Analysis of mutation affecting antifungal property of a fluorescent Pseudomonas sp. during cotton-Rhizoctonia interaction

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ABSTRACT: Pseudomonas sp. strain EM 85, a rhizosphere isolate, showed in vitro antifungal activity against a number of soil borne plant pathogens and produced multiple antifungal compounds. A mutant AN 21 generated from the wild type strain by chemical mutagenesis showed no antifungal property. The wild type and the mutant strains were analysed in detail with respect to the antifungal activity. Both the strains produced similar levels of siderophores, HCN and fluorescent pigments. The mutant, however was unable to produce an antifungal antibiotic and failed to inhibit the fungal growth. In vivo experiment also established that antifungal antibiotic was responsible for the disease control ability of the strain.

Key words: Biological control, fluorescent pseudomonad, cotton, Rhizoctonia solani

Soil pseudomonads are excellent biological control agents against diseases caused by soil borne plant pathogenic fungi. They are efficient root colonisers on crop plants. The mechanism of plant disease control by soil pseudomonads include production of antibiotics, siderophores, volatile compounds like HCN and ammonia, induction of systemic resistance and competition for nutrients (Dowling and O'Gara, 1994; O'Sullivan and O'Gara, 1992). Siderophores are iron chelating ligands produced by bacterial strains when the availability of Fe in the surrounding medium become low. Ammonia and HCN are produced at specific conditions as well as at specific growth stages of the bacteria. The contribution of each of these compounds in the disease controlling ability of the producer strain vary among different species and strains. Mutations, both biological and chemical have been used for elucidating the mechanism of biological control by fluorescent pseudomonads (Bakker et al., 1986; Schippers et al., 1987; Loper 1988; Keel et al., 1992; Thara and Gnanamanickam, 1994; Bangera and Thomashow, 1996; Chaterjee et al., 1996).

MATERIALS AND METHODS
Organisms
Pseudomonas sp. strain EM 85 was isolated from maize rhizosphere and its biological control efficiency has been proved under pot culture conditions (Pal, 1996). The strain was reported to be having multiple antifungal characteristics like production of siderophores, volatile compounds and antibiotics. A virulent strain of Rhizoctonia solani obtained from ITCC, New Delhi was used in this study. Fungal strain was cultivated on potato dextrose agar (PDA) and the bacterial strains on Luria agar broth and PDA/ potato dextrose broth (PDB).

Inhibition assay
In vitro inhibition assay was performed on Potato Dextrose Agar medium. A heavy inoculum from an actively growing Pseudomonas strain EM 85 was applied using a loop on the edge of a PDA plate. A fungal agar disc of 5 mm diameter was cut out using a cork borer from an actively growing fungal culture grown on PDA and kept at the centre of the plate inoculated with the bacterial culture. Plates were incubated at 28°C for two to five days and observed for zone of inhibition of fungal growth around the bacterial streak.

Chemical mutagenesis of the wild type strain
To 10 ml of actively growing bacterial culture in 30 ml tubes, nitrosoguanidine was added @ 100 μg/ml and incubated for 2 h with shaking. The cells were then pelleted and washed twice with sterile water and resuspended in 10 ml LB. Mutations were fixed by incubating the culture overnight and the mutant bank was stored at -70°C after addition of glycerol at a final
concentration of 25 ml/l. Single colonies obtained from this were used for inhibition assay. Failure to inhibit the growth of the fungus was taken as the mutant phenotype.

Inhibition of fungal growth by cell free extract

The biochemical nature of cell free extract of the wild type strain and the mutant was studied by an inhibition assay as described below. Single colonies of the wild type and mutant strains were separately inoculated into 5 ml of PDB in 30 ml tubes and incubated overnight with shaking. Cells were harvested at 10,000 rpm for 15 min and the supernatant collected aseptically, filter sterilised and the volume was reduced to 200 µl using a concentrator. Wells of 10 mm diameter were made towards the edge of PDA plates by removing the agar disc from the medium using a cork borer. The wells were then partially sealed with molten agar. When the agar solidified, 100 µl of the concentrated cell free extract was put carefully into the well and allowed to percolate. Co-inoculation of the plate with actively growing *Rhizoctonia solani* agar disc was also done and observed for inhibition of fungal growth after three days of inoculation.

Extraction of antifungal compound

Antifungal factor(s) were extracted according to the method of Levy *et al.*, (1988). Ten PDA plates each were heavily inoculated with the wild type and mutant strains. Agar was collected from these plates after incubation for 48 h, made into small pieces and treated with 2 volumes of acetone in a 11 flask for 24 h with shaking. The contents in the flask were collected, filtered through muslin cloth, centrifuged and the supernatant collected. Acetone was evaporated in a rotary evaporator. The aqueous fraction was extracted twice with diethyl ether and the ether fraction was evaporated to dryness. The dried material was dissolved in 1 ml acetonitrile and 100 µl used for inhibition assay against *R. solani* as the above experiment. Growth inhibition of the fungus was observed after 2 days of incubation.

Siderophore production

Wild type and the mutant strains were tested for production of siderophores on universal chrome azurol solution agar (CAS) medium (Schwyn and Neilands, 1987). Cell suspensions from freshly streaked plates were spot inoculated on to CAS A medium and incubated for 24 h. Presence of a yellow halo around the colonies indicated siderophore production. The siderophore activity and siderophore specific activity were done according to Page and Huyer (1984).

HCN production

HCN production was detected as per the procedure described by Castric (1977) on King's B medium amended with glycine. HCN production was indicated by yellow to deep brown colour change of a filter paper dipped in 0.5% picric acid and 2% sodium carbonate placed inside the lid of the sealed petriplate.

