Identification of siderophore producing and cynogenic fluorescent Pseudomonas and a simple confrontation assay to identify potential bio-control agent for collar rot of chickpea

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Abstract In soil, plant roots coexist with bacteria and fungi that produce siderophores capable of sequestering the available iron. Microbial cyanogenesis has been demonstrated in many species of fungi and in a few species of bacteria (e.g., Chromobacterium and Pseudomonas). Fluorescent Pseudomonas isolates P29, P59, P144, P166, P174, P187, P191 and P192 were cyanogenic and produced siderophores in the presence of a strong chelater 8-Hydroxyquinoline (50 mg/l). A simple confrontation assay for identifying potential antagonists was developed. Fluorescent Pseudomonas isolates P66, P141, P144, P166 and P174 were antagonistic against both Rhizoctonia solani and Sclerotium rolfsii. Vigorous plant growth was observed following seed bacterization with P141, P200 and P240. In field experiments, seed bacterization with selected bacterial isolates resulted in reduced collar rot (S. rolfsii) incidence.

Keywords Fluorescent Pseudomonads · Collar rot · HCN · Siderophores · Confrontation assays

Introduction

Rhizosphere inhabiting fluorescent Pseudomonads are one of the most dominant and potentially most promising group of plant growth promoting rhizobacteria involved in the bio-control of plant diseases (Haas and Défago 2005; Glick 2014). They maintain soil health by employing a wide variety of mechanisms, including nitrogen fixation, enhanced solubilization of phosphate and phytohormone production that positively impact plant health (such as auxins and cytokinins) (Penrose and Glick 2003; Mirza et al. 2006). Pseudomonas spp. produce an arsenal of antimicrobials (including hydrogen cyanide (HCN), pyoluteorin, phenazines, pyrrolnitrin, siderophores, cyclic lipopeptides and 2,4-diacetylphloroglucinol (DAPG) (Thomashow and Weller 1996; Weller 2007). They also are able to promote plant growth and induce systemic resistance (ISR) in plants (Raaijmakers et al. 2009; Glick 2014). In the present study we evaluate fluorescent Pseudomonas isolates for siderophore (CAS assay-plate screening, CAS assay-spectrophotometric analysis, hydroxyquinoline test, tetrazolium test, FeCl\textsubscript{3} test and Arnow’s assay) and HCN production. Pseudomonas spp. have been employed efficiently as commercial biocontrol agents (Loper and Lindow 1987; Walsh et al. 2001). However, there is always a scope for isolating better, locally adapted strains for deployment as biocontrol agents. Hence, we have screened local isolates of fluorescent Pseudomonads for developing formulation and possible commercialization for the management of collar rot of chickpea (S. rolfsii Sacc), one of the major biotic factors contributing towards low production (55–95% mortality of chickpea seedlings).

Materials and methods

Microorganisms and culture conditions

The experimental material consisted of purified 29 isolates of fluorescent Pseudomonas spp. from soils (rhizospheric and non-rhizospheric) of different geographical locations.
of Chhattisgarh. Isolation of fluorescent pseudomonads was
done by adopting serial dilution method on King’s B (KB)
medium. After incubation at 28 °C for 2 days, fluorescent
pseudomonad colonies from plates were identified under
UV light (366 nm). Isolates were characterized on the basis
of biochemical tests as per the procedures outlined in
Bergery’s Manual of Systematic Bacteriology (Sneath et al.
1986). Isolated colonies of fluorescent *Pseudomonas* were
further streaked onto KB agar plates to obtain pure cul-
tures. The isolates were maintained (at −80 °C on King’s
B broth (Himedia) containing 30% (v/v) glycerol) in the
culture collections of the Department of Plant Molecular
Biology and Biotechnology, Indira Gandhi Krishi Vish-
wavidyalaya, Raipur, Chhattisgarh, India, and revived on
King’s B slants when required.

**Siderophore production**

Siderophore production (qualitative and quantitative) was
determined by (CAS assay (Schwyn and Neilands 1987).
Specific tests were carried out for identification of
hydroxamate and Catecholate types of siderophores fol-
lowering the standard methods (Arnow 1937). Chrome
azurol S solution was prepared and added to melted King’s
B agar medium in the ratio 1:15. Spot inoculation at the
centre of the CAS plate was done from actively growing
cultures of *Pseudomonas*. Colonies exhibiting an orange
halo after 3 days of incubation (28 ± 2 °C) were consid-
ered positive for siderophore production and the diameter
of the orange halo was measured.

