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Screening of beneficial properties of rhizobacteria isolated from Saffron (*Crocus sativus* L) rhizosphere

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Plant growth promoting rhizobacterias (PGPRs) are free living soil bacteria that colonize root surfaces and have the capacity to enhance plant growth directly or indirectly. A total of 23 bacterial strains were isolated from saffron rhizoshere soil during the flowering stage of corms. All these isolates were screened for their plant growth promoting traits like production of IAA, phosphate solubilisation activity and siderophore production. The maximum percentage of the bacterial isolates was of Gram negative rod shaped type. A total of six isolates were capable of showing one or more than one of the activities like IAA production, Siderphore production and phosphate solubilisation activity. The *Bacillus subtilis* showed highest IAA production of 360 µg/ml while as *Pseudomonas* ssp., was found to be highly efficient in terms of phosphate solubilisation production (460 µg/ml) and siderophore production (62%). It was concluded from the results that these rhizobacterial strains isolated could be a promising source for plant growth promoting agent in increasing the growth of cormlets vis a vis enhancing the yield of saffron.

Key words: IAA, PGPR, phosphate solubilisation, rhizosphere, siderophore, saffron.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) representing about (2 to 5%) of total rhizobacterial community defined as the indispensable part of rhizosphere biota that when grown in association with the host plants can stimulate the growth of the host (Vessey, 2003). PGPR seemed as successful candidates in getting established in soil ecosystem due to their high adaptability in a wide variety of environments, faster growth rate and biochemical versatility to metabolize a wide range of natural and xenobiotic compounds (Bhattacharyya and Jha, 2012). They promote plant growth either by direct or indirect mechanisms (Farina et al., 2012), provide nitrogen and make available phosphorus to the plant, produce phytohormones like indoleacetic acid (Arshad and Frankenberger, 1992). IAA, the product of L-tryptophan metabolism by several microorganisms inhabiting the rhizosphere because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils is one of the most physiologically active hormones among the auxins (Strzelczyk and Pokojska-Burdziej, 1984; Lynch, 1985). Diverse soil microorganisms including bacteria (Muller et al., 1989), fungi (Stein et al., 1985) and algae (Finnie and Van Staden, 1985) are capable of producing physiologically active quantities of auxins, which may exert pronounced effects on plant growth and establishment.

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Abbreviations: IAA, Indole acetic acid; **PGPR,** plant growth promoting rhizobacteria; **PSB,** phosphate solublising bacteria.

Siderophores are other types of compounds secreted by PGPR's in response to iron deficiency that helps the transportation of ferric iron into plant cells from insoluble forms by either mineralization or sequestration (Lankford, 1973). They not only improve rhizosphere colonization of the producer strain but also play a definite role in iron nutrition of plant (Vansuyt et al., 2007); antagonism against phytopathogens (Chincholkar et al., 2007) and in induced systemic resistance in plants (De Meyer et al., 1999).

Phosphate solubilizing bacteria (PSBs) inhabiting the rhizosphere are considered as promising biofertlizers since they can supply plants with 'phosphorus' from sources otherwise poorly available by several mechanisms and hence a viable substitute to chemical phosphate fertilizers (Khan et al., 2006; Chaiharn et al., 2008). They are known for secretion of organic acids and phosphates to solubilise insoluble phosphate to soluble forms (Ahemad and Khan, 2011). The importance of saffron stigma production in the economy of our state encouraged us to isolate and screen new PGPRs on the basis of their ability to produce IAA for enhancing the corm size, siderophore production to quench iron and availability of phosphorus to the corms. The saffron soil is unique in itself found in some areas of J&K, India; particularly in Pampore area of Kashmir province and is known both for maximum area under saffron cultivation and also for the bulk as well as good quality saffron production.

This work has brought some very important strains in focus which is highly efficient plant growth promoters especially some opportunistic organisms like *Acinetobacter* ssp. and *Pantoea* ssp.; however, recent literature included that it could be reliable and relevant to use this organisms of non-clinical origin for enhancing the plant growth (Hebbar et al., 1999).

MATERIALS AND METHODS

Chemicals required

Luria-Bertani (LB) agar medium and broth, biochemical kits (KB002, KB013 and KB001), nutrient agar and broth, $FeCl_3$, percholric acid, ortho-phosphoric acid, IAA, tryptophan, $SnCl_2$, conc. HCl, ammonium molybdate, KH_2PO_4, hexa-decyltrimethy ammonium bromide (HDTMA), King's B broth, Pikovskaya's broth. All the chemicals used were purchased from *Hi-Media* Mumbai Pvt. Ltd.

