Biocontrol of charcoal rot in sorghum by fluorescent pseudomonads associated with the rhizosphere

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A B S T R A C T

Charcoal rot of sorghum caused by Macrophomina phaseolina is a disease of economic importance for which a high level of genetic resistance is not available. Therefore, we made an effort to manage this disease with fluorescent pseudomonads associated with field-grown sorghum crops. One hundred and twenty-six fluorescent Pseudomonas spp. from different sorghum growing regions of India were isolated, selected and evaluated for in vitro antifungal activities and three selected strains were further tested for management of charcoal rot under field conditions. Antifungal activities (inhibition of growth, biomass, microsclerotia production, spore germination) of the secondary metabolites and the cell-free culture filtrates of the selected fluorescent pseudomonad strains (SRB129, SRB288 and Pseudomonas chlororaphis SRB127) were studied in detail. SRB127, SRB129 and SRB288 inhibited mycelial growth of M. phaseolina ranging from 30.5 to 76.5% in dual culture assay. The cell-free culture filtrates of these strains at 20% (v/v) concentration significantly reduced the formation and germination of microsclerotia of M. phaseolina. In the field, P. chlororaphis SRB127 emerged as an effective biocontrol agent of charcoal rot of sorghum. The bacterium, when applied as seed treatment, reduced the charcoal rot incidence by >40%, crop-lodging by >20%, and increased grain mass. P. chlororaphis SRB127, when grown in a gnotobiotic sand system, effectively colonized the sorghum root and formed microcolony-like cell-aggregates in some parts of the root. Under glasshouse conditions the bacterium survived in the sorghum rhizosphere without a significant reduction in population. We conclude that the use of a selection of effective strains of bacteria can be a useful component of integrated management of charcoal rot in sorghum.

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1. Introduction

Charcoal rot of sorghum is caused by the fungus Macrophomina phaseolina (Tassi) Goid. It is a major disease in dry regions of Asia, Africa, the Americas and Australia. In India, the disease causes significant yield loss in post-rainy (Rabi) sorghum (Sorghum bicolor) occupying more than 5 million ha in the states of Maharashtra, Karnataka and Andhra Pradesh. Germination of sclerotia of M. phaseolina might be triggered by root exudates from sorghum seedlings (Smith, 1969). The germinated sclerotia can infect the primary root and cause seedling blight (Livingston, 1945). If infection occurs before the emergence of secondary roots, the plants die. Less severely infected seedlings, however, survive and establish secondary roots and grow to mature plants (Partridge et al., 1984). M. phaseolina infection leads to rotted roots followed by rotting of stalks, resulting in lodging of the plant at later stages. Charcoal rot can cause 100% lodging, and up to 64% loss in grain yield under conditions favouring disease incidence (Muguhoho and Pande, 1984), besides adversely affecting the quality of stover (dry fodder). In India as in many semi-arid tropical countries sorghum stover is nearly as valuable as grain. Lodging of the crop at flowering stage severely affects grain filling and causes loss in seed mass and quantity (Anahosur et al., 1987). In the diseased stalk, the pathogen produces many toxins. The toxin phaseolinone, produced by M. phaseolina, can cause anaemia in mice (LD50 0.98 g kg−1 body wt) (Bhattacharya et al., 1994). Thus, the disease has indirect implications on animal health. High level of genetic resistance for the disease is not available especially in high yielding cultivars on which the disease becomes severe and destructive (Das et al., 2008b). Protection of root from infection of the fungus with microbial inoculants is targeted for eco-safe management of this disease.

Biological suppression has proved to be a reliable component of integrated disease management of phytopathogenic fungi following greenhouse (Paulitz and Belanger, 2001) and field demonstration of several biocontrol agents. Among the bacterial biocontrol agents, fluorescent Pseudomonas spp. have emerged as the largest and
potentially promising group of rhizobacteria involved in the biocontrol of plant diseases (O’Sullivan and O’Gara, 1992). These bacteria are ideally suited as soil inoculants because of their rapid and aggressive colonization of the root. This feature alone is suggested as a disease control mechanism by preventing the invasion of detrimental soil microorganisms onto the root surface (Altman, 1970). Secondary metabolites like antibiotics, cyanide and siderophores have also been reported to be associated with the antagonism exerted by fluorescent Pseudomonas spp. It should be recognized, however, that there are problems of variable results obtained in different soil types (Burr and Caesar, 1984; Hase et al., 2001) and the inadequate survival of strains (Hase et al., 2000) on seeds prior to planting, preventing the commercial use of many of these biological control agents. Past research indicated that microbial isolates which originated from drought-stressed environments proved superior when used as inoculants under such conditions. Efficacy of bacterial biocontrol agents in managing charcoal rot has been demonstrated in maize (Pal et al., 2001). In an earlier study, we demonstrated that selected isolates of bacteria from the sorghum rhizosphere could stimulate seedling growth and suppress charcoal rot of sorghum in potted plants (Das et al., 2007). In the present study, attempts are made to identify antifungal fluorescent Pseudomonas spp. effective against charcoal rot of sorghum in vivo.