**Hydroxyquinoline mediated siderophore test**

For selection of *Pseudomonas* isolates with high ability to
siderophores, isolates were inoculated on King’s B medium
supplemented with a strong chelator 8-Hydroxyquinoline
(50 mg/l) (De Brito et al. 1995). Inoculated isolates were
incubated at 28 ± 2 °C for 48–72 h; only those bacteria
that produce a more avid iron chelator will grow.

**Arnow’s assay**

Arnow’s assay was used for quantification of catechol type
siderophore. For qualitative estimation of siderophores,
actively growing cultures of *Pseudomonas* were inoculated
to 20 ml King’s B medium in 50 ml tubes and incubated
for 3 days at 28 ± 2 °C. The bacterial cells were removed
by centrifugation at 3000 rpm for 5 min. Three ml of the
culture supernatant was then mixed with 0.3 ml of 5 N HCl
solution, 1.5 ml of Arnow’s reagent (10 g NaNO₂, 10 g
Na₂MoO₄·2H₂O dissolved in 50 ml distilled water) and
0.3 ml of 10 N NaOH. After 10 min the presence or
absence of pink colour was observed and noted.

**Tetrazolium test**

This test is based on the capacity of hydroxamic acid to
reduce tetrazolium salt by hydrolysis of hydroxamate
groups using a strong alkali. The reduction and release of
alkali shows red colour to a pinch of tetrazolium salt when
1–2 drops of 2 N NaOH and 0.1 ml of test sample are
added. Instant appearance of a deep red colour indicated
the presence of hydroxamate siderophore.

**FeCl₃ test**

One ml of the culture supernatant was mixed with freshly
prepared 0.5 ml of 2% aqueous FeCl₃ and observed for the
presence and absence of deep red colour.

**HCN production**

The production of HCN was estimated by the method of
Wei et al. (1991). The cultures were grown on KM
plates supplemented with 4.4 g/l glycine as a precursor
and the filter paper strips soaked in saturated picric acid
solution were exposed to the growing *Pseudomonas*
isolates. The plates were incubated for 7 days at
28 ± 2 °C and observations were recorded as change in
the colour of filter paper to brown as positive indicator
for HCN production.

**Confrontation assay**

Fluorescent *Pseudomonas* isolates were multiplied on
King’s B broth and incubated for 2 days at 28 °C till the
fluorescent pigment appeared in the broth. Petri-plates
containing pre-sterilized potato dextrose agar (PDA)
medium were inoculated with plant pathogenic fungi
*Sclerotium rolfsii* or *Rhizoctonia solani* (in the centre)
and incubated at 252 °C for 3 days till the fungus
completely covered the entire plate. Bipartite interac-
tions were performed following a simple confrontation
assay which was developed during the course of inves-
tigation. To identify prospective bio-agent, a simple
confrontation assay was developed wherein edge of glass
funnel was deployed for bio-agent inoculum deposition
surrounding pre-inoculated fungal pathogen. The edge of
a glass funnel was sterilized by dipping in alcohol fol-
lowed by flaming. Broth containing young growing cell
(3-day-old) of fluorescent *Pseudomonas* was dispensed
in sterile petri dish and picked at the edge of the funnel
by dipping. Care was taken to remove the excess
inoculum by gently shaking the funnel. Inoculation was
done by gently touching the edge of the funnel (con-
taining fluorescent *Pseudomonas*) which encircled the
pre-inoculated plant pathogenic fungi on agar plug.
equidistantly (Fig. 1). Inhibition zone was measured after 72 h of incubation at 28 ± 2 °C. Percent inhibition of pathogens by *Pseudomonas* isolates over control was calculated using the formula of (Vincent 1947): 

\[
\text{Percent inhibition} = \frac{(\text{Growth of pathogen in control} - \text{growth of pathogen with } Pseudomonas \text{ isolate/growth of pathogen in control}) \times 100}
\]

Proposed technique has the following advantages: (1) uniform inoculum deposition during all combinations of bipartite interactions. (2) Replica-plating can be done of the inoculum picked on the edge of the funnel. (3) Ability to evaluate the antagonistic potential of a sporulating bio-inoculant (e.g. *Trichoderma* sp.). The only disadvantage with this technique is that for each bio-inoculant in the broth a separate plate is required to dispense the inoculums.

