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Effect of Iron Availability on Induction of Systemic Resistance to *Fusarium* Wilt of Chickpea by *Pseudomonas* spp.

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Selected isolates of *Pseudomonas fluorescens* (*Pf*4-92 and *Pf*RsC5) and *P. aeruginosa* (*Pa*RsG18 and *Pa*RsG27) were examined for growth promotion and induced systemic resistance against *Fusarium* wilt of chickpea. Significant increase in plant height was observed in *Pseudomonas* treated plants. However, plant growth was inhibited when isolates of *Pseudomonas* were used in combination with *Fusarium oxysporum* f. sp. *ciceri* (*Foc*Rs1). It was also observed that the *Pseudomonas* spp. was colonized in root of chickpea and significantly suppressed the disease in greenhouse condition. Rock wool bioassay technique was used to study the effect of iron availability on the induction of systemic resistance to *Fusarium* wilt of chickpea mediated by the *Pseudomonas* spp. All the isolates of *Pseudomonas* spp. showed greater disease control in the induced systemic resistance (ISR) bioassay when iron availability in the nutrient solution was low. High performance liquid chromatography (HPLC) analysis indicated that all the bacterial isolates produced more salicylic acid (SA) at low iron (10 μM EDDHA) than high iron availability (10 μFe^{3+} EDDHA). Except *Pa*RsG27, all the three isolates produced more pseudobactin at low iron than high iron availability.

KEYWORDS: ***Fusarium oxysporum* f. sp. *ciceri*, *Fusarium* wilt, Pseudobactin, *Pseudomonas* spp., Salicylic acid**

Infection of plants with necrotizing pathogens induces systemic resistance to subsequent attacks by pathogens. This resistance is called as systemic acquired resistance (SAR; Ryals *et al.*, 1996). The capacity of plants to express broad-spectrum, SAR after primary infection with a necrotizing pathogen is well known. Some rhizosphere microorganisms, which do not cause necrosis, were also reported to induce systemic resistance (ISR; Pieterse *et al.*, 1996). Phenotypically, ISR resembles pathogen-induced SAR, which is effective against a broad spectrum of plant pathogens. This form of induced disease resistance is commonly referred to as rhizosphere bacteria mediated ISR (van Loon *et al.*, 1998). Some other rhizosphere bacteria e.g. *P. fluorescens* and *P. aeruginosa* are also present on the root surface, where plant exudates provide nutrients and caused ISR for different plant diseases. Several mechanisms have been suggested for disease control by these bacteria, viz. siderophore - mediated competition for iron, competition for carbon, production of HCN, ammonia, antibiotics, volatile compounds etc. or by competing with pathogens for nutrients or colonization space (Glick, 1995).

Some *Pseudomonas* spp. can also produce a non-fluorescens siderophore called pyochelin, which consist of a salicylic-substitute cysteinyl peptide (Cox *et al.*, 1981). Salicylic acid considered to be an intermediate in pyochelin synthesis (Bucheuer and Cox, 1988), which is produced under iron limiting condition (Visca *et al.*, 1993). Buysens *et al.* (1996) demonstrated that *P. aeruginosa* 7NSK2 showed plant growth promotion and suppressed *Pythium* induced disease though the production of pyochelin and pyoverdin. In iron-limiting conditions *P. aeruginosa* strain produced three siderophores-pyochelin (Hofte *et al.*, 1990), pyoverdin and salicylic acid (Buysens *et al.*, 1996).