2. Materials and methods

2.1. Bacterial cultures

One hundred and twenty-six fluorescent Pseudomonas spp. (FP) were used for the study. The bacteria were from sorghum rhizospheres in five districts of Maharashtra and Karnataka, India (Fig. 1). Firstly, the bacteria (irrespective of genera) with an antagonistic effect on M. phaseolina were isolated from 34 rhizosphere samples following a two-layer agar plate method for isolation of antagonistic bacteria from soil (Rupela et al., 2003). M. phaseolina was spread plated on quarter-strength (1/4) potato dextrose agar (PDA); its surface was dried in a laminar flow for 4 h and then another layer (2–3 mm thick) of 1/4 PDA was poured over it, on which an appropriate dilution of the rhizosphere sample was spread plated. The plates were incubated at 28 ± 2 °C for 4–6 d. The antagonistic bacteria that produced a clear zone, inhibiting fungal growth, were picked up and purified. From this pool of antagonistic bacteria the fluorescent Pseudomonas spp. were identified, based on fluorescent pigment production on King’s B agar medium. Fluorescent Pseudomonas spp. were selected because this group of rhizobacteria is known to be potentially promising for biocontrol of plant diseases (O’Sullivan and O’Gara, 1992). The isolates were designated as ‘SRB’ (sorghum rhizobacteria) followed by the isolation number (e.g., SRB129). The charcoal rot pathogen used in the study was isolated from a diseased sorghum stalk and its pathogenicity was confirmed by Koch’s postulates.

2.2. In vitro antifungal activity

In vitro antifungal activity of 126 fluorescent Pseudomonas spp. against M. phaseolina was evaluated by dual culture assay on PDA. The isolates that inhibited growth of M. phaseolina >30% (based on diameter of clearing zone) were selected for further studies. There were 10 such strains (SRB27, SRB79, SRB84, SRB125, SRB127, SRB129, SRB183, SRB226, SRB278 and SRB288). Antifungal activity of these 10 strains was further determined by dual culture assay on...
sorghum root dextrose agar (SDA) [sorghum root extract 10% (v/v), dextrose 20 g, agar 18 g, distilled water 1 L, pH 6.8], and by a paired Petri dish technique (for antifungal volatiles). Antifungal activity of cell-free culture filtrates (CCF) of three highly effective antifungal strains (SRB127, SRB129 and SRB288) was tested by inhibition of radial growth, biomass, microsclerotia production of the fungus and in vitro microsclerotia germination assay.

2.2.1. Dual culture assay

The bacterial culture was inoculated as a line on one edge of a 90-mm diameter Petri dish containing SDA and at the centre of the plate, a 6-mm diameter disc from an actively growing fungus was inoculated. Dual cultured plates with fungus at the centre and without any bacterial inoculation served as control. The plates were incubated at 28 ± 2 °C with a 12 h photoperiod. After 5 d incubation, the radius of the fungal colony was measured and percentage of growth inhibition was calculated in comparison to the control.

2.2.2. Effects of volatile metabolites

The effect of volatile metabolites of FP on growth of M. phaseolina was studied by a paired Petri dish technique (Gagne et al., 1991). A fresh 24 h-old bacterial culture was uniformly inoculated on the surface B agar plate. In another set, PDA plates were inoculated at the centre with a 6-mm fungal disc from a 3-d-old culture. The PDA plate with the fungus (downward facing) was then paired with the Petri dish containing the bacteria (upward facing) and sealed with parafilm. Uninoculated PA plates paired with PDA plates inoculated with fungus only served as control. The paired plates were incubated at 28 ± 2 °C and fungal colony diameters were measured after 6 d.

2.2.3. Production of siderophores

Siderophores act as antimicrobial compounds by increasing competition for available iron in the rhizosphere. Selected bacterial strains (SRB27, SRB79, SRB84, SRB125, SRB127, SRB129, SRB183, SRB226, SRB278 and SRB288) were tested for production of siderophores, qualitatively on chrome azurol-S agar (CAS) as described by Schwyn and Neilands (1987).