Sampling process and bacterial isolation

The rhizosphere saffron soil samples were collected in the month of September to October 2010 during the flowering of saffron corms from the saffron field in Pampore area, J&K, India for isolation of rhizobacterial strains with beneficial traits like Siderophore production, phosphate solubilization and IAA production. The corms were uprooted and the soil adhering to the roots which represent rhizosphere soil were shaken from the roots and collected in sterilized plastic bags. The soil samples were then transported to the microbiology laboratory of the Centre of Research for Development (CORD), University of Kashmir, for immediate processing. To isolate bacteria, 1 g of soil sample was transferred to 9 ml distilled water and was serially diluted. Diluted suspensions were spread plated on LB agar medium and were incubated at 28°C for 24 h. Representative colonies were randomly selected from the countable plates and re-streaked onto new plates of the different media to obtain pure colonies. A total of 23 isolates obtained in this manner were maintained on agar slants. Because many isolates were morphologically indistinguishable in culture, preliminary characterization procedures including cytochrome oxidase (Kovacs, 1956), oxidative fermentation (Hugh and Leifson 1953), catalase and motility tests were conducted. Based on these preliminary microscopic/macroscopic characterizations, six (06) isolates were chosen from the original 23 isolates and were subjected to biochemical tests using strain specific biochemical kits (*Hi-media*).

The three types of kits used as per the strain were KB002 for Gram negative rods, KB013 for gram Positive bacillus and KB001 for Enterobacteriaceae. The strains were identified as per the chart sheet of the kits to the nearest value. Pure cultures of isolates stored at -80°C in nutrient broth supplemented with 200 mg/g glycerol were used for the screening of growth promoting activities.

Quantitative estimation of IAA production

IAA production was detected by the modified method as described by Brick et al. (1991). The bacterial cultures were grown for 24 h in LB broth medium supplemented with 300 µg/ml of tryptophan. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of salkowski reagent (50 ml, 35% percholric acid, 1 ml of 0.5 M $feCl_3$ solution). Development of pink color indicates IAA production and optical density was measured at 530 nm with the help of spectrophotometer (Systronics 106). Concentration of IAA produced by the cultures was measured with the help of standard graph of IAA (*Hi media*) obtained in the range of 100 to 1000 µg/ml.

Quantitative estimation of phosphate solubilization

The amount of soluble phosphate was measured by the colorimetric method as described by King (1932) with some modifications. Isolates of the phosphate solubilizing microorganisms (100 µl of the overnight grown in case of bacteria and were inoculated to 100 ml of Pikovskaya's broth in 250 ml flasks in case of bacteria with equal number of uninoculated controls. The flasks were incubated on an orbital shaker at 28±2°C for 05 days. The amount of phosphate released in the broth in flasks was estimated after 5 days of inoculation. The broth cultures of bacteria were centrifuged at 10,000 rpm for 20 min. The supernatant (1 ml) was mixed with 10 ml of chloromolybdic acid (7.5 g of ammonium molybdate in 150 ml distilled water to which 162 ml of conc. HCl was added). The volume was made up to 1 L with distilled water and the volume was made to 45 ml with distilled water. 0.25ml of chlorostannous acid (25 g of $SnCl_2.2H_2O$ in 100 ml conc. HCl and rising to 1 L with distilled H_2O) was added and the volume was made up to 50 ml with distilled H_20 water. The concentration of phosphate was determined by the absorbance of the color blue at 610 nm. A standard calibration curve was performed with a solution of KH_2PO_4.

Percentage of siderophore production

Siderophore production by the isolated rhizobacterial strains was detected as described by Schwyn and Neilands (1987) with several modifications. The assay utilized the ternary complex chrome azurol S/iron (III)/hexa-decyltrimethy ammonium bromide (HDTMA) as an

indicator. Change in the dye color from blue to orange indicated the production of siderophore. A loopfull of frozen culture was transferred to 3 ml of King's Bbroth and the strains were cultured for 48 h at room temperature with shaking at 150 rpm. Cultures were centrifuged at 3000 rpm/30 min and 500 µl of the supernatant were mixed with 500 µl of CAS solution. The color changed from blue to orange at the rate of production of siderophores. The optical density was measured by a spectrophotometer at 630 nm after 20 min of incubation.

$$\% \text{ decolorization} = \frac{A_r - A_s}{A_r} \times 100$$

Where, A_r = Absorbance of reference and A_s = absorbance of sample.