Pseudobactins, a yellow-green, fluorescent siderophore are also produced under iron limiting conditions by some *Pseudomonas* spp. (Leong, 1986). They are consisting polypeptide chain containing hydroxamate groups linked to a fluorescent quinoline chromophore (Hofte, 1993). It was demonstrated that the pseudobactin for the plant growth promoting strain *P. putida* WCS358 highly specific for the uptake of iron. It was reported that ISR is influenced by iron availability (Leeman *et al.*, 1996; Press *et al.*, 2001). Leeman *et al.* (1996) reported ISR induced by *P. fluorescens* WCS374 against *Fusarium* wilt of radish is inversely related to iron availability of the plant substrate. Similarly, Press *et al.* (1997) also reported that ISR caused by *Serratia marcescens* strain 90-166 against cucumber anthracnose was reduced when iron is made available in the planting medium. ISR mediated by rhizobacteria was improved significantly when external iron

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availability to the host plant was reduced through addition of iron chelator EDDHA (Press *et al.*, 2001). They also reported that when the iron concentration of a planting mixture decreased suppression of cucumber anthracnose significantly improved by *S. marcescens* strain 90-166. It had also been reported that several strains of rhizobaceria produced salicylic acid in environment when iron is not abundant (Meyer and Hofte, 1997; Press *et al.*, 1997).

We isolated some strains of *P. fluorescens* and *P. aeruginosa* from chickpea and rice rhizosphere soil and they were assessed for their effect on charcoal rot and *Fusarium* wilt of chickpea (Srivastava *et al.*, 2001; Saikia *et al.*, 2003). Some of these strains were found effective and exhibited systemic resistance against charcoal rot (Srivastava *et al.*, 2001) and *Fusarium* wilt of chickpea (Saikia *et al.*, 2003) as well as sheath blight of rice (data not publish). Our previous studies indicated that shoot and root length was significantly increased in *P. fluorescens* treated chickpea plants and the reduction in disease severity was more pronounced when chemical inducers were applied with *P. fluorescens* (Saikia *et al.*, 2003). It was exhibited that exogenously supplied salicylic acid stimulated systemic resistance against *Fusarium* wilt and reduced the disease severity significantly. We also reported that *P. fluorescens* could produce SA and induced systemic resistance to chickpea against *F. o.* f. sp. *ciceri*. Here, we determined whether induction of systemic resistance by *Pseudomonas* spp. against *Fusarium* wilt of chickpea was dependent on iron availability or not and observed the effect of iron availability on siderophores production (SA and pseudobactin) by *Pseudomonas* isolates.

Materials and Methods

Plant material, bacterial isolates and pathogen. Seeds of chickpea (*Cicer arietinum* L.) cultivar JG-62 highly susceptible to *F. o.* f. sp. *ciceri* were surface sterilized as described earlier (Saikia *et al.*, 2003). Isolates of *P. fluorescens* and *P. aeruginosa* were isolated from the rhizosphere soil of chickpea and rice from different area of Varanasi and Guwahati (India) and grown on KB medium. Isolation and identification was done according to the method as described earlier by Yeole and Dube (1997) and isolates of *P. fluorescens* and *P. aeruginosa* were designated as *Pf*4-92, *Pf*RsC5, *Pa*RsG18 and *Pa*RsG27. These isolates were grown in KB medium in 500 *ml* Erlenmeyer flasks on a rotary shaker (50 rpm) for 24 h at 28±2°C and cell concentration was adjusted to 10^8 cells ml^{-1}. The pathogen, *F. o.* f. sp. *ciceri* Rs1 (*Foc*Rs1) was obtained from the Laboratory of Applied Mycology, Department of Botany, Banaras Hindu University, Varanasi, India.

Plant growth promotion, disease induction and root colonization. Surface sterilized chickpea seeds were sown in earthen pots (18 cm dia., 3 seeds pot^{-1}) filled with *Foc*Rs1 infested and *P. fluorescens* inoculated soil. Inoculum of *Foc*Rs1 was prepared in sterile sand: maize meal medium (50 g + 1.5 g maize meal + 10 *ml* water) incubated for 15 days at 28±2°C. The inoculum (5% w/w) was mixed thoroughly in double autoclaved sandy loam chickpea field soil, the isolates of *Pseudomonas* spp. were poured separately (30 *ml* pot^{-1}, ca. 10^8 cells ml^{-1}) to the pots as per following treatments. (i) control - pots containing sterilized soil, (ii) *Foc*Rs1 control - pots containing soil infested with inoculum of the pathogen (1.0 g pot^{-1}, 10^3 cfu g $soil^{-1}$), (iii) *Pseudomonas* spp. treatment - the seeds sown in pots containing either of 4 isolates of *Pseudomonas* spp. and (iv) the soil containing isolates of *Pseudomonas* spp. + *Foc*Rs1. Experiment was carried out as complete randomized designs (CRD) in a greenhouse. Disease severity was examined for the next 27 days by the formula (Saikia *et al.*, 2003); % of DS = (Infection length in control − infection length in treatment) × 100/Infection length in control. Shoot and root length of plants was measured before the plants were removed at 45 days and population levels of strains of *P. fluorescens* were also determined using five randomly selected plantlets 1 week after challenge inoculation with *Foc*Rs1.