2.2.4. Effects of CCF on biomass, radial growth and microsclerotia production of M. phaseolina

Overnight-grown bacterial culture (500 μl) was added to 50 ml of Luria–Bertani (LB) broth in a 250 ml flask and incubated at 28 ± 2 °C for 48 h. The incubated culture was centrifuged for 10 min at 10,000 rpm at 4 °C and the supernatant was filter sterilized to obtain the CCF. To study the effects of CCF on fungal biomass, the CCF was added to 30 ml of autoclaved, pre-cooled potato dextrose broth (PDB) in a 250 ml flask to a final concentration of 10, 25 and 50% (v/v). Each flask was inoculated with a 6 mm disc of actively growing fungus and incubated at 28 ± 2 °C for 5 d. Mycelial mass was harvested on a filter paper, oven dried, and the biomass recorded. Each treatment was replicated thrice and the experiment was repeated twice.

To study the effects of the CCF on radial growth of fungus, the CCF was added to autoclaved PDA medium (with 3% agar) to a final concentration of 10, 25 and 50% (v/v), just before pouring the plate. After the medium was set, a 6 mm diameter disc from an actively growing fungus was inoculated at the centre of the plate. In the control plate, the CCF was substituted with 10, 25 and 50% (v/v) sterile LB broth. The plates were incubated at 28 ± 2 °C with a 12 h photoperiod. Radius of the fungal colony was measured after 5 d. Each treatment was replicated thrice and the experiment was repeated twice.

The same plates, after recording the fungal radial growth, were used for estimation of the density of microsclerotia of M. phaseolina. Density was estimated by visual observations under a light microscope on a 1–5 scale (1 = low and 5 = high density) (Das et al., 2008a).

2.2.5. Effects of CCF on germination of microsclerotia of M. phaseolina

Germination of microsclerotia of M. phaseolina was quantified by direct microscopic observation following application of experimental treatments in vitro. A suspension of microsclerotia was prepared by macerating a 6-mm disc of fungal growth containing microsclerotia, in PDB. A reaction mixture of 100 μl (consisting of required quantity of CCF and microsclerotia) at a final concentration of 20, 40 and 80% of CCF (v/v) was placed on a cavity slide and mixed well. A microsclerotia suspension with required quantity of sterile LB broth served as control. The slides were placed in a humid chamber and incubated in the dark for 48 h at 30 ± 2 °C. Immediately after the incubation period was over, a drop of lactophenol-cotton blue was added to each slide to prevent further growth. Germinated microsclerotia were identified by morphological features of germ hyphae (Robert, 2006). Each treatment was replicated thrice and the experiment was repeated twice.

2.3. Field experiment

Three selected strains of fluorescent Pseudomonas spp. (SRB127, SRB129 and SRB288) were evaluated under field conditions for efficacy to control charcoal rot. Two Rabi sorghum cultivars, CSV8R (highly susceptible) and M35-1 (moderately resistant to charcoal rot), were used in the experiment. An experimental plot in which sorghum was grown every year for the last 10 years and occurrence of charcoal rot was very common was selected for this experiment at the farm of Centre on Rabi Sorghum, Solapur in Maharashtra. Seeds were surface sterilized with 0.01% HgCl2 for 3 min, washed thrice with sterile distilled water and treated with a t alc-based formulation of selected biocontrol agents (2 × 107 cfu g−1, at 8 g kg−1 seed). The treatments were: 1, SRB127; 2, SRB129; 3, SRB288; 4, SRB129 + SRB288; 5, carbendazim at 0.2% a.i.; and 6, control (sterile t alc powder). The field experiment was laid out in split plot design with treatment as main-plot and cultivar as sub-plot and was carried out during the post-rainy (Rabi) season in the year 2006. Each genotype was sown in four rows of 5 m length (sub-plot size: 5 m × 1.8 m) and replicated four times. Irrigation to the crop was withheld at flowering stage to induce adequate moisture stress required for development of charcoal rot. Incidence of charcoal rot and lodging was recorded at crop maturity (115 d after emergence). Each plant in a plot was split open longitudinally along with roots with a knife for ensuring the presence or absence of charcoal rot symptoms. Percentage incidence of charcoal rot and lodging was calculated for each plot. One thousand grains were counted from a grain sample representative of the bulk grains of a plot and weighed in a balance to obtain 1000-grain weight. Data on disease incidence, lodging incidence and 1000-grain weight were analyzed statistically, using a statistical software package (SPSS, version 10.0).