In vivo biological control activity

The biological control efficiency of the wild type and the mutant strains of the bacteria in controlling *Rhizoctonia solani* induced damping off of cotton seedlings was assessed in pot cultures. Pots were filled with unsterile soil and fungal inoculum prepared in sand-maize powder mixture (Rajendran and Ranganathan, 1996) was added @100 g/pot to the upper 10 cm of the soil one week prior to planting. Ten seeds of cotton (cv. Pusa 761) pre-soaked in sterile water for 10 h were sown in each pot by dibbling. Overnight culture of the bacterial inoculum grown in PDB was applied @ 2 ml on to the seeds in the planting hole, before covering the seeds with soil. Observations on damping off was taken one week after sowing.

RESULTS AND DISCUSSION

*In vitro* inhibition of growth of fungus on PDA on co-inoculation with the wild type bacterial strain was taken as evidence for the antifungal activity of the biocontrol strain. The fungal hyphae failed to grow over the bacterial streak even after one week incubation. Growth inhibition zone of 7-10 mm was found around the wild type bacterial colony. The phenotype of wild type strain was designated as Aft<sup>t</sup>. Treatment with nitrosoguanidine for 2 h resulted in 99.42 % killing of the bacteria and was sufficient to create enough number of mutations in the surviving population. Colonies from the mutant bank were used for screening for the antifungal defective mutants. A colony

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of plants survived&lt;sup&gt;*&lt;/sup&gt;</th>
<th>% increase over control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. solani</em> + EM 85 (wild)</td>
<td>7.5a</td>
<td>57.89</td>
</tr>
<tr>
<td><em>R. solani</em> + AN 21 (mutant)</td>
<td>3.25b</td>
<td>-31.58</td>
</tr>
<tr>
<td><em>R. solani</em> (control)</td>
<td>4.75b</td>
<td>-</td>
</tr>
<tr>
<td>No inoculation with</td>
<td>9.25c</td>
<td>94.74</td>
</tr>
</tbody>
</table>

*R. solani*

*Mean of four replications having 10 seeds each; CD (P = 0.05): 1.23 ; Digits followed by same alphabets do not differ significantly
which completely failed to inhibit the fungal growth was obtained and was named as AN 21 and the phenotype designated as Aft- (Fig. 1).

Comparison of the wild type and the mutant strains showed that there was no change in the colony morphology of both the strains on nutrient agar, PDA or minimal medium. The generation time of the wild type strain and the mutant was found out by plotting the growth curve by viable count method in broth culture. The generation time of the wild type and the mutant was found to be 52 and 54 min respectively. The growth rate of the two was showing similar trend in nutrient broth and PDB.

A concentrated cell free extract of the wild type strain showed inhibition of growth of *R. solani* on PDA whereas, that obtained from mutant AN 21 failed to do so, showing that the fungal inhibitory factor(s) was lacking in case of the mutant strain. Abolition of inhibitory activity in case of the mutant strain may be attributed to mutations either on the genes governing the antifungal factor(s) production or those governing protein transport enabling the bacterium to secrete the toxin into the medium. When the bacterial cells were physically disrupted by sonication, and the supernatant thus obtained was tested likewise, then also the mutant extract failed to inhibit the growth of the fungus. These observations indicated that in the present study, the defect may not be due to mutation in any protein transport genes but due to its effect on the genes governing the production of inhibitory factor(s). Further there was fungal growth inhibition with the crude antibiotic preparation obtained from the wild type strain, whereas that obtained from the mutant AN 21 failed to do so. As the mutant AN 21 completely lost the fungal growth inhibition property, the same was used for a genetic analysis for identification of genes governing the antifungal toxin production. A genomic library of the wild type strain was constructed and using a functional complementation approach the wild type genomic DNA region complementing the defective character was isolated (Anith et al., 1998). It has been reported that mutations with respect to the antifungal property may result in pleiotropic effect resulting in the alteration of many other related characters like production of pigment, HCN, siderophore etc. (Laville et al. 1992; Corbel and Loper, 1995). A detailed analysis of the mutant was done with respect to these characters.

Yellow halo of similar intensity was produced on CAS A medium by strain EM 85 and AN 21 after 24 h of incubation. Quantitative measurement of siderophore production in low iron medium revealed that both the wild type and the mutant strain released equal amount of siderophore for iron chelation. Siderophore production when expressed as total siderophore activity (TSA) gave values of 43 and 40 for wild type and mutant respectively. The wild type strain expressed a total siderophore specific activity of 102 TSA units/mg of protein and that of mutant was 98 TSA units/mg of protein. Both the wild type and mutant strain produced HCN showing browning of the filter paper.

In the *in vivo* assay, damping off was observed on the emerging seedlings one week after sowing. The number of surviving plants in each treatment was noted for the assessment of disease suppressive ability of the strains. The results obtained are given in Table 1. The statistical analysis of the data showed that there was a significant reduction in disease incidence over the control when the wild type strain was used but the mutant failed to suppress the disease at all.

The presence of multiple antifungal traits in a single biological control strain is always advantageous. However, for further improvement of the strain the efficiency and the relative importance of each of these factors in controlling the disease should be studied through genetic manipulation and mutation. In this experiment we have found out that the agent responsible for the antifungal activity of the fluorescent *Pseudomonas* strain EM 85 is an antifungal antibiotic or a similar toxin since the mutant strain was able to produce other antifungal metabolites.
REFERENCES


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