Role of fluorescent pseudomonads in the suppression of root rot and damping-off of cotton

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Rhizoctonia solani Kuhn, which causes the root rot and damping-off in cotton, is the primary pathogen associated with the seedling disease complex. It can live in soil for long periods. In the presence of host debris, this pathogen is difficult to manage by chemical means or by breeding for resistance (10). In recent years, biological control of soil-borne plant pathogens has been successfully achieved using antagonistic fluorescent pseudomonads (FP) through ‘seed bacterization’ (4,5,11). Fluorescent pseudomonads colonize the root system. They also produce a variety of antagonistic secondary metabolites that include siderophores (7), antibiotics (3) and volatile compounds such as cyanide (12). These attributes make fluorescent pseudomonads effective biocontrol agents. Chet (2) suggested that activity may not be restricted to only one of these characters. An efficient biocontrol agent may affect the pathogens by a combination of mechanisms. In this study, we evaluated the antagonistic potential of fluorescent pseudomonad isolates in the suppression of R. solani in cotton.

Fluorescent pseudomonads were isolated (6) from the rhizosphere of healthy cotton seedlings using King’s B medium or Pseudomonas agar for fluorescein (PAF). The rhizosphere soil samples were taken from different geographic locations and from different cotton species, viz., Gossypium hirsutum, G. arboreum, and G. barbadense. The isolates were additionally characterized by standard microbiological tests (6). The fungus R. solani was isolated from the infected cotton seedlings showing the symptoms of collar rot. The aggressiveness of the fungus was tested by soil inoculation on cv. H-777 (G.hirsutum).

The antagonism of fluorescent pseudomonads to R. solani was tested in vitro by the dual culture technique on PAF medium. The PAF plates inoculated with only fungus served as control. To study the effect of iron (Fe³⁺) on the inhibition of fungal growth by FP isolates ferric chloride, sterilised using bacteriological filter was incorporated at a final concentration of 100 μg/ml of PAF medium.

Production of cyanide as one of the volatile metabolites was tested using the method of Lorck (8). The influence of volatile secondary metabolites of FPs on the growth of R. solani was studied by paired petri dish technique (6). FPs were inoculated on PAF and PAF with glycine (4.4g/L). The fungus was inoculated on PDA. Each PDA plate with the fungus(above) was paired with a petri dish containing the bacteria (below) and sealed with parafilm. Plates inoculated with fungus only, paired with uninoculated PAF or PAF with glycine plates served as controls. The fungal growth was measured as colony diameter (mm) on the 3rd and 6th day of incubation.

For bioassay of antifungal substance the isolate FP-43 was used. From 7-day old growth on PAF medium the bacteria were scraped and removed. The agar from each of these plates was cut into one square cm pieces and extracted with 20 ml acetone for each plate. The yellowish green fluorescent extracts were then filtered through two layers of muslin cloth. The pooled acetone extract (300 ml) was reduced to one-third of the original volume at 38-40°C under low pressure. Saturated sodium chloride solution (25 ml) was added to this acetone extract acidified with HCl to pH 1-2. This was re-extracted with 100 ml of ethyl acetate by vigorous shaking in a separating funnel and the volume was reduced to 1 ml at 35°C. A 0.5 ml sample was streaked on thin layer plates (0.5 mm) coated with silica gel GF 60. These plates were then developed in chloroform- methanol (100:1) solvent system. The bands were observed under UV light, scraped off individually and eluted with acetone.
Table 1. Antagonistic suppression of damping off of cotton caused by *Rhizoctonia solani* by fluorescent pseudomonads in greenhouse

<table>
<thead>
<tr>
<th>Fluorescent pseudomonad isolates</th>
<th>Mean diseased plants (%)</th>
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<tbody>
<tr>
<td>FP-25</td>
<td>72.0 (58.8)*</td>
</tr>
<tr>
<td>FP-28</td>
<td>36.0 (36.4)</td>
</tr>
<tr>
<td>FP-33</td>
<td>62.0 (52.0)</td>
</tr>
<tr>
<td>FP-43</td>
<td>10.0 (14.6)</td>
</tr>
<tr>
<td>FP-47</td>
<td>24.0 (23.8)</td>
</tr>
<tr>
<td>Control</td>
<td>88.0 (73.9)</td>
</tr>
</tbody>
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CD (p=0.05) 18.6
CD (p=0.01) 25.2

*Figures in the parentheses indicate Arc Sine transformed values

Table 2. Distribution of fluorescent pseudomonad on cotton roots

<table>
<thead>
<tr>
<th>Distance along roots from base</th>
<th>CFU/cm of root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FP-43</td>
</tr>
<tr>
<td>1st cm</td>
<td>74.6 x 10⁶</td>
</tr>
<tr>
<td>2nd cm</td>
<td>10.0 x 10⁶</td>
</tr>
<tr>
<td>3rd cm</td>
<td>13.6 x 10⁶</td>
</tr>
<tr>
<td>4th cm</td>
<td>10.6 x 10⁶</td>
</tr>
</tbody>
</table>

*Each seed was coated with 60 x 10¹⁰ CFU of fluorescent pseudomonad. The seeds were incubated in a soil-free moist chamber. The antagonist population on each piece of root (1 cm in length) was measured by dilution plating on PAF medium supplemented with rifampicin.