2.4. Root colonization and survival of bioagent

2.4.1. Root colonization

SRB127-rif1, the spontaneous mutant of SRB127, was obtained by plating the cell suspension (1 × 108 cfu ml−1) on LB agar medium with rifampicin (100 μg ml−1). The plates were incubated at 28 ± 2 °C for 72 h and mutant colonies were picked up. The mutants were evaluated for stability of resistance by subculturing 20 times on LB agar medium with rifampicin (100 μg ml−1). Root colonization of SRB127-rif1, the rifampicin-resistant mutant of Pseudomonas chlororaphis SRB127, was studied in a gnotobiotic sterile sand system (Simons et al., 1996). Surface sterilized and pre-germinated sorghum seeds were bacterized with 72 h old bacterial culture (1 × 107 cfu ml−1) for 15 min, air dried and sown in a sterile sand column (one seed per column) under aseptic conditions.
conditions. Root sampling was done by uprooting seedlings 3 and 6 d after sowing. Each root was cut into pieces of 10 mm length each starting from the base and each piece was numbered. Each root piece was suspended in 10 ml of 10 mM phosphate buffer, pH 7.2. The suspension was incubated at 30 °C for 1 h, on a rotary shaker at 150 rpm. After serial dilution, the suspension was plated on LB agar medium with rifampicin (100 μg ml⁻¹). Incubated plates were incubated at 28 ± 2 °C for 48 h. The observed colonies were counted and expressed as log₁₀ cfu cm⁻¹ root.

For scanning electron microscopic studies, root samples from sorghum seedlings raised from bacterized seed in the gnotobiotic sand system were used. The samples were transferred to vials and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4 °C and post fixed in 2% aqueous osmium tetroxide in the same buffer for 2 h. After the post fixation, samples were dehydrated in a series of graded alcohol and dried samples were mounted over the stubs with double-sided conductivity tape. Finally, a thin layer of platinum metal was applied over the sample using an automated sputter coater (JEOL JFC-1600) for about 3 min and scanned in scanning electron microscope (Model: JEOL-JSM 5600) at various magnifications at RUSKA Lab, College of Veterinary Science, Hyderabad, India.

2.4.2. Survival of SRB127-rif1 in sorghum rhizosphere

Survival and multiplication of SRB127-rif1 were tested in the sorghum rhizosphere of two sorghum cultivars (cv CSV8R and cv M35-1) in a M. phaseolina infested potting mixture under glasshouse conditions. Talc-based formulations (containing 2 × 10⁻⁷ cfu g⁻¹) of the rifampicin resistant mutants were used for seed bacterization. Survival of the bioagent in the sorghum rhizosphere was determined at regular intervals 12, 24 and 36 d after sowing (DAS). Three to five plants were uprooted at each sampling and a 5 cm portion from the middle of the root along with the tightly adhering potting mixture was suspended in 50 ml of 10 mM phosphate buffer, pH 7.2. The bacterial population in the root was estimated by serial dilution (as described in Section 2.4.1), and expressed as log₁₀ cfu g⁻¹ root. Each treatment was replicated thrice.

2.5. Identification of Pseudomonas strains SRB127

Species level identification of the Pseudomonas strain SRB127 was done as per Hildebrand et al. (1992) following standard biochemical and cultural methods (e.g., fluorescent pigment on King’s B and King’s A agar, levan production, oxidase test, gelatin hydrolysis, arginine dihydrolase test, growth at 4 and 41 °C, sugar utilization, pigment on YGCA slant, etc.). The strain SRB127 was identified as P. chlororaphis.

2.6. Data analysis

All the experiments had four replications and repeated once, unless mentioned otherwise. Data were analyzed with SPSS (version 10.0) software package.

3. Results

3.1. In vitro antifungal activity

All of the 126 FP strains evaluated, showed in vitro antifungal activity against the charcoal rot pathogen, M. phaseolina. Percentage inhibition of growth ranged from 1.9 to 76.5% in dual culture assay on PDA (Fig. 2). Inhibition ranged from 30.5 to 76.5% for the top 10 isolates (SRB27, SRB79, SRB84, SRB125, SRB127, SRB129, SRB183, SRB226, SRB278 and SRB288), and 56.5–76.5% for the top three isolates (SRB127, SRB129, SRB288).

3.1.1. Dual culture assay

All of the 10 selected bacterial strains showed antifungal activities when tested in dual culture assay on PDA and SDA. SRB127 had maximum inhibitory effect (76.5, 80.5%), followed by SRB129 (60.0, 69.8%), and SRB288 (56.5, 64.8%), respectively, on PDA and SDA medium (Table 1). Antifungal effects were greater on SDA medium (40–80.5%) than on PDA (30.5–76.5%).

3.1.2. Effects of volatile metabolites on vegetative growth of M. phaseolina

Antifungal volatile metabolites of seven bacterial strains (SRB27, SRB79, SRB125, SRB127, SRB226 and SRB288) significantly reduced the radial growth of M. phaseolina (p < 0.01) (Table 1). SRB125 inhibited growth most (diameter 43.5 mm) followed by SRB288 (diameter 47.3 mm) and SRB127 (diameter 48.8 mm), while volatiles of SRB183 and SRB84 had no significant inhibitory effect.

