltd, India pH, organic carbon (% oxidizable OC), available phosphate (P_2O_5), available potassium (K_2O), ammonical-nitrogen (NH_3-N), and nitrate nitrogen (NO_3-N) contents.

Plant growth promotion studies under net house conditions

The pot trial was performed at Pantnagar (244 m, 28.97°N, 79.41°E), a Tarai region of Indian Shiwalik Himalayas, during the month of August to October as described previously by Rani et al. (2012). For the experiment, local varieties of chickpea, blackgram, greengram, pigeon pea and finger millet were used. Seeds sterilized with 1% sodium hypochlorite for 3 min and washed thrice with sterile distilled water were bacterized (10^8 cells/seed) using carboxymethyl cellulose (Katiyar and Goel, 2003; Singh et al., 2012). Seven seeds per pot were sown in 20-cm-diameter pots filled with 3-kg non-sterilized sandy-loam soil having pH 7.5 without any external fertilizer input. Non-bacterized seeds served as control. Pots were kept in a net house having natural fluctuating temperature range from 30 ± 5°C during the day to 15 ± 5°C during the night, for 90 days (Rani et al., 2012). All the analysis was carried out in triplicate. Agronomical parameters (shoot length, root length, fresh weight, dry weight), leaf nitrate reductase activity, total leaf chlorophyll content and leaf P content was measured at 30, 45,

60 and 90 days after sowing (das). Simultaneously, rhizospheric soil samples were collected for bacterial community analysis, using sterile spatula in sterile polythene bags and transported to laboratory under sterile and cold conditions. Each soil sample was collected in triplicates which were later mixed to make a single composed sample per treatment.

Nitrate reductase activity, chlorophyll assay and estimation of leaf P content

The nitrate reductase activity of plant flag leaves was measured according to previous reports (Rani et al., 2012; Singh et al., 2012). The total chlorophyll content of plant flag leaves was measured as described previously (Rani et al., 2012). P content of plant flag leaves was measured according to Fiske and Subbaraw (1925). The plant flag leaves were sampled at 30, 45 and 60 days.

Statistical analysis

The pot experiment was performed with three replicates per treatment. Data were analyzed by ANOVA. Mean difference of the treatments was considered to be significant at the 5% level. STPR-15 software was used to calculate analysis of variance which was programmed by department of Mathematics, Statistics and Computer Science G.B.P.U.A.&T. Pantnagar.

Bacterial community analysis

Effect of bio-inoculant on native micro-flora was assessed by using qPCR and PCR-DGGE techniques. Rhizospheric soil samples (not deeper than 15 cm) were collected from the rhizosphere of each crop plants at 0, 30, 45, 60 and 90 days, using sterile spatula in sterile polythene bags and transported to laboratory under sterile and cold conditions. The soil samples from the replicate pots (3) were mixed to make a single composed sample per site. All the samples were analyzed chemically as per described earlier in the manuscript.

Total soil DNA extraction

Metagenomic DNA was extracted from each 0.5 g (fresh weight) soil sample by using the Powersoil™ DNA isolation kit (Mobio Lab. Inc., USA) according to the manufacturer's instructions. After extraction, DNA samples were quantified spectrophotometrically at 260 nm and used immediately for further analysis.

Real time PCR (qPCR) analysis

Copy number of 16S rDNA and *nif*H from collected soil samples were quantified by the primer set 16S F/R(5' CCTACGGGAGGCAGCAG 3' and 5' ATTACCGCGGCTGCTGG 3', respectively) and PolF/R (5'-TGC GAY CCS AAR GCB GAC TC-3' and 5'-ATS GCC ATC ATY TCR CCG GA-3', respectively), using iCycleriQ™ Multicolor (Bio-Rad Lab, Hercules, USA) real-time polymerase chain reaction (qPCR) machine as described previously (Prema et al., 2009; Soni and Goel, 2010).

Bacterial community analysis

Variable region 3 (V3) of the 16S ribosomal RNA (rRNA) gene was amplified by the primer set 357f-GC (*Escherichia coli* position, 341-357, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGG-3′, underline of sequence denotes GC clamp) and 518r (*E. coli* position, 518-534, 5′-ATTACCGCGGCTGCTGG-3′). Denaturing gradient gel electrophoresis (DGGE) was performed with 8% (w/v) acrylamide gel containing a linear chemical gradient ranging from 40 to 60% denaturant as described earlier (Soni and Goel, 2010).

Bacterial strain deposition

P. jesenii MP1 strain used in this study has been deposited in the "National Bureau of Agriculturally Important Microorganisms (NBAIM) microbial repository" recognized by Biodiversity Authority of India, under accession number B-01444.

RESULTS AND DISCUSSION

P. jesenii strain MP1 has shown the capability to enhance plant growth as substantiated by the presence of various PGP traits (Table SM1). Moreover, it can grow on N_2 free media and subsequently gave an amplification of 360 bp*nif*H fragment confirming its diazotrophic trait too. Detection of nitrogen fixers, by conventional methods *viz.* acetylene reduction assay, is sometimes ineffectual and inconclusive (Franche et al., 2009). Therefore, *nif*H gene is most often used as biomarker for nitrogen fixation.