RESULTS AND DISCUSSION

PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion (Glick, 1995; Lalande et al., 1989). There are many papers related to the advantages and screening of PGPR from crop plants but as such no information about screening and using PGPR for saffron corm development is available in our country; however, Sharaf-Eldin et al. (2008) studied the effect of *Bacillus subtilis* FZB24 on saffron (*Crocus sativus* L.) corms under *ex-vitro* conditions in Eygpt and reported that inoculation of *B. subtilis* FZB24 significantly increased leaf length, flowers per corm, weight of the first flower stigma, total stigma biomass and significantly decreased the time required for corms to sprout and the number of shoots. The associated rhizobacteria were isolated from the roots of the flowering corms of saffron on Laureia Bertani (LB) agar medium. The colonies showing different morphological characteristics on the plates were selected for biochemical characterization.

A total of 06 isolates of rhizobacteria were selected and were identified on the basis of biochemical reactions using strain specific biochemical kits (*Hi media*) as well as normal biochemical tests. The rhizobacterial strains isolated were identified on the basis of the biochemical reactions were as *Acinetobacter lwofii*, *Acinetobacter haemolyticus*, *Bacillus subtilis*, *Psuedomonas* ssp, *Pantoea* ssp. and *Klebsella* ssp. (Table 1). The data obtained show a greater abundance of gram negative rod shaped species in the saffron soil rhizosphere and is in agreement with the previous study by Donate-Correa et al. (2004) who reported higher level of gram negative bacteria in the rhizosphere of *Chamaecytisus proliferus* relative to gram positive species. Some enterobacteriaceae like *Klebsella* sp. (Sajjad et al., 2001) and *Pantoea* ssp. (Rennie et al., 1982) and *Pantoe agglomerans* from wheat rhizosphere (Amella et al., 1999) have been-reported in association with sugarcane or other grasses; similarly, *Acinetobacter* ssp. was isola-ted from wheat rhizosphere (Sarode et al., 2009) and *Pennisetum glaucum* rhizosphere (Rokhbakhsh-Zamin et al., 2011) with having strong PGPR activity. Three ap-proaches like screening of rhizobacteria for *in vitro* IAA and siderophore production and the ability to solubilize inorganic phosphate were simultaneously employed to select effective PGPR to be used as bio fertilizers. All the 06 isolates isolated were tested for the IAA production and the efficiency for producing IAA among strains varied significantly.

The *B. subtilis* (360 µg/ml), *Pantoea* ssp. (320 µg/ml) and *A. lwofii* (275 µg/ml) were categorized as high IAA producers while as *Psuedomonas* ssp. produces about 230 µg/ml followed by *A. haemolyticus* (160 µg/ml) and *Klebsella* ssp. (102 µg/ml). The *B. subtilis* was found to be best strains as for as IAA production was considered and is a reliable and efficient prospect for enhancing the growth and development of corms. The production of IAA is reported to be more common among rhizobacterial strains (Arora and Gaur, 2001) (Table 2). The scrutiny of IAA production by the isolates showed that almost 83.33% of the strains studied were able to synthesize the auxin that coincides with previous study (Leinhos and Vacek, 1994) which has validated the presence of large proportions of microorganisms in the rhizosphere capable of producing this hormone.

As earlier reported (Patten and Glick, 1996), the unevenness in the amounts produced was enormous among different species so, as IAA production has been related with an enhancement in vegetative growth (Cleland, 1990). The *Acinetobacter* ssp. obtained from the rhizosphere of *P. glaucum* have been reported to produce IAA (10 to 13 µg/ml) (Rokhbakhsh-Zamin et al., 2011). The isolated rhizobacterial strains were also tested for phosphate solubilisation activity and the *Pseudomonas* ssp. was found to be highly efficient in terms of phosphate solubilisation production (460 µg/ml) followed by *B. subtilis* (395 µg/ml) occurred after 5 days of incubation.

Similarly, for other rhizobacterial ssp, the phosphate solubilisation activity was in the following order that is, *Pantoea* ssp. (210 µg/ml), *A. lwofii* (160 µg/ml), *A. haemolyticus* (80 µg/ml) and *Klebsella* ssp. (110 µg/ml) (Table 2). The genus *Pseudomonas* is currently considered important groups that are able to solubilise the phosphate *in vitro* as very efficient phosphate solubilizers (Kucey, 1983; Peix et al., 2003). As earlier, *Pseudomonas* ssp. (Illemer and Schinner, 1992) and *Bacillus* ssp. (Arora and Gaur, 1979) have been reported to solublise inorganic phosphate. The different patterns of phosphatase activity are widespread in bacteria belonging to different groups and production of these enzymes is often controlled by complex regulatory mechanisms, so that the enzyme activity is detectable

Table 1. Characteristics features of rhizobacterial species isolated from Saffron (*Crocus sativus* L) rhizosphere soil.