3.1.3. Production of siderophores

All 10 selected strains produced siderophores in CAS medium. SRB129 (18.3 mm), SRB84 (18.0 mm), SRB127 (17.0 mm), SRB79 (17.0 mm) and SRB125 (16.8 mm) produced significantly large zones of siderophores (p < 0.01) compared to other strains (SRB278, SRB27, SRB226, SRB183 and SRB288).

3.1.4. Effects of CCF on biomass, radial growth and microsclerotia production of M. phaseolina

The effect of CCF on biomass, radial growth and microsclerotial production of M. phaseolina varied with the concentration of CCF. CCF of SRB127 and SRB129 significantly reduced fungal biomass at all three concentrations (i.e., 50, 25 and 10%), while SRB288 did only at the highest concentration (i.e., 50%). At 50% concentration of CCF, reductions of fungal biomass were 60.8, 52.6 and 20.8% by SRB127, SRB129 and SRB288, respectively (Table 2). At a concentration of 50% CCF all three bacterial strains significantly reduced radial growth (SRB129: 65.5, SRB127: 37.4 and SRB288: 34.1%) (Table 2). At a concentration of 25% CCF, only SRB129 reduced the radial growth significantly, while the other two strains (SRB127 and SRB288) were not effective.

CCF of all three selected strains at the 25% or higher concentration significantly reduced the production of microsclerotia by M. phaseolina. Reduction in density of microsclerotia was significantly

![Image](https://via.placeholder.com/150)
rot and lodging was significantly high in the susceptible cultivar, significantly reduced lodging (30.4%), carbendazim (28.4%) and SRB129 (21.3%). Effects of the strains SRB288, all other treatments (SRB127, SRB129, SRB278 Parbhani, MS, SRB79 Parbhani, MS, SRB84 Solapur, MS, SRB125 Dharwad, KA, SRB127 Solapur, MS, SRB129 Solapur, MS, SRB183 Rahuri, MS, SRB226 Dharwad, KA, SRB278 Parbhani, MS, SRB288 Bijapur, KA Control – – – – – – Mean diameter of the halo zone of siderophore production (eight replications from two experiments) on chrome azurol-S agar plate measured at 48 h.

Table 1

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Source</th>
<th>Growth inhibition on dual culture (%)</th>
<th>Colony diameter (mm) on pair plating&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diameter of siderophore zone (mm)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDA</td>
<td>SDA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRB27</td>
<td>Solapur, MS</td>
<td>30.1d</td>
<td>43.0d</td>
<td>69.3d</td>
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<td>SRB79</td>
<td>Parbhani, MS</td>
<td>30.5d</td>
<td>41.0d</td>
<td>85.8e</td>
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<td>33.8d</td>
<td>40.0d</td>
<td>86.8ef</td>
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<td>51.8c</td>
<td>43.5a</td>
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<td>SRB127</td>
<td>Solapur, MS</td>
<td>76.5a</td>
<td>80.5a</td>
<td>48.8b</td>
</tr>
<tr>
<td>SRB129</td>
<td>Solapur, MS</td>
<td>60.0b</td>
<td>69.8b</td>
<td>66.0c</td>
</tr>
<tr>
<td>SRB183</td>
<td>Rahuri, MS</td>
<td>36.5c</td>
<td>40.3d</td>
<td>89.8f</td>
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<tr>
<td>SRB226</td>
<td>Dharwad, KA</td>
<td>37.3c</td>
<td>45.5c</td>
<td>63.5c</td>
</tr>
<tr>
<td>SRB278</td>
<td>Parbhani, MS</td>
<td>43.3c</td>
<td>46.8cd</td>
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<td>SRB288</td>
<td>Bijapur, KA</td>
<td>56.5b</td>
<td>64.8b</td>
<td>47.3b</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>89.0f</td>
<td>–</td>
</tr>
</tbody>
</table>

Means with different letters within the same column differ significantly at p < 0.05.

<sup>a</sup> A 6-mm fungal disc from 3-d-old culture was used as inoculum. The paired plates with test bacteria were incubated at 28 ± 2 °C and fungal colony diameter was measured after 6 d.

<sup>b</sup> Mean diameter of the halo zone of siderophore production (eight replications from two experiments) on chrome azurol-S agar plate measured at 48 h.

higher at 50% concentration than at 25%. The low concentration (i.e., 10%) CCF had no effect on microsclerotia production.