In vitro seed germination assay confirm the efficacy of MP1 strain to enhance the seed germination of all the crops (Table 1). The maximum germination rate of treated seeds was observed in black gram (22%), followed by green gram (21%). In pigeon pea and finger millet the increment in seed germination was 18%, while, in case of chickpea, it was minimum (17%). Significant difference in plant growth was observed among inoculated and uninoculated treatments. The effect of MP1 strain on agronomical parameters of chickpea, black gram, green gram, pigeon pea and finger millet was significantly higher with an increase in shoot length upto 41.5, 136.8, 49.0, 20.7 and 39.3%; root length upto 83.3, 81.2, 14.1, 66.7 and 80.0%; fresh weight upto 102.2, 97.4, 81.7, 84.4 and 97.4% and dry weight upto 261.8, 125.0, 150.0, 149.6 and 225.0%, respectively, over control (Table 1). In general, better plant growth was observed in chickpea and black gram than other crops. Moreover, the most prominent effect of bio-inoculant on the crops was observed up to 45 days with a slight deviation in case of black gram and green gram. The increment in agronomical parameters of the MP1 treated plants in comparison with respective control could be correlated with enhanced crop productivity (Rani et al., 2012; Singh et al., 2012). An overwhelming number of studies have revealed the successful implementation of the PGPR having profound effects on seed germination, seedling vigor, plant growth and development, nutrition, diseases and productivity (Kogel et al., 2006; Rani et al., 2012; Singh et al., 2012). However, exploration of cold adapted bacterial strains for plant growth promotion in indigenous plants is still lacking. Katiyar and Goel

Table 1. Two- way ANOVA depicting the effect of *P. jesenii* MP1 strain on growth of various crops under greenhouse conditions.

Crops		*In vitro* seed germination assay (% germination of the seeds)	Days	Shoot length (cm)[a]	Root length (cm)[a]	Plant fresh weight (g plant^{-1})[a]	Plant dry weight (g plant^{-1})[a]
Pigeon pea	C	65 ± 0.52	30	43.00±1.5	6.00±1.10	2.76±0.08	0.75±0.02
			45	58.83±1.1	8.16±.1.80	5.51±0.16	1.37±.0.04
			60	68.16±0.3	9.67±1.31	10.35±0.43	3.01±0.06
			90	68.50±2.0	11.17±1.10	11.67±0.33	4.50±0.09
	T	80 ± 0.27	30	50.00±1.2(16.3)[b]	10.00±0.90(66.7)[b]	4.30±0.28(55.8)[b]	1.41±0.30(88.0)[b]
			45	71.00±1.9(20.7)[b]	11.00±0.84(34.8)[b]	10.16±0.26(84.4)[b]	3.42±0.50(149.6)[b]
			60	73.00±1.5(7.1)[b]	11.50±1.20(18.9)[b]	13.65±0.75(31.9)[b]	5.20±0.19(72.8)[b]
			90	74.60±1.3(8.9)[b]	12.30±0.74(10.1)[b]	18.00±0.8 (54.2)[b]	6.16±0.27(36.9)[b]
Gram	C	70 ± 0.31	30	23.80±0.9	6.00±0.44	1.74±0.15	0.52±0.06
			45	23.80±0.9	6.00±0.44	3.99±0.15	0.62±0.06
			60	30.17±1.4	9.60±0.75	7.37±0.19	1.10±0.03
			90	32.17±1.7	11.17±0.91	8.36±0.29	1.90±0.07
	T	85 ± 0.58	30	31.17±1.8(31.0)[b]	11.00±0.53(83.3)[b]	3.52±0.26(102.2)[b]	1.16±0.12(123.0)[b]
			45	33.67±2.4(41.5)[b]	11.00±0.34(83.3)[b]	10.30±1.20(158.1)[b]	2.00±0.19(222.5)[b]
			60	40.33±1.9(33.7)[b]	11.50±0.50(19.8)[b]	11.5±0.29(56.0)[b]	3.98±0.08(261.8)[b]
			90	41.83±1.3(30.0)[b]	12.30±0.64(10.1)[b]	16.00±0.58(79.4)[b]	5.50±0.13(189.4)[b]
Green Gram	C	75 ± 0.64	30	19.80±1.6	5.30±0.68	4.65±0.18	0.52±0.04
			45	30.00±2.3	6.30±0.45	6.02±0.33	0.75±0.26
			60	32.30±1.7	7.16±0.16	7.50±0.16	1.44±0.49
			90	33.30±0.7	8.00±0.46	10.27±1.20	2.5±0.78
	T	95 ± 0.72	30	29.50±1.1(49.0)[b]	6.00±0.21(13.2)[b]	7.13±1.15(53.3)[b]	1.3±0.07(150.0)[b]
			45	33.30±1.3(11.0)[b]	7.00±0.35(11.1)[b]	8.46±0.46(36.4)[b]	1.35±0.11(80.0)[b]
			60	35.00±2.0(8.4)[b]	8.17±0.36(14.1)[b]	10.89±0.76(45.2)[b]	2.33±0.06(61.8)[b]
			90	35.80±1.2(7.5)[b]	8.50±0.44(6.3)[b]	18.67±0.43(81.7)[b]	2.83±0.07(13.2)[b]
Black Gram	C	70 ± 0.79	30	16.67±1.2	6.00±0.29	4.99±0.02	0.44±0.02
			45	17.67±1.1	8.00±0.29	7.09±0.03	0.58±0.03
			60	19.00±1.4	10.50±0.35	11.13±0.07	0.79±0.07
			90	20.50±2.1	11.30±0.18	12.83±0.10	1.50±0.10
	T	90±0.85	30	17.67±1.1(6.0)[b]	10.00±0.31(66.6)[b]	8±1.53(60.3)[b]	0.99±0.05(125.0)[b]
			45	21.50±1.2(21.7)[b]	14.50±0.17(81.2)[b]	14±1.43(97.4)[b]	1.09±0.08(87.9)[b]
			60	45.00±2.3(136.8)[b]	18.17±0.13(73.0)[b]	18.33±0.67(64.6)[b]	1.13±0.06(43.0)[b]
			90	47.16±1.1 (130.0)[b]	20.00±0.12(76.9)[b]	19.19±0.44(49.4)[b]	1.83±0.18(22.0)[b]