Iron availability on ISR. Rock wool bioassay technique was used for this experiment. Surface sterilized seeds of chickpea were sown in sand; after 7 days, seedlings were transferred to locally made rock wool cubes ($4\ cm^2 \times 4.5$ cm deep; one seed per cube) in such a way that the root system was divided over two cubes (Leeman *et al.*, 1995). *Pseudomonas* spp. were inoculated on the lower part of the root system, at the root tips and 3 days later, inoculum of the pathogen, *F. o.* f. sp. *ciceri* prepared in sand: maize meal medium (Saikia *et al.*, 2003) was applied to the part of the root system near the stem. The populations of the bacteria and the pathogen remained spatially separated throughout the experiment to avoid direct interaction between the bacteria and pathogen. These plants were grown in green house and watered with sterile deionised water. Half-strength Hoagland's nutrient solution (pH 7; Hoagland and Arnon, 1938) with iron (Fe-EDDHA 10 μM) was applied in the root base compartment where pathogen was inoculated. Ten μM EDDHA (low iron availability) or F^{3+}- saturated EDDHA (12 μM $FeCl_3$ + 10 uM EDDHA; for high iron availability) was substituted for the iron source of nutrient solution for study of the effect of iron availability on *Pseudomonas* spp. mediated ISR. This nutrient solution was applied to the root tip compartment of bacterized zone. Three week after pathogen inoculation, plants were harvested and percentage of disease severity was recorded

(Saikia *et al.*, 2003).

Iron availability on production of pseudobaction and SA. To study the *in vitro* growth and production of the siderophore, *Pseudomonas* isolates were grown in standard succinate medium (SSM) (pH 7.0) for 48 h at 28± 2°C on a rotary shaker. To observe the effect of iron on the production of pseudobaction and SA, different concentration (0~30 μM) of filter-sterilized $FeCl_3$ was added to SSM medium. Bacterial growth was measured by UV-spectrophotometer (Thermospectronic, USA) at 650 nm. In control, no bacterial isolates was inoculated. Pseudobactin was extracted from ammonium sulphate saturated supernatant with phenol-chloroform (1 : 1, w/v) followed by column chromatography as described by Raaijmakers *et al.* (1994). The concentration of pseudobactin was determined spectrophotometrically using a molar extinction coefficient of 14,000 $M^{-1}cm^{-1}$ at 400 nm and pH 7.1.

For measurement of SA, above liquid culture was centrifuged at 2800 g for 20 min at 4°C and then supernatant was acidified to pH 2. The solution was filtered through nylon membrane under vacuum and partitioned twice with 2 *ml* $CHCl_3$, finally dried under nitrogen stream at 40°C. Each sample was re-suspended in 1 *ml* of 23% methanol in 20 mM sodium acetate buffer (pH 5) and then the samples were analyzed with HPLC (Yalpani *et al.*, 1991) at 280 nm with a Bondapak C18 column (3.9 mm × 30 cm), with a mobile phase flow rate at 0.5 *ml* min^{-1}. SA was separated isocratically with 23% methanol (v/v) in 20 mM sodium acetate buffer (pH 5). Ten μl of each sample was injected in to the column. Retention time of isolated SA was compared with standard SA (Sigma). The SA was estimated μg ml^{-1} bacterial culture. All the experiments were repeated twice with 5 replicates for each treatment.