3.1.5. Effects of CCF on germination of microsclerotia of M. phaseolina

Effect of CCF on microsclerotia germination was inversely related to its concentration. At all three concentrations tested (i.e., 20, 40 and 80%), CCF of SRB127, SRB129 and SRB288 significantly reduced microsclerotia germination (p < 0.01) (Fig. 3). At the lowest concentration tested (i.e., 20%), CCF of SRB127 reduced microsclerotia germination significantly more (53%) than the CCF of strain SRB288 (39%). At 40 and 80% concentration, all were effective and the effect of CCF did not vary among the three bacterial strains. At the highest concentration of CCF (80%), the reduction of germination was 82, 79 and 72%, respectively, for SRB127, SRB129 and SRB288.

3.2. Charcoal rot suppression under field conditions

Charcoal rot and lodging varied among the treatments. Except SRB288, all other treatments (SRB127, SRB129, SRB129 + SRB288 and carbendazim), when applied as seed dressing, significantly reduced charcoal rot (p < 0.05) compared to the control (Table 3). Among the treatments, reduction of charcoal rot (over the control) was highest in SRB127 (40.4%) followed by SRB129 + SRB288 (30.4%), carbendazim (28.4%) and SRB129 (21.3%). Effects of the treatments on crop-lodging were less pronounced compared to that on incidence of charcoal rot. The reduction in lodging due to treatments ranged from 7.3% (SRB288) to 21.4% (SRB127) compared to 16.4% (SRB288) to 40.4% (SRB127) for charcoal rot incidence. Seed treatment with SRB127, SRB129 + SRB288 and carbendazim significantly reduced lodging (p < 0.05). The incidence of charcoal rot and lodging was significantly high in the susceptible cultivar, CSV8R (44.7 and 64.5%) compared to the moderately resistant, M35-1 (25.0 and 56.3%). Effect of treatment on 1000-grain weight was significant (p < 0.05) only for the treatment SRB127. The effects of SRB127 on charcoal rot and lodging were on par with that of the fungicide (carbendazim). Combined application of SRB129 with SRB288 resulted in a synergistic effect on charcoal rot or lodging compared to their single applications. There was no significant interaction between cultivar and bioagent for the parameters studied (charcoal rot, lodging and seed weight).

3.3. Root colonization and survival of SRB127 in sorghum rhizosphere

3.3.1. Root colonization by SRB127-rif1

SRB127-rif1, the rifampicin resistant mutant of P. chlororaphis SRB127, applied as seed treatment with 72 h old culture at 1 x 10<sup>7</sup> cfu ml<sup>-1</sup> colonized the primary root of sorghum seedlings. The population of SRB127-rif1 was highest at the first-basal-section of the root (log<sub>10</sub> 7.2 cfu cm<sup>-1</sup> at 3 d and log<sub>10</sub> 7.4 cfu cm<sup>-1</sup> 6 d after seed bacterization) and gradually decreased in the subsequent

**Table 2**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>CCF (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mycelial dry mass (mg)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Colony diameter (mm)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Microsclerotia density (1–5 scale)&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
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<td>87.0ef</td>
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</tr>
<tr>
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<td>54.0ab</td>
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<td>SRB288</td>
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<td>73.0cd</td>
<td>3.0c</td>
</tr>
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<td>SRB288</td>
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<td>90.3cd</td>
<td>55.7b</td>
<td>2.0ab</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>131.7e</td>
<td>82.3efd</td>
<td>4.7d</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>117.0de</td>
<td>77.7cde</td>
<td>5.0d</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>113.7de</td>
<td>84.7efd</td>
<td>4.5d</td>
</tr>
</tbody>
</table>

Means with different letters within the same column differ significantly at p < 0.05.

<sup>a</sup> CCF of stationary-phase culture was added to the autoclaved and pre-cooled PDA or PDB.

<sup>b</sup> A 6-mm fungal disc was inoculated in a flask containing 30 ml PDB and incubated at 28 ± 2 °C for 5 d.

<sup>c</sup> The M phaseolina inoculated PDA plates were incubated at 28 ± 2 °C and fungal colony diameter was measured after 6 d.

<sup>d</sup> Microsclerotia density was estimated by visual observations under a light microscope on a 1–5 scale (1 = low and 5 = high density).

Fig. 3. Effect of cell-free culture filtrates (CCF) of three selected strains of fluorescent *Pseudomonas*, SRB129, SRB127 and SRB288, on the germination of microsclerotia of *M. phaseolina*.

Germination was quantified by direct microscopic observation following application of treatment in vitro. Each data point is the mean of nine replications from three experiments. Bar represents standard error.
sections towards the tip (Fig. 4). Population cm\(^{-1}\) root was higher on the 6 d-old root compared to the corresponding root section at 3 d. A scanning electron micrograph of the root showed that the colonizing bacterial cells multiplied on sorghum root and formed cell-aggregates in selected parts of the root (Fig. 5).