Table 1. Contd.

Ragi	C	72±0.82	30	14.00±0.6	5.00±0.10	0.96±0.15	0.29±0.08
			45	15.83±0.4	6.67±0.11	1.24±0.09	0.32±0.07
			60	19.17±0.7	8.00±0.15	1.37±0.11	0.67±0.04
			90	20.30±0.2	9.50±0.11	1.83±0.08	1.50±0.13
	T	88±0.67	30	19.50±0.2(39.3)[b]	9.00±0.26(80.0)[b]	1.66±0.12(72.9)[b]	0.66±0.12(127.5)[b]
			45	20.00±0.3(26.3)[b]	10.60±0.64(58.9)[b]	1.99±0.15(60.4)[b]	1.04±0.15(225.0)[b]
			60	21.50±0.6(12.2)[b]	11.00±0.23(37.5)[b]	2.06±0.08(50.3)[b]	1.43±0.08(113.4)[b]
			90	24.30±0.8(19.7)[b]	12.83±0.27(35.1)[b]	2.67±0.13(45.9)[b]	1.83±0.13(22.0)[b]
A (Treatment)	SEM			0.37	0.16	0.15	0.06
	CD			1.04	0.44	0.42	0.17
B (Days)	SEM			0.52	0.22	0.21	0.09
	CD			1.47	0.62	0.59	0.24
C (Crops)	SEM			0.58	0.25	0.24	0.09
	CD			1.64	0.69	0.67	0.27
AXB	SEM			0.73	0.31	0.30	0.12
	CD			2.08	0.88	0.85	.34
AXC	SEM			0.83	0.35	0.34	0.13
	CD			2.33	0.98	0.95	0.38
BXC	SEM			1.17	0.49	0.48	0.19
	CD			3.29	1.39	1.34	0.54
AXBXC	SEM			1.65	0.69	0.67	0.27
	CD			4.65	1.96	1.89	0.77

a: Mean of three replicates; b: Values in parentheses indicate percent increase over treatment. Data were analyzed statistically at the 5% (p>0.05) level of significance.

(2003) assessed the inoculation effect of phosphate-solubilizing cold-tolerant mutant of *P. fluorescens* on mungbean in sterilized and unsterilized soil and observed that inoculated plants resulted in better plant growth in both soils. Biochemical parameters of the MP1 treated plants were also found to be enhanced significantly (Figure 1).

The total chlorophyll content of the treated plants of all the crops was maximum at 45 days. Nitrate reductase activity was maximum on 30 days in chick pea, pigeon pea and finger millet, while on 45 days in black gram and green gram. The leaf P content was also found to increase significantly in MP1 treated plants especially in finger millet, followed by chick pea, green gram, pigeon pea and black gram on 60 days (Figure 2). These results showed that MP1 treated plants had better nutrient uptake efficiency in comparison with their respective controls.

Members of the rhizosphere microbiome can significantly influence the nutrient status of plants. Well-known examples are the rhizobia, the mycorrhizal fungi and *Pseudomonas* sp. that facilitate N and P uptake (Miransari, 2011; Rani et al., 2012).