Results

Plant growth promotion, disease induction and root colonization. A marked increase in plant height was observed in *Pseudomonas* treated plants (Table 1). For example, 15.6% increase in plant height was obtained in the plants treated with *Pf*4-92. *Pa*RsG24 was found the least effective in growth promotion activity among the isolates. In general, *Foc*Rs1 inhibited the plant growth when used in combination with *Pseudomonas* isolates or alone (Table 1). For example, 37.5% and 16.6% reduction of plant height was recorded when the plants were grown in *Foc*Rs1 infested and *Foc*Rs1 plus *Pf*4-92 inoculated pots, respectively. The isolates of *Pseudomonas* systemically induced resistance against *Fusarium* wilt of chickpea and suppressed the wilt disease by 34~45% compared to control. Maximum reduction (45%) in disease was observed with *Pf*4-92, followed by *Pf*RsC5 (41.4%) and *Pa*RsG18 (36.4%; Table 1).

Table 1. Effect of *Pseudomonas* isolates on chickpea growth and *Fusarium* wilt disease

Treatment*	Disease severity (%)	Plant height (cm)
*Pf*4-92	0.0	37.0±2.2
*Pf*RsC5	0.0	36.3±1.3
*Pa*RsG18	0.0	35.0±2.0
*Pa*RsG27	0.0	34.2±1.0
*Pf*4-92 + *Foc*Rs1	30.0±1.5	24.0±1.1
*Pf*RsC5 + *Foc*Rs1	32.2±2.1	23.3±0.7
*Pa*RsG18 + *Foc*Rs1	35.0±1.7	23.0±1.4
*Pa*RsG27 + *Foc*Rs1	36.3±2.0	22.5±0.6
Control	0.0	32.0±1.7
*Foc*Rs1 control	55.0±2.6	20.0±1.5

Critical difference (C.D.) = 3.43 for disease severity; 5.25 for plant height; C.D. computed at $P = 0.05$. **Pf*4-92: *Pseudomonas fluorescens* 4-92; *Pf*RsC5: *Pseudomonas fluorescens* RsC5; *Pa*RsG18: *Pseudomonas aeruginosa* RsG18; *Pa*RsG27: *Pseudomonas aeruginosa* RsG127; *Foc*Rs1: *Fusarium oxysporum* f. sp. *ciceri* Rs1.

All the isolates of *Pseudomonas* colonized roots of chickpea, however, bacterial populations were not significantly different before and after challenge with *Foc*Rs1. Population densities were from 4.06 to 5.08 log CFU gm^{-1} when determined before challenge with *Foc*Rs1 and bacterial densities determined 1 week after challenge with *Foc*Rs1 were from 5.01 to 5.58 log CFU gm^{-1}. In both the cases, population density was highest in isolate *Pf*4-92 than others (Fig. 1). No bacteria were isolated in blank control.

Iron availability on ISR. Except PaRsG27, all other isolates suppressed the disease significantly ($P = 0.05$) both at low (10 μM EDDHA) and high iron (10 μM

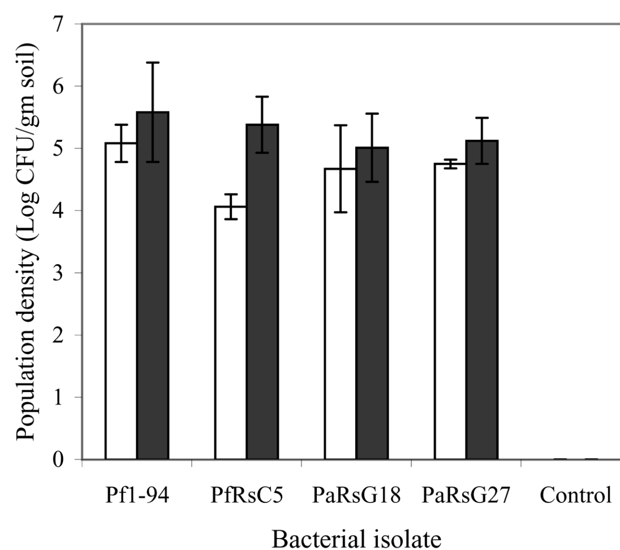

Fig. 1. Chickpea root colonization by *Pseudomonas* spp. Bars represent: □ = before inoculation and ■ = after inoculation; Ɪ =S.D.