**Field trial for testing selected fluorescent *Pseudomonas* isolates against chickpea collar rot**

For field trials seed bacterization was done with stock cultures (used for confrontation assays) of selected fluorescent *Pseudomonas* isolate. Slurry for seed bacterization was prepared @ 5 ml of bacterial culture + 3 g of talcum powder/kg of chickpea seeds. Care was taken for uniform coating of all the seeds, which were dried in shade and then sown in a field naturally infested with *S. rolfsii*. Sowing was done in 2 M × 7 M plots. Care was taken to carry out all normal agricultural practices. Observations were recorded on germination, plant vigour, mortality and bundle and grain weight.

**Results**

**Qualitative and quantitative assay for siderophore production**

In the present investigation, 29 isolates of *Pseudomonas* were screened by six different siderophore assays viz., CAS assay-plate screening, CAS assay-spectrophotometric analysis, hydroxyquinoline test, tetrazolium test, FeCl$_3$ test and Arnow’s assay. All isolates exhibited an orange halo after 3 days of incubation (28 ± 2 °C) on CAS agar plate and, therefore, were considered positive for siderophore production. Intensity of orange halo and diameter showed wide variation among the isolates, which ranged from 11.50 (isolate P56) to 64.50 (isolate P191) mm. Isolates P29, P59, P66, P141, P144, P166, P174, P187, P191, P192, P200, P207 P229 and P260 were identified as producer of more avid iron chelator as they were tested –ve in King’s B medium supplemented with a strong chelater 8-Hydroxyquinoline (50 mg/l). Three isolates (P7, P43 and P45) tested positive in Arnow’s assay (detects catechol type of siderophores). We observed that isolates P43 and P45 tested –ve in HQ test but were +ve for other siderophore tests. All isolates produced deep red colour on addition of hydroxamate type of siderophores (reduce tetrazolium salt by hydrolysis of hydroxamate group in presence of strong alkali). FeCl$_3$ test was positive for isolates P7, P23, P29, P43, P59, P66 P74, P80, P123, P141, P144, P174, P180, P187, P192, P200, P207 P229 and P260. Only one isolate (P7)
Table 1: Quantitative and qualitative estimation of siderophores by different tests, HCN production and antagonistic activity of fluorescent *Pseudomonas* isolates against *Rhizoctonia solani* and *Sclerotium rolfsii*