Bacterial application significantly changed soil physiochemical properties ((Table SM2). The initial pH of the soils was neutral (7.5 ±0.1), which was increased to maximum (pH 9.0±0.1) on 45 days, and thereafter, decreased to pH 8.0 to 7.5 (±0.1) on crop maturity, regardless to bacterial treatment. Contrary to earlier reports (Orhan

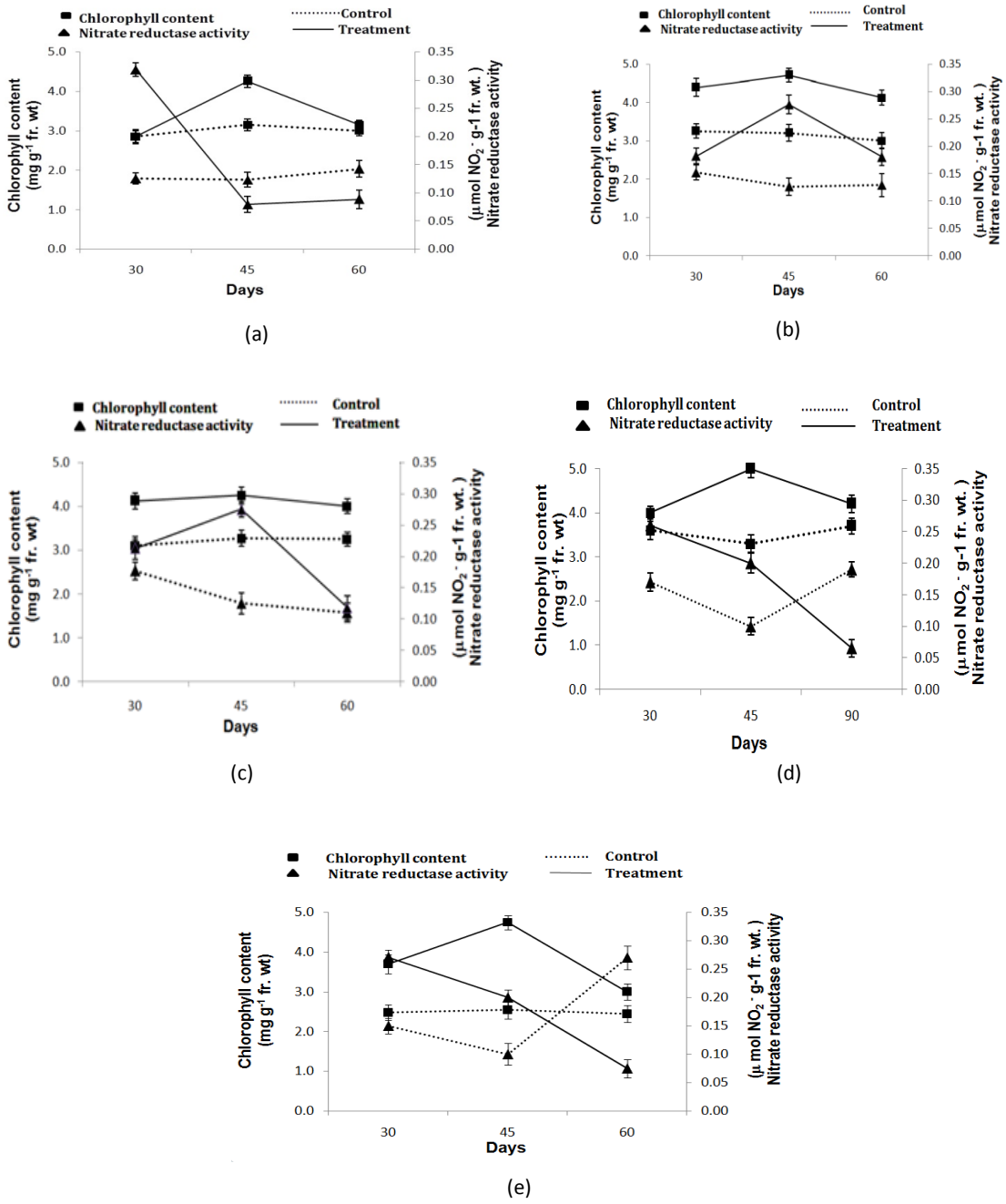

Figure 1. Impact of MP1 strain inoculation on chlorophyll content and nitrate reductase activity of chickpea (a), black gram (b), green gram (c), pigeon pea (d) and finger millet (e), respectively

et al., 2006; Das and Singh, 2014), increase in pH may be explained by the combined effect of different plant exudates and/or bacterial activities. It was observed that bacterial diversity was highest in neutral soils and lower in acidic soils (Fierer and Jackson, 2006).

Most rhizobacterial species are organotrophs and therefore, their growth is greatly affected by the availability and accessibility of the available carbon (Rousk and Baath, 2007). The total organic carbon (TOC) content in soil was “0.505-0.750% oxidizable OC’ which was decreased on 90 days in black gram and finger millet, regardless of bacterial treatment. Contrary to it, MP1 treatment in chickpea, green gram and pigeon pea, increased the TOC on 90 days to “0.750-1.00% oxidizable OC”; thereby, helping the growth of other soil bacteria.