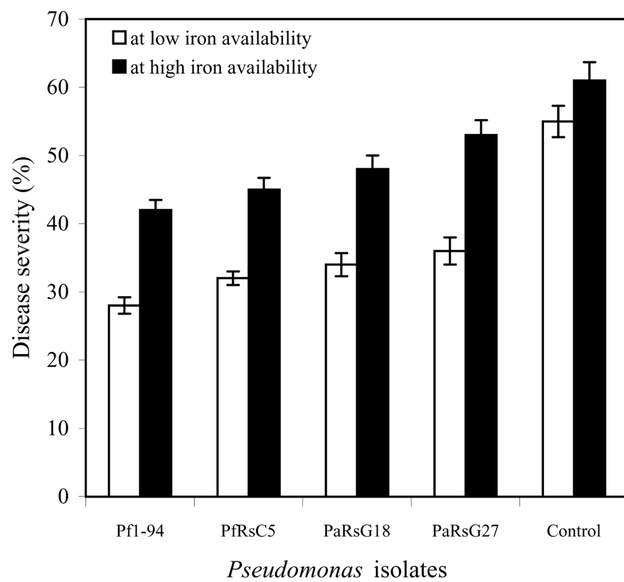

Fig. 2. Percentage of disease severity in the ISR at low (10 μM EDDHA) and high 10 μM (Fe-EDDHA) iron availability.

Fe^{3+} EDDHA) availability (Fig. 2), however, more disease reduction was observed at low iron than high iron availability. *Pa*RsG27 suppress the disease significantly (P = 0.05) only at low iron availability.

Iron availability on production of pseudobaction and SA. Pseudobactin was produced by all the isolates of *Pseudomonas* except *Pa*G27. Isolate *Pf*4-92 produced more pseudobactin than other isolates, as detected at 400 nm (Fig. 3a). No pseudobactin was detected in *Pa*G27 culture. All the isolates of *Pseudomonas* produced SA in SSM medium. More pseudobactin and salicylic acid (SA) was produced at low iron (10 μM EDDHA) than high iron availability (10 μFe^{3+} EDDHA). HPLC analysis also indicated that *Pf*4-92 produced comparatively more SA (29 μg ml^{-1}) than the other isolates (Fig. 3b). In control, where no bacterial strain was inoculated, neither pseudobactin nor SA was detected (Fig. 3a & 3b).

The production of pseudobactin as well as SA production was affected by iron availability in the medium. The production of these siderophore decreased with increasing iron ($FeCl_3$) concentration. More pseudobactin and SA was produced by the isolates when iron was not present in the medium (Fig. 4a & 4b). A rapid reduction of pseudobactin production was observed between the concentrations of $FeCl_2$ from 5 μM to 15 μM. No pseudobaction production was observed after 20 μM concentration of $FeCl_3$. SA production was also progressively decreased with the increasing of $FeCl_3$ concentration. SA production was recorded up to 25 μM concentration of $FeCl_3$. However, after this concentration of $FeCl_3$ no SA was detected.