Characteristic feature	JCORD01	JCORD04	JCORD06	JCORD08	JCORD09	JCORD23
Gram staining	-	-	+	-	-	-
Morphology	Bacilli	Bacilli	Bacilli	Cocobacilli	Cocobacilli	Bacilli
Motility	M	NM	M	NM	NM	M
Oxidase	+	-	-	-	-	-
Catalase	+	+	+	+	+	+
Lactose	-	+		-	-	V
Amylase	-	-	+	-	-	-
Glucose	+	+	+	-	-	+
Xylose	Nd	-	+	-	-	+
Mannitol	Nd	-	+	-	-	+
Sucrose	-	-	+	-	-	-
Galactose	-	-	-	-	-	+
β-galactosidase	Nd	nd	+	_	_	-
Gelatinase	Nd	Nd	Nd	+	-	V
Arabinose	-	+	+	-	-	
Manose	-	-	-	-	-	+
Rhamnose	-	+	-	-	-	+
Maltose	-	-	-	-	-	+
Trehalose	-	-	+	-	-	+
Adonitol	Nd	+	-	-	-	-
H2S	-	Nd	Nd	-	-	-
Simmons citrate	+	+	+	+	V	+
Urea	V	Nd	-	-	-	-
Nitrate reduction	+	nd	-	-	-	+
Methyl red	-	V	+	-	-	-
Voges-Proskauer	-	+	_	-	-	+
Indole	-	-	-	-	-	+
Organisms	*Psuedomonas* sp.	*Klebsella* sp.	*B. subtilis*	*A. haemolyticus*	*A. iwoffii*	*Pantoea* sp.

V = Variable; Nd = not detected; NM = non motile; M = motile.

Table 2. *In vitro* IAA production, phosphate solubilisation and % siderophore production of rhizobacterial species isolated from Saffron (*Crocus sativus* L) rhizosphere.

Isolate name	IAA production (µg/ml)	Soluble phosphate (µg/l)	Siderophore production (%)
Psuedomonas ssp.	230.2	450	64
Klebsella sp.	-	110	31
Bacillus subtilis	360	395	59
Accinetobacter haemolyticus	160.0	80	-
Accinetobacter lwoffii	275.87	160	45
Pantoea sp.	310	210	52

only under specific environmental conditions (Wanner, 1987). The principal mechanism for the regulation of phosphatases production is the regulation by inorganic phosphate (Pi) concentration (that is, phosphate repress-sible phosphatases) that has been best studied in the alkaline phosphatase (*gene phoA*) of *E. coli*, which is suddenly and fully induced when the Pi concentration decreases from 100 to 0.16 mM (Rosenberg, 1987). These findings indicate that most of alkaline phospha-tases found in the family *Enterobacteriaceae* are Pi-repressible, while many of the acid phosphatases are pi-irrepressible.

In *Pseudomonas fluorescens* MF3, it was determined that the expression of the *apo*gene, which encodes an

acidic phosphatase enzyme, was regulated by the growth temperature. The findings of a co-regulation mechanism for these genes (whose expression is maximal at 17.58°C) as a response to the growth temperature (Burini et al., 1994). Several PSB could also promote plant growth as they renders phosphate into solution more than they needed for their metabolism and the surplus can be absorbed by plant (Kloepper et al., 1989). The study also focuses on the screening of rhizobacterial strains on the basis of their potential to produce siderophore compounds that may directly/indirectly influence the plant growth and are considered as good candidates for siderophore production. Of all the six isolates tested for siderophore production, the highest percentage (%) for siderophore production of 62% was recorded for *Psuedomonas* ssp. followed by *B. subtilis* (59%) while as *Pantoea* ssp. (52%), *A. lwofii* (45%) and *Klebsella* ssp. (31%) also showed good siderophore production activity; however, *A. haemolyticus* did not show any activity (Table 2). Siderophores chelate the ferric ions with a high specific activity and serve as vehicles for the transport of iron (Fe3+) into the microbial cell. Most of the siderophores have hydroxamate, catechol orcarboxylate ligands (Hofte, 1993).

In line to our findings of multiple plant growth promoting activities among PGPR have been reported by some other investigators (Gupta et al., 1998; Dey et al., 2004; Abd El-Azeem et al., 2007). Also, number of studies has demonstrated that production of siderophore by PGPR was most effective in controlling the plant root pathogens (Diaz et al., 2002; Dey et al., 2004). The potential to produce siderophores by microorganisms in improving iron availability to plants was also reported by some workers (Bar-Ness et al., 1992; Rroco et al., 2003; Sharma et al., 2003). From the results, it could be concluded that the tested strains have some important plant growth promoting traits that can be used as biofertelizers and application of these rhizobacterial strains may provide some benefit to saffron growers by speeding corm growth (earlier shoot emergence) and increasing stigma biomass.

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