In the case of available P (APH), MP1 treatment in each crop, increase the availability of P from 22-56 to

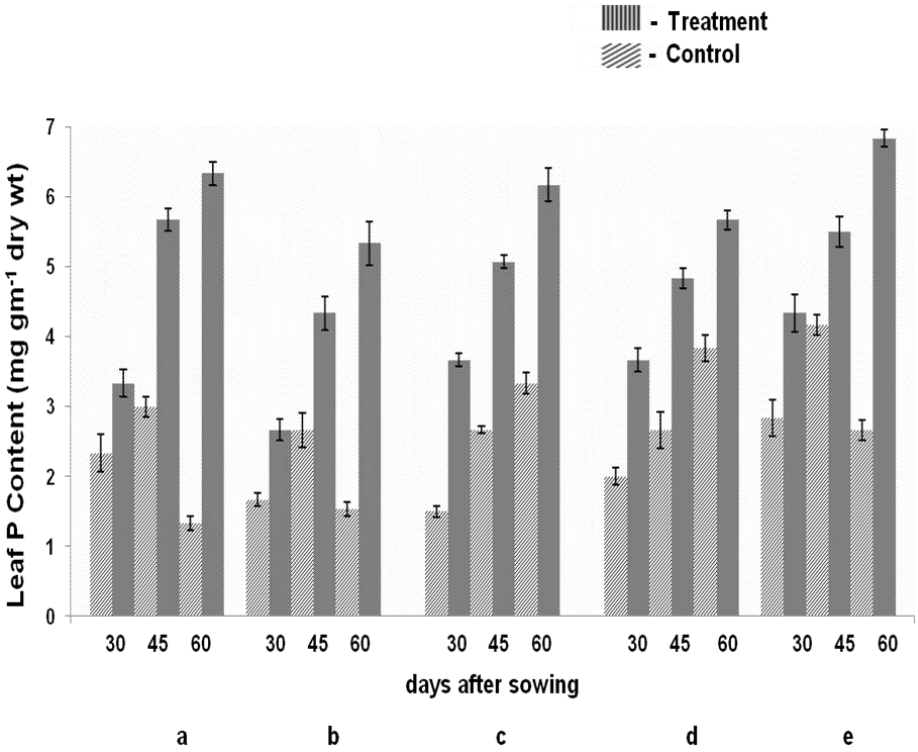

Figure 2. Impact of MP1 strain inoculation on leaf P content of chickpea (a), black gram (b), green gram (c), pigeon pea (d) and finger millet (e), respectively.

56-73 kg ha^{-1} on 45 days and then maintain its concentration at 22-56 kg ha^{-1}; while, in untreated plants a APH concentration remained constant (22-56 kg ha^{-1}) up to 60 days and thereafter decreased to < 22 kg ha^{-1}, excepting finger millet, where it was found to increase to 56-73 kg ha^{-1} on 45 days. Similarly, N content of the soil was analyzed in two forms: "ammonical N (AN) and nitrate N (NN) content". A common trend of increasing AN content from ≤ 15 to 16 ≤ 73 kg ha^{-1} onwards to 60 days was observed in the soils of treated plants; however, that of untreated plants showed no changes in their AN content. Soil NN content of the untreated plants was found to remain constant (≤ 04 kg ha^{-1}) up to 45 days and thereafter increased to 05 ≤ 15 kg ha^{-1}on 60 and 90 days, in all the crops except finger millet, where it remained the same (≤ 04 kg ha^{-1}) till 90 days. Contrary to it, soil NN content of the MP1 treated plants, was found to increase from ≤ 04 kg ha^{-1} (initial) to 05 ≤ 15 kg ha^{-1} (30 and 45 das) to 16 ≤ 20 kg ha^{-1} (60 and 90 das), except finger millet where the increment was only up to 5 ≤ 15 kg ha^{-1}. Increment in soil NN content on later days indicates the lesser requirement of N at crop maturity. Furthermore, its higher amount in the soil of pulses than that of cereal, could be correlated with the fact that the earlier are frequently nodulated by symbiotic N_2 fixers which ultimately contributes to the soil N reservoir. Available potassium (APT)" content of the crops was also found to increase in MP1 treated plants, although, no generalized trend was observed. Orhan et al. (2006) analyzed the effects of PGPR on growth and nutrient contents in organically growing raspberry and observed the significant increase in soil nutrients. Therefore, enhanced N and P content on application of MP1 strain signified its importance in reducing the N and P fertilizers in Himalayan soils.

In the present study, qPCR and PCR-DGGE techniques were explored just to show the effect of MP1 strain inoculation on abundance and composition of native soil bacterial diversity. MP1 strain treatment in all the crops significantly increased the 16S rDNA and *nif*H abundances, calculated on the basis of respective standard curves with slope (0.973 and 0.986) and R^2 value (0.958 and 0.984). In the treated plants of green gram, black gram and finger millet, 16S rDNA abundance was maximum at 45 days; while, in chick pea and pigeon pea, it was maximum at 30 and 60 days, respectively (Figure 3). Untreated controls showed gene abundance maxima at 60 days after sowing in all the crops except black gram, where it was 45 days. Similarly, in the case of *nif*H, abundance was maximum at 30 days after sowing in MP1 treated plants and at 60 days after sowing in untreated controls of each crop. It may be due to the soil NN status which was found to increase after 30 days and therefore, negatively affect the soil diazotrophic