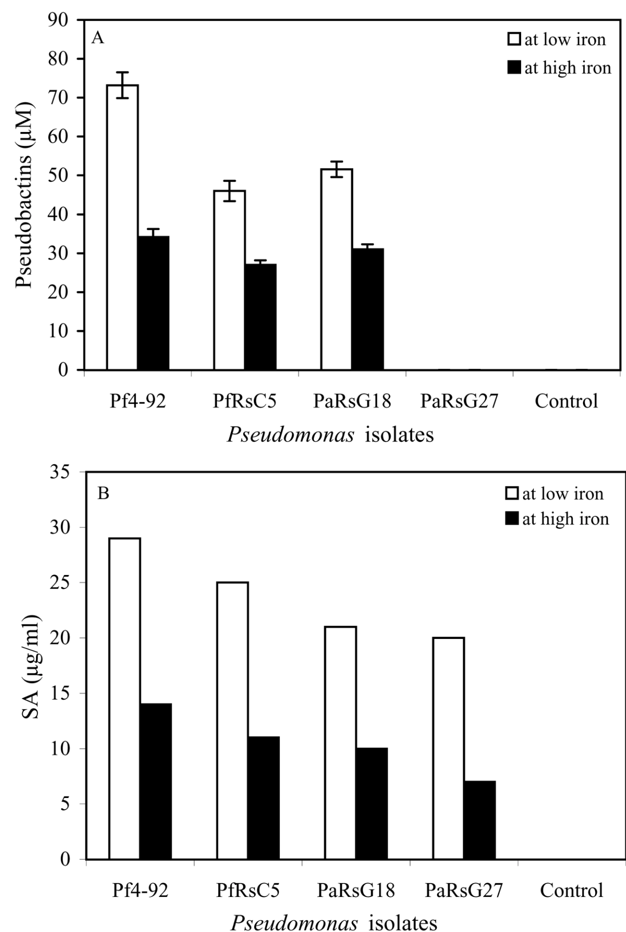

Fig. 3. Production of pseudobactins (A), salicylic acid (B) by *Pseudomonas* spp.

Discussion

Pseudomonas spp. have been widely used for plant growth promotion as well as for the control of plant diseases (Kloepper, 1992; Meena *et al.*, 2000; Saikia *et al.*, 2003; Siddiqui and Shaukat, 2004). The results demonstrate that the isolates of *Pseudomonas* increased the growth and controlled the severity of wilt disease of chickpea. Plant growth promotion by *Pseudomonas* spp. is studied in detail and several mechanisms such as phosphate solublization, production of siderophore and plant hormones, greater rhizosphere competence etc. has been suggested for their growth promotion activity (Glick, 1995; van Loon *et al.*, 1998). The phenomenon of induction of disease resistance may be dependent on SA accumulation or jasmonic acid pathway (van Loon *et al.*, 1998). Significant level of disease control was observed by the isolates of *Pseudomonas* inoculation (Table 1). These results are in agreement with the work of Meyer and Hofte (1997), Meena *et al.* (2001), Saikia *et al.* (2003).

In this study, we observed that the isolates of *Pseudomonas* colonized in root system of chickpea (Fig. 1). Plant

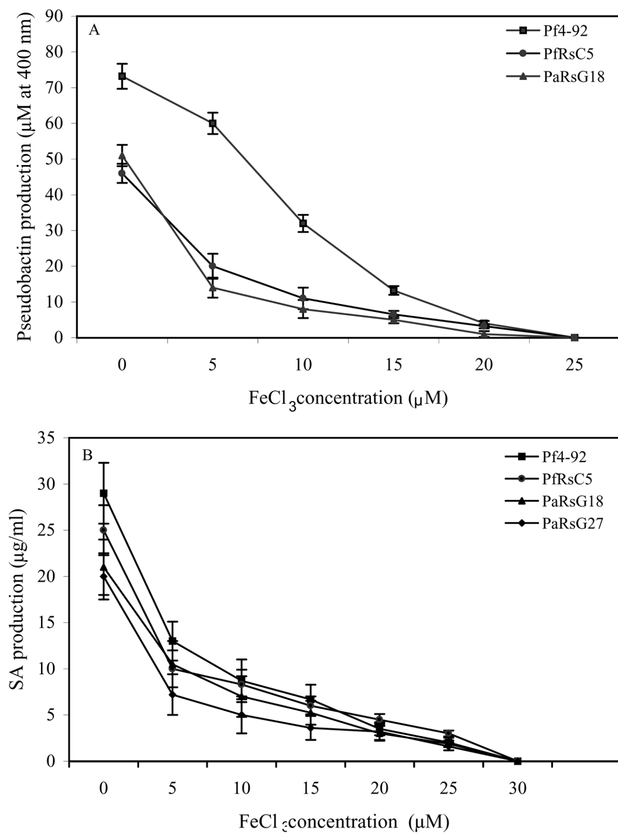

Fig. 4. Effect of $FeCl_3$ concentration on pseudobactin production (A) and SA production (B).

