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* (Pf4-92 and PfRsC5) and *P. aeruginosa* (PaRsG18 and PaRsG27) 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* (FocRs1). 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 µM FeEDDHA). Except PaRsG27, 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 cysteinyil 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 rhizobacteria 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/8-92, PfRsG5, PdRsG18 and PdRsG27. 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 (FocRs1) 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 FocRs1 infested and _P. fluorescens_ inoculated soil. Inoculum of FocRs1 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 dual autoclaved sandy loam chickpea field soil, the isolates of _Pseudomonas_ spp. were poured separately (30 ml pot^-1_, ca. 10^7 cells ml^-1_) to the pots as per following treatments. (i) control - pots containing sterilized soil, (ii) FocRs1 control - pots containing soil infested with inoculum of the pathogen (1.0 g pot^-1_, 10^8 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. + FocRs1. 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 = (Infected 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 FocRs1.

**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 × 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 Fe^2+– saturated EDDHA (12 µM FeCl_3 + 10 nM 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.
Iron availability on production of pseudobactin 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 pseudobactin and SA, different concentration (0–30 µM) of filter-sterilized FeCl3 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 CHCl3, 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 (Y alpani 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 Pf4-92. PaRsG24 was found the least effective in growth promotion activity among the isolates. In general, FocRs1 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 FocRs1 infested and FocRs1 plus Pf4-92 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 Pf4-92, followed by PfRsC5 (41.4%) and PaRsG18 (36.4%; Table 1).

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

| Treatment* | Disease severity (%) | Plant height (cm) |
|------------|----------------------|------------------|
| Pf4-92     | 0.0                  | 37.0±2.2         |
| PfRsC5     | 0.0                  | 36.3±1.3         |
| PaRsG18    | 0.0                  | 35.0±2.0         |
| PaRsG27    | 0.0                  | 34.2±1.0         |
| Pf4-92 + FocRs1 | 30.6±1.5 | 24.0±1.1         |
| PfRsC5 + FocRs1 | 32.2±2.1 | 23.3±0.7         |
| PaRsG18 + FocRs1 | 35.7±1.7 | 23.0±1.4         |
| PaRsG27 + FocRs1 | 36.3±2.0 | 22.5±0.6         |
| Control    | 0.0                  | 32.0±1.7         |
| FocRs1 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. *Pf4-92: Pseudomonas fluorescens 4-92; PfRsC5: Pseudomonas fluorescens RsC5; PaRsG18: Pseudomonas aeruginosa RsG18; PaRsG27: Pseudomonas aeruginosa RsG127; FocRs1: 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 FocRs1. Population densities were from 4.06 to 5.08 log CFU gm–1 when determined before challenge with FocRs1 and bacterial densities determined 1 week after challenge with FocRs1 were from 5.01 to 5.58 log CFU gm–1. In both the cases, population density was highest in isolate Pf4-92 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 M EDTA).
Iron availability on production of pseudobactin and SA. Pseudobactin was produced by all the isolates of *Pseudomonas* except *PaG27*. Isolate *Pf4-92* produced more pseudobactin than other isolates, as detected at 400 nm (Fig. 3a). No pseudobactin was detected in *PaG27* 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 μM Fe<sup>3+</sup>EDDHA). HPLC analysis also indicated that *Pf4-92* produced comparatively more SA (29 µg ml<sup>−1</sup>) 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<sub>3</sub>) 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<sub>3</sub> from 5 μM to 15 μM. No pseudobactin production was observed after 20 μM concentration of FeCl<sub>3</sub>. SA production was also progressively decreased with the increasing of FeCl<sub>3</sub> concentration. SA production was recorded up to 25 μM concentration of FeCl<sub>3</sub>. However, after this concentration of FeCl<sub>3</sub>, no SA was detected.

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

**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 solubilization, 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...
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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. P4-92 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 FeCl3.

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 PaRsG27 strain, it also significantly (P = 0.05) suppressed the disease. It is not clear by which disease-suppressive mechanisms the PaRsG27 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 availability 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 exhibit ISR activity.

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