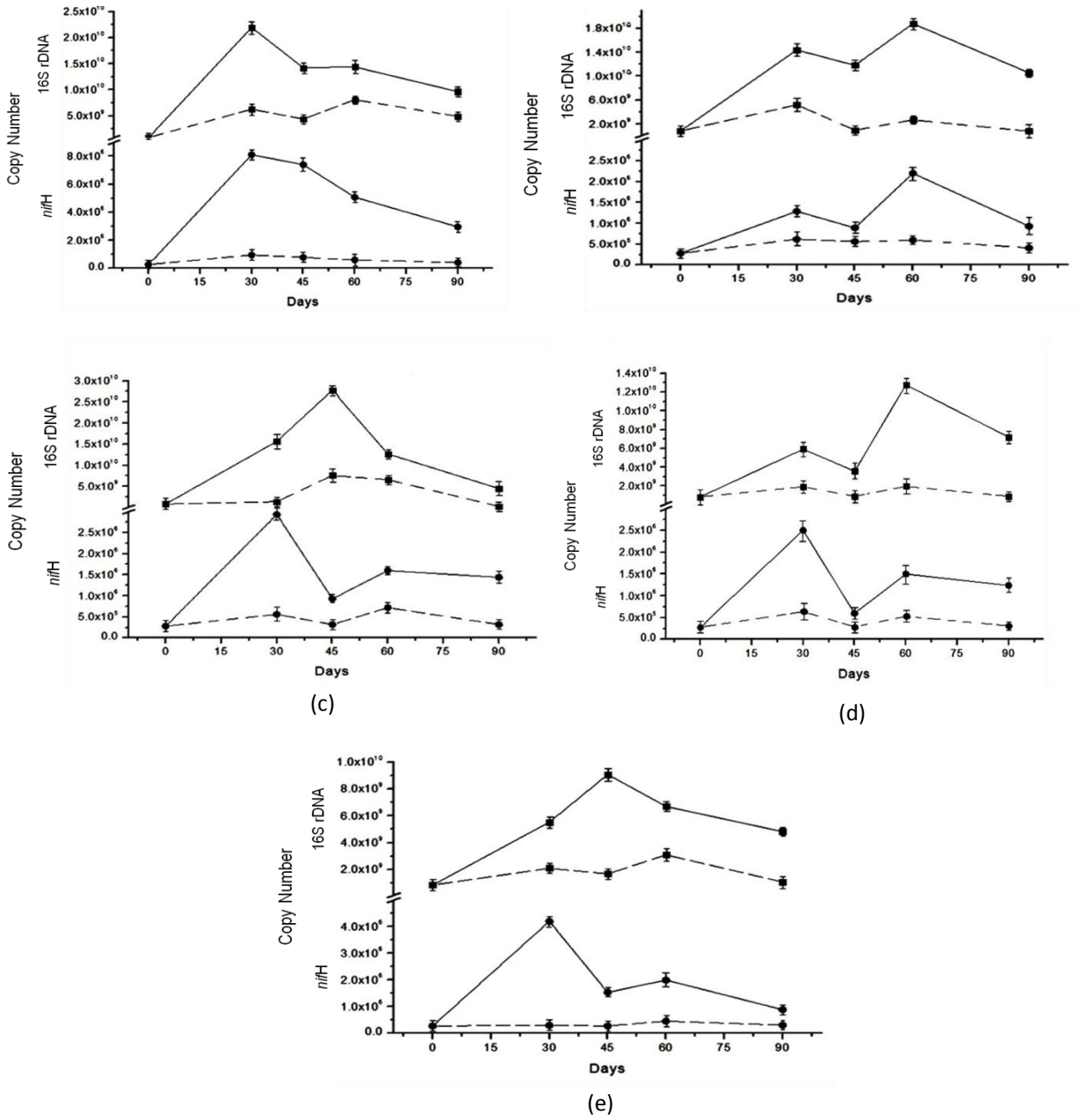

Figure 3. Dynamics of 16S rDNA and *nif*H copy number in the rhizosphere of chickpea (a), black gram (b), green gram (c), pigeon pea (d) and finger millet (e), respectively

population (Parmar and Dufresne, 2011). In all the cases, gene abundances were found to decrease towards harvesting of the crops; yet, greater than their untreated controls. Furthermore, rhizospheric bacterial communities from each crop during different time intervals were compared based on the DGGE patterns of partial 16S rRNA gene amplified using a bacteria-specific primer set. DGGE patterns are shown in Figure 4 along with respective intensity curves (IC), generated by software Quantity One (BioRad). No difference in patterns was observed in sample duplicates (data not shown). The patterns and intensities of bands were mainly affected by number of days after sowing; however, a few bands were influenced by type of crop and bacterial inoculation. Increasing number of peaks in IC revealed the increment in Rhizospheric bacterial communities, significantly after 30 days of sowing except green gram and pigeon pea. It could be attributed to the time required for bacterial adaptation, once adapted, contributes to the diversity.