| Isolates | Quantitative siderophore units | Qualitative siderophore test | HCN production | % inhibition | R. solani | S. rolfsii |
|----------|--------------------------------|------------------------------|----------------|-------------|-----------|-----------|
|          | CAS ± Arnow’s FeCl₃ Tetrazolium HQ |                             |                |             |           |           |
| P66      | 78.555 ± 0.39ₐ                  | 63.5 ± 1.5ₐ                  | ++ +           | 68.89 ± 1.1₁ᵃ | 82.78 ± 2.7₈ᵇ |          |
| P141     | 80.15 ± 0.15ₐ                  | 55 ± 1.8ₐ                    | + +             | 52.78 ± 2.7₈ᶜ | 43.335 ± 2.2₂²ᵇ |          |
| P200     | 49.475 ± 2.11¹                  | 45.5 ± 1.5₈ₑ                 | + +             | 60 ± 5.5₈ᵇ   | 28.89 ± 1.1₁¹   |          |
| P229     | 22.365 ± 1.32¹                  | 36 ± 1.₇ᵇ                   | + +             | 19.445 ± 2.7₈¹ | 28.885 ± 4.4₄⁵ᶠ |          |
| P260     | 71.05 ± 2.63ᵇ                  | 61.5 ± 0.₅ᵃ                  | ++ +           | 37.775 ± 5.5₆ᵇ | 32.775 ± 3.3₃ᵇⁱ |          |
| P2       | 37.25 ± 1.06ᵈ                  | 35 ± 1.₇ᵇ                   | + +             | 40.5 ± 0.₅ᶜ | 17.25 ± 0.₅ʲ |          |
| P3       | 30.2 ± 0.2₈ᵉ                  | 52.5 ± 2.₅ᵇₑ                 | + +             | 32.95 ± 0.₄⁵ᵇ | 17.1 ± 0.₄ᵢ |          |
| P43      | 70.6 ± 0.₈₈ᵇ                  | 41 ± 1.₆₈ḡ                  | + +             | 26.2 ± 1.₂ᵢ | 33.95 ± 0.₆₅ᵇᵃ |          |
| P45      | 24.15 ± 0.₂₁ʲ                 | 16.5 ± 1.₅ᶜ                 | + +             | 13.05 ± 0.₅₅ᵏ | 25.1 ± 0.₇ʲ |          |
| P130     | 47.5 ± 0.₃₂ᶠ                  | 31.5 ± 1.₅₅ᶜ                 | + +             | 25.15 ± 0.₁₅ⁱ | 31.85 ± 0.₇₅ᵇᵃ |          |
| P6       | 47.5 ± 0.₇₁ᵗ                  | 40 ± 2.₅₆ᵇ                 | + +             | 21.825 ± 0.₅₈ | 45.15 ± 0.₇₅ᵇ |          |
| P123     | 43.78 ± 0.₃₁ᵍ                 | 39 ± 2.₉ᵇ                   | + +             | 33.15 ± 0.₆₅ᵇ | 34 ± 0.₇ʰ |          |
| P150     | 43.78 ± 0.₃₁ᵍ                 | 42.5 ± 2.₅₆ᵗₑ               | + +             | 38.2 ± 0.₇ᵇ | 44.95 ± 0.₅₅ᵇ |          |
| P23      | 48.25 ± 0.₃₅ᶠ                  | 42 ± 2.₅₆ᵗₑ                 | + +             | 39.375 ± 0.₆₃ᵇ | 45.05 ± 0.₆₅ᵇ |          |
| P56      | 27.5 ± 0.₇₁ᵗ                  | 11.5 ± 1.₅ᵗ                  | + +             | 10.8 ± 0.₅ᵗ | 50.65 ± 0.₆₅ᶠ |          |
| P184     | 40.615 ± 0.₈₇ᵗ                | 40.5 ± 0.₅₆ᵗᵉ               | + +             | 18 ± 0.₅ᶠ | 50.75 ± 0.₇₅ᵇ |          |
| P206     | 36.16 ± 0.₂₃ʲ                 | 51 ± 1.₈ᶜ                   | + +             | 46.875 ± 0.₆₃ᵇ | 72.8 ± 0.₆ |          |
| P132     | 23.835 ± 0.₂₃¹                | 41 ± 1.₇ₑ                   | + +             | 13.15 ± 0.₆₅ᵏ | 61.95 ± 0.₈₅ᵈ |          |
| P7       | 52.9 ± 0.₄ᶜ                  | 41.5 ± 1.₅₇ₑ                 | + +             | 21.625 ± 0.₃₈ | 61.8 ± 0.₇ᵈ |          |
| P192     | 36.115 ± 0.₁₆ᶜ               | 54.5 ± 1.₅ᵇ                  | + +             | 28.2 ± 0.₇ᵈ | 56.25 ± 0.₆₅ᵉ |          |
| P191     | 47.945 ± 0.₀₈ᵀ                | 64.5 ± 1.₅ᵃ                  | + +             | 30.8 ± 0.₈ᵉ | 72.6 ± 0.₄ |          |
| P59      | 60.5 ± 0.₇₁ᶜ                  | 39.5 ± 1.₅₈ᵇ                 | + +             | 36.625 ± 0.₃₈ᵇ | 47.3 ± 0.₆¢ |          |
| P29      | 60.5 ± 0.₇₁ᶜ                  | 54.5 ± 1.₅ᵇ                 | + +             | 43.85 ± 1.₃₅ₑ | 87.3 ± 0.₆₆ |          |
| P187     | 60.115 ± 0.₁₆ᶜ              | 51.5 ± 1.₅ᵇₑ                 | + +             | 44.325 ± 0.₅₇ₑ | 74.05 ± 0.₇₅