For assaying the antifungal activity, a small well (8 mm in diameter) was made at the centre of the PAF plate. Around this well, four fungal plugs were inoculated at equal distances (3cm) from centre. After 24 h of incubation, 0.1 ml of acetone eluate was placed in the well and left for 4 d. The plates with fungus and 0.1 ml acetone served as control. The plates were then examined for the mycelium-free zone around the well.

Acid de-linted cotton seeds were treated with five selected FP isolates viz., FP-25, FP-28, FP-33, FP-43 and FP-47. Seeds were soaked for 24h in 45-h-old growth of bacterial cells suspended in 0.1M MgSO₄ to which 1% (W/V) carboxymethyl cellulose (sodium salt) was added. The seeds were then air dried on a blotting sheet. Seeds treated similarly but without bacteria served as control. Field-collected soil was mixed with farm yard manure (3:1 ratio) and sterilised by 1% formalin treatment. The upper 6 cm soil from each pot (20 cm in diameter) was thoroughly mixed with 100 ml of water containing 0.5g mycelial fragments of *R. solani*. Bacteria charged seeds were then sown @10 seeds/pot. Five replications were kept for each treatment. The average percentage of diseased plants in treated and control pots were recorded on the tenth day after germination. The experiment was laid out in a completely randomized design and the data were subjected to ANOVA.

The transport and colonization of antagonistic FP isolates (FP-43 and FP-47) was studied on the roots of germinated seeds in a soil-free moist chamber. To monitor the population on root surface rifampicin resistant antagonists were used. Surface-sterilised cotton seeds were heavily coated with rifampicin resistant mutants (60x10¹⁰ CFU/ seed). After air drying, the seeds were kept in a moist petridish at 30°C. Six seeds were kept for each treatment. After five days, the roots of the germinated seedlings were cut into pieces (1 cm long). The population of antagonists in each root segment was studied by serial dilution on PAF supplemented with 100 ppm of rifampicin.

Forty-eight fluorescent bacterial isolates were established from the rhizosphere of different species of cotton. The bacteria were found to be gram-negative, gelatine liquifiers, oxidase- and arginine dihydrolase-positive. They produced yellowish green fluorescent pigment on King's B medium. The fungus *R. solani* was found to be highly virulent on cotton. It produced typical rotting symptom at the collar region of the seedlings.

Sixteen isolates were found to inhibit the growth of *R. solani* in dual culture on PAF medium. The radius of inhibition zone ranged from 2 mm in FP-44 to 11.5 mm in FP-43. In most cases the antagonistic effect was drastically reduced in ferric chloride-amended PAF medium indicating the production of some antifungal compound(s) only under iron-limiting condition. Further, there was a total suppression of fluorescent pigment production by FP isolates in iron-supplemented medium. This indicates the relation between fungal growth suppression and fluorescent pigment production.

All the 16 isolates which showed antagonistic activities in dual culture plate were found to produce volatile cyanides. The effect of volatile metabolites produced by the isolates, FP-25, FP-28, FP-33, FP-43, and FP-47, on the growth of *R. solani* was studied. All
the five isolates significantly \((p=0.05)\) suppressed the fungal growth when compared to control (Fig. 1a).

The present study obtained two sets of evidence for the production of siderophore as antifungal compound by the FP isolates. Firstly, incorporation of iron \((\text{FeCl}_3)\) in an iron-depleted medium (for example, PAF) suppressed the production of fluorescent green pigment and drastically reduced the antagonism exhibited by FPs in dual culture. Secondly, the addition of an aqueous solution of iron reagent \((0.1\text{M FeCl}_3\) in \(0.1\text{N HCl}\)) to the yellowish-green culture filtrate caused an immediate colour change to reddish-brown, accompanied by a total disappearance of fluorescence. Meyer and Abdallah (9) showed that these changes were due to complexes formed by siderophores with \(\text{Fe}^{3+}\). In our study, thin layer chromatography of the ethyl acetate extract produced four bands. One fraction \((\text{RF} = 0.18)\) gave a reddish-brown reaction with iron reagent. Acetone eluates of this fraction produced 3.6 cm diameter inhibition zone against \(R.\ solani\).

Fluorescent pseudomonads have been successfully used as biological control agents against many soil-borne plant pathogens. The present data show that in bacteria-charged seeds the disease incidence was significantly less \((p=0.05)\) with isolates FP-43, FP-47, FP-28 (Table 1). Isolate FP-43 and FP-47 reduced the disease to 10% and 24%, respectively from 88% in control treatment. Isolate FP-28 reduced the disease to 36%.

More than \(10^7\) CFU of FP-43 per cm root could be detected throughout the root system (Table 2). In FP-47 also, a high population \( (>10^7\ \text{CFU/cm})\) was detected up to 3rd cm of root from the base; beyond this, the population declined. The presence of fluorescent pseudomonads at higher population levels along the root indicates their mobility from seeds and later, multiplication along the root system.

Our studies demonstrate that fluorescent pseudomonads can be successfully used for the management of root rot and damping-off in cotton in India. This can be made through proper ‘seed bacterization’ with FPs. The results also suggest that more than one factor is associated with antagonistic activity of the fluorescent pseudomonads.
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REFERENCES


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