growth promoting bacteria including *Pseudomonas* spp. have been reported to stimulate the development of healthy root system and rapidly colonized root system of plant was also reported by Bolton *et al*. (1990). The inductions of physiological changes by application of *Pseudomonas* spp. have been demonstrated in several plants, which reduced the disease symptoms of a wide range of pathogens (Leeman *et al.*, 1995; Liu *et al*., 1995; Hoffland *et al.*, 1996). Our results also observed that isolates of *Pseudomonas* spp. enhanced chickpea growth promotion and suppressed the disease.

Rock wool bioassay for ISR study, both *P. fluorescens* and *P. aeruginosa* could reduce more disease severity when the iron availability in the nutrient solution was low (Fig. 2). The results indicate that *Pseudomonas* mediated ISR is dependent on iron availability. In presence of high iron availability i.e. addition of Fe-EDDHA to the nutrient solution reduced the disease suppressive activity of the *Pseudomonas* isolates. This observation was in agreement with those reported by Leeman *et al.* (1996) and Press *et al.* (2001). Leeman *et al.* (1996) observed that when radish plant was treated with *P. putida* strain WCS358 did not show ISR under conditions of high iron availability. Similarly, the other strain *P. fluorescens*WC374 was more effective in suppressing disease through ISR under low iron availability.

The amount of production of SA and pseudobactin differed among the isolates. *Pf*4-92 which showed maximum ISR produced more SA and pseudobactin than other strains. It indicates that production of the siderophore is correlated with ISR activity. Leeman *et al.* (1996) also observed positive correlation between siderophore production and ISR activity. Many other workers have showed role of SA in ISR with different plants (Meyer and Hofte, 1997; Chen *et al.*, 1999; Audenaert *et al.*, 2002; Saikia *et al.*, 2003). The findings indicate that SA and pseudobactin production is negatively regulated by iron under culture conditions and depends upon the concentration $FeCl_3$.

Production of SA was recorded in microbial decomposed of corn and rye residues (Chou and Patrick, 1976) and also in rhizosphere soil of mung bean and corn, but, SA was not detected in non-rhizosphere soil (Pareek and Gour, 1973). It suggests that saprophytic and rhizosphere microorganisms are involved in SA biosynthesis. In present study, apparently, it seems that at low iron availability more salicylic acid was produced and it may be responsible for the additional reduction of disease in the ISR rock wool bioassay under low iron. However, the possibility cannot be denied that the pseudobactin was involved in the ISR as well. Although, the pseudobactin was not detected in *Pa*RsG27 strain, it also significantly ($P = 0.05$) suppressed the disease. It is not clear by which disease-suppressive mechanisms the *Pa*RsG27 strain was able to suppress the disease. In some bioassay, pseudobactin negative mutant of *P. putida* WCS358 could suppress *Fusarium* wilt disease of radish (Leeman *et al.*, 1996). However, Boer *et al.* (2003) reported that the pseudobactin negative strains *P. putida* was no longer suppressed disease significantly. We think that effect of iron availably on ISR caused by *Pseudomonas* spp. against *Fusarium* wilt of chickpea is a new finding of this paper.

Overall findings suggest that - (i) *Pseudomonas* spp. can control the *Fusarium* wilt of chickpea as well as promote the growth and colonized the roots of chickpea; (ii) ISR caused by *Pseudomonas* spp. against *Fusarium* wilt of chickpea is related with iron availability; (iii) though both SA and pseudobactin taking part in ISR, *Pseudomonas* isolate which do not produce pseudobactin can also exihibit ISR activity.

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