### 3.3.2. Survival of SRB127-rif1 in sorghum rhizosphere

Cells of SRB127-rif1, the rifampicin resistant mutant of *P. chlororaphis* SRB127, survived in the rhizosphere of sorghum cultivars (cv CSV8R and M35-1) when tested in *M. phaseolina* infested potting mixtures under glasshouse conditions (Fig. 6). SRB127-rif1 maintained its population in the rhizosphere of both the test cultivars without significant reduction in population from 12 to 36 DAS. However, at 72 DAS, the population was quite low (log\(_{10}\) 3.71 cfu g\(^{-1}\)) compared to that at 24 DAS (log\(_{10}\) 4.00 cfu g\(^{-1}\)).

### 4. Discussion

Biological suppression is a reliable component of integrated disease management (Paulitz and Belanger, 2001). Protection of root from infection of the fungus with microbial inoculants was targeted for eco-safe management of root rot in several crops (Pal et al., 2001; Kishore et al., 2005; Fernando et al., 2007). In the present study, fluorescent *Pseudomonas* spp. from different charcoal rot prone regions of India were isolated, selected and evaluated for their efficacy against charcoal rot in sorghum. Frequency of isolation of antifungal FP strains was quite high because the strains were selected based on initial screening for antagonistic activity on

Table 3

<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>Charcoal rot (%)</th>
<th>Lodging (%)</th>
<th>1000-Grain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSV8R (S) M35-1 (MR)</td>
<td>CSV8R (S) M35-1 (MR)</td>
<td>CSV8R (S) M35-1 (MR)</td>
</tr>
<tr>
<td>SRB127</td>
<td>34.7 19.1 26.9 55.3 51.9 53.6 31.7 30.5 31.1</td>
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<td></td>
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<tr>
<td>SRB129</td>
<td>47.6 23.5 35.5 62.7 58.3 60.5 28.8 25.7 27.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRB288</td>
<td>45.4 30.0 37.7 70.3 56.1 63.2 27.8 29.3 28.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRB129 + SRB288</td>
<td>38.2 24.6 31.4 63.5 52.7 59.0 30.0 26.3 28.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbendazim at 0.2%</td>
<td>43.9 20.7 32.3 59.5 56.6 58.1 30.2 27.7 28.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58.3 32.0 45.1 74.1 62.3 68.2 27.6 27.1 27.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean of eight replications on two cultivars. NS = non-significant at 5% level of significance.

\(^a\) Seeds were treated with talc-based formulation of the biocontrol agent containing \(2 \times 10^7 \text{cfu} \, \text{g}^{-1}\) carrier material (at 8 g kg\(^{-1}\) seed) before sowing. S = susceptible and MR = moderately resistant.

*M. phaseolina*. The selected bacterial strains differed in their antifungal activities depending on growth-media (PDA, SDA) and metabolites (antibiotics, volatiles). Such variations are common among strains of fluorescent *Pseudomonas* spp. (O'Sullivan and O'Gara, 1992). Substituting potato extracts (of PDA) with the extract of sorghum root (in SDA) increased the inhibitory effects of the bacterial strains on *M. phaseolina*. Presence of antifungal compounds in sorghum roots (Odufna, 1978) might be one of the reasons for this.

Fluorescent *Pseudomonas* spp. produce an array of extracellular metabolites in the culture medium with antifungal activities like antibiotics, enzymes and cyanide. Many such metabolites play significant role in biological control of plant diseases (Dowling and

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Fig. 4. Colonization of sorghum root by SRB127-rif1 in gnotobiotic sterile sand system. Bacterial numbers (log\(_{10}\) cfu cm\(^{-1}\)) were estimated on primary roots of sorghum seedling at 3 and 6 d-old. Each data point is the mean of six replications from two experiments. Bar represents standard error.

Fig. 5. Scanning electron micrographs showing colonization by the bacterial biocontrol agent SRB127-rif1 on the root of 6 d-old sorghum seedling grown in gnotobiotic sand system. (A) Colonization pattern at the basal portion, and (B) at the middle portion of the root. The formation of cell-aggregates in some parts of the root is indicated by circle. The arrow indicates the bacterial cell undergoing division. The bar represents 5 μm.