Another reason could be the soil health. Initially, the

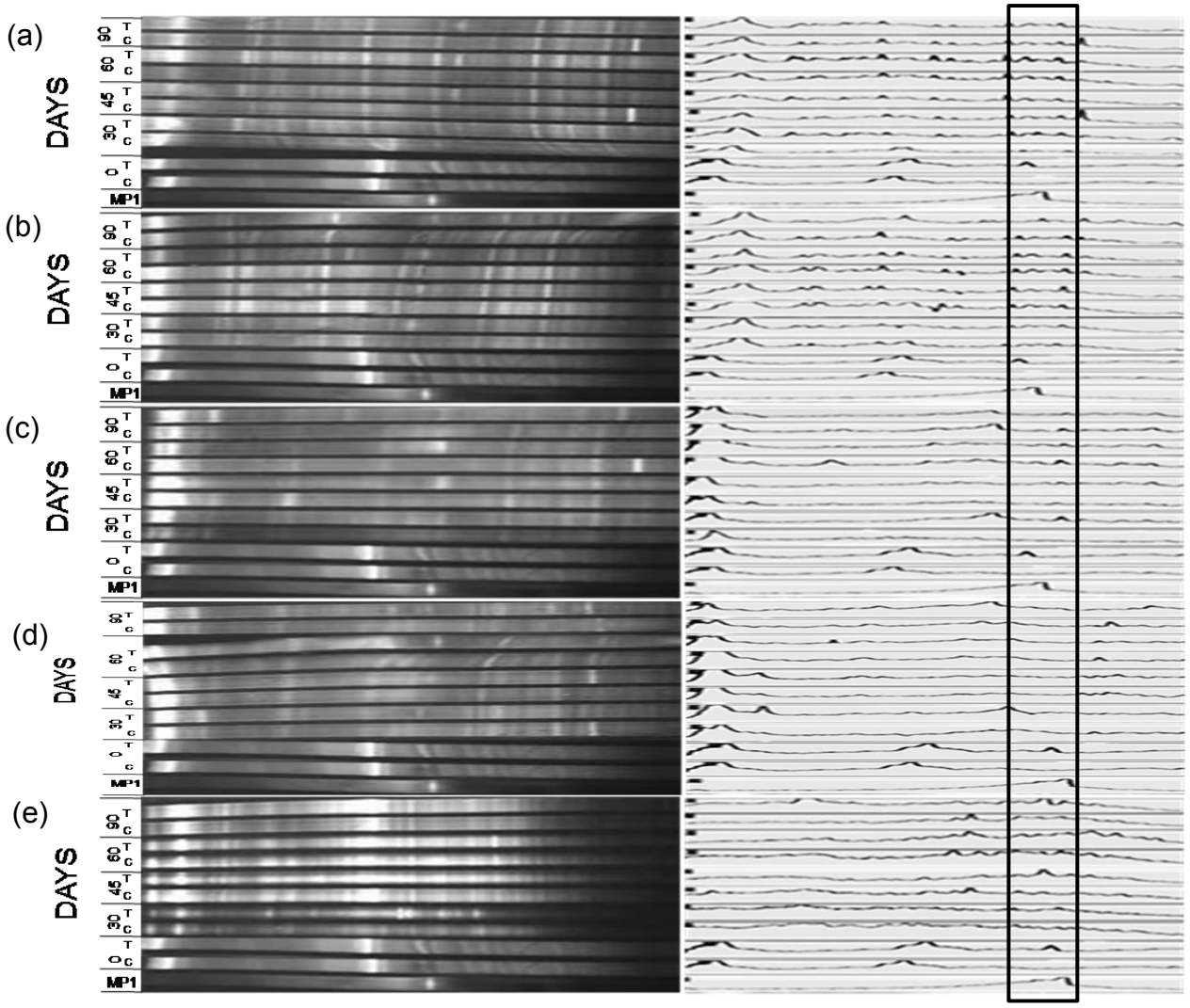

Figure 4. DGGE patterns of partial 16S rDNA of bacterial communities in rhizosphere of five crops *viz*. chickpea (a), black gram (b), green gram (c), pigeon pea (d) and finger millet (e), respectively. C and T are control and treatment, respectively. MP1 represent partial 16S rDNA DGGE pattern of *P. jesenii* strain MP1. Highlighted part indicates the probable persistence of bio-inoculant in respective rhizosphere

nutrient status of the soil was low and therefore, plants restricted its rhizospheric microbial diversity to its minimum; as supported by the fact that at higher diversity the increase in productivity decreases because resources become limiting, resulting in the classic asymptotic diversity-productivity pattern (Schnitzer and Klironomos, 2011). Moreover, during this period, most of the crops require external fertilizers, even nodulating pulses too, due to lack of functional nodules. In this perspective, MP1 strain could be very promising because it was dominant up to 30 days and probably, take care of the plant's growth and development. Once, soil nutrient reservoir increased after 30 days, bacterial diversity was increased and simultaneously, the rate of persistence of MP1 strain was declined, as revealed by aligning its DGGE band position with the crops. Cook et al. (1995) postulated that plants may modulate the rhizosphere microbiome to their benefit by selectively stimulating microorganisms with traits that are beneficial to plant growth and health. The effect of inoculant on soil microbial communities has been studied earlier using qPCR (Babic et al., 2008), ribosomal intergenic spacers analysis (RISA) (Schumpp and Deakin, 2010), DGGE (Herrmann et al., 2012) and other techniques. Trabelsi et al., (2011) revealed that the perturbation of the community due to inoculation with a rhizobial strain is higher than that due to chemical fertilization. It suggests that the introduction of exogenous bacteria in a community is likely to produce more long-term effects than external chemical supply. For future studies, unraveling the rhizosphere microbiome holds

potential to improve crop protection and to uncover numerous yet unknown soil microorganisms, functions and genes for diverse applications.

Conclusion

Application of *P. jesenii* MP1 strain as a bio-inoculant in four important WIH pulses *viz.* chickpea, black gram, green gram and pigeon pea enhanced their growth and respective soil nutrients status. In cereal (finger millet), MP1 strain was not so effective, although better than respective untreated control. Therefore, being a psychrotolerant, *P. jesenii* MP1 strain can withstand extremities of temperature fluctuations and play an important role in growth and yield enhancement of native Himalayan pulses under individual cropping pattern.

Conflict of Interests

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

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Supplementary material

Growth promotory properties

Phosphate solubilization

The bacterial strain was checked for phosphate solubilizing ability on Pikovskaya (PVK) agar medium (Pikovskaya, 1948) incorporated with tricalcium phosphate as the sole source of phosphate. Formation of a clear halo zone around the index (S.I.). Formation of a clear halo zone around the

Table SM1. Growth promotory properties of *P. Jesenii* MP1 strain.

S/N	Accession No.	Strain name	Phosphate Solubilization (SI)	IAA Production	Siderophore production
1	JX310329	*Pseudomonas jesenii* strain MP1	+ve (2.54)	+ve	+ve

(+) indicates +ve response; (-) indicates -ve response; SI: solubilization index = colony diameter + halozone diameter/colony diameter (Edi Premono et al., 1996).

Table SM2. Physiochemical characteristics of the soils collected on subsequent days of sowing

Crops		Days	pH	Soil physiological parameters[a]																						
				OC[b]						APH[c]				APT[d]				AN[e]			NN[f]					
				L	ML	M	MH	H	B	L	M	MH	H	L	M	H	VH	L	M	H	VL	L	M	H		
Gram	C[g]	0	7.5			■					■			■				■			■					
		30	8.0				■				■			■				■			■					
		45	9.0		■						■			■				■			■					
		60	8.0	■							■			■				■				■				
		90	8.0	■						■						■		■				■				
	T[h]	0	7.5			■					■			■				■			■					
		30	8.5				■				■					■		■				■				
		45	9.0	■								■			■			■				■				
		60	8.0	■							■				■				■				■			
		90	8.0				■				■						■		■				■			
Black Gram	C[g]	0	7.5			■					■			■				■			■					
		30	8.0			■					■				■			■			■					
		45	9.0	■							■				■			■				■				
		60	8.0	■							■						■	■				■				
		90	7.5	■						■					■			■				■				
	T[h]	0	7.5			■					■			■				■			■					
		30	8.5				■				■				■			■				■				
		45	9.0			■						■			■			■				■				
		60	8.0	■							■						■		■				■			
		90	7.5	■							■				■				■				■			
Green Gram	C[g]	0	7.5			■					■			■				■			■					
		30	8.0					■			■			■				■			■					
		45	9.0			■					■				■			■				■				
		60	8.0		■						■				■				■				■			
		90	7.5	■						■						■			■				■			
	T[h]	0	7.5			■					■			■				■				■				
		30	8.5				■				■				■			■				■				
		45	9.0	■								■			■			■				■				
		60	8.0		■						■					■				■				■		
		90	7.5				■				■				■					■				■		

phosphate ($Ca_3(PO_4)_2$) by observing the solubilization bacterial growth after seven days of incubation at 28°C indicates phosphate solubilizing ability. S.I. was calculated on PVK plates by the formula: Solubilization index = Colony diameter + Halozone diameter/Colony diameter (Edi Premono et al., 1996).

Siderophore production

The chrome azurolsulfonate (CAS) assay (Schwyn and Neilands, 1987) was used for screening siderophores production, since; it is comprehensive, exceptionally responsive, and most convenient. For the qualitative assay, MP1 strain was spot inoculated onto the blue agar and incubated at 28°C for 24 to 48 h. The results were interpreted based on the colour change due to transfer of the ferric ions from its intense blue complex to the siderophore. The sizes of yellow orange haloes around the growth indicated siderophore activity.

IAA production

For qualitative estimation of IAA production, Tryptone soy broth is used. Tryptone soy broth (5.0 ml) tubes with and without tryptophan (200 µl/ml) were inoculated with loopful of actively growing bacterial cultures aseptically and incubated for 48 h at 28°C under shaking conditions. Cultures were centrifuged at 10,000 rpm for 10 min. 2 ml of Salkowski reagent was added in 1 ml supernatant. The mixture was incubated at 28°C for 25 min. Development of pink colour shows IAA production.

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