Fig. 6. Population of SRB127-rif1, rifampicin resistant mutants of SRB127, in the rhizosphere of sorghum cultivar M35-1 and CSV8R in *M. phaseolina* infested potting mixture. Data points are the mean of six replications from two experiments. Bar represents standard error.
In the present study, CCF of the selected FP strains (SRB127, SRB129 and SRB288) showed significant inhibitory effects on biomass, radial growth and microsclerotia formation. CCF of SRB127 was highly inhibitory even at low concentrations (at 10%, v/v) and reduced reproduction ability of the pathogen. Such anti-fungal metabolites of the bacterial antagonists might inhibit and inactivate the pathogen in the natural environments and aid in biological control of soil-borne pathogens. The ability of biocontrol pseudomonads to produce the antifungal compound 2,4-diacetylphloroglucinol was associated with superior disease suppression activity in the *Pythium*–cucumber and *Fusarium*–tomato pathosystems (Rezzonico et al., 2007). The phenazine-1-carboxamide (PCN) producing strain PCL1391 (*P. chlororaphis*) suppressed disease in the tomato/*Fusarium oxysporum* system (Chin-A-Woeng et al., 1998). Protective activity of the antibiotic aeruginol, isolated from the cultural filtrate of *Pseudomonas fluorescens* MM-B16, was reported against *Phytophthora* disease of pepper (Lee et al., 2003).

Reduction of inoculum density of the pathogen in the soil helps in reducing the incidence of soil-borne diseases. Microsclerotia, incubated in the presence of CCF of the selected FP strains, lost viability (<20% germination). The antispore metabolite of the CCF might have contributed to reduction of pathogen propagules from the infection court. Chin-A-Woeng et al. (2005) reported inhibition of germination of fungal spores by CCF of *Pseudomonas* strains GSE18 and GSE19 and these strains effectively controlled collar rot of groundnut in potted plants.

*P. chlororaphis* SRB127 emerged as a promising biocontrol agent for charcoal rot of sorghum. Different mechanisms, including production of extracellular antibiotics, volatile substances and siderophores, and effective colonization in root and survival in sorghum rhizosphere together contributed to the disease control. Biocontrol efficacy of *P. chlororaphis* strains against fungal root rot has been demonstrated in tomato (Chin-A-Woeng et al., 1998) and canola (Fernando et al., 2007). It is understood that none of the mechanisms of biological control (e.g., antibiotics, competition, parasitism, and induced resistance) are necessarily mutually exclusive and frequently several modes of action are exhibited by a single biocontrol agent (Whipps, 2001). Two strains (SRB127 and SRB129) with similar antifungal activities (radial growth, biomass, microsclerotia production and siderophore zone) failed to suppress charcoal rot with similar effectiveness. This suggested that apart from antibiotics, other mechanisms could be involved in suppression of charcoal rot in sorghum. Lack of definite correlation between the in vitro antifungal activity and ability to control collar rot disease was reported in groundnut (Kishore et al., 2005). Combined application of SRB129 and SRB288 resulted in a synergistic effect on reduction of charcoal rot and lodging. Similar synergism was observed in suppression of root infection by *M. phaseolina* in tomato (Imran and Shaukat, 2002). In addition to suppression of charcoal rot, seed treatment with *P. chlororaphis* SRB127 reduced lodging of the crop and increased 1000-grain weight. In sorghum, grain mass is strongly associated with lodging and charcoal rot (Anahosur et al., 1987). Statistically significant interactions between bioagent and cultivar were absent. It would be interesting to test these strains for effects on other cultivars.

Root colonization is needed to deliver the beneficial bacteria at the right place and time on the root. Poor root colonization may result in decreased biocontrol activity (Schippers et al., 1987). *P. chlororaphis* strain SRB127-rif1, when inoculated on seed surface, successfully colonized the growing root of sorghum seedlings forming cell-aggregates or microcolonies. This implied that the SRB127-rif1 might have multiplied on sorghum roots, a property desirable for survival and functioning of a biocontrol agent. Pseudomonads are known as good colonizer of plant root system owing to their competitive advantage. Well known pseudomonads biocontrol strains WCS365, CHAO and F113 showed profuse colonization and formed microcolonies on tomato root (Chin-A-Woeng et al., 1997). Survival of introduced biocontrol agents in the rhizosphere is of primary importance for biological control of plant diseases. *P. chlororaphis* SRB127-rif1, when inoculated in *M. phaseolina* infected the potting mixture through seed bacterization, survived in the sorghum rhizosphere without significant reduction in population up to 36 DAS.

The present study was successful in selecting effective strains of bacteria that can be a useful component of integrated disease management. It is suggested that the *P. chlororaphis* strain SRB127 could emerge as a promising biocontrol agent for the control of charcoal rot of sorghum. In the absence of high levels of genetic resistance, especially in high yielding cultivars, this bioagent could be effective in controlling charcoal rot and related loss in grain and stover quality in sorghum. Achieving successful biological control is a challenge as there may be variation in results on different soil types and environments (Burr and Caesar, 1984). Further testing of these promising strains for efficacy in multiple locations, involving different soil types and sorghum genotypes, will provide useful information on adaptation and application. Suitable combinations of multiple strains could be studied for possible synergistic effects.

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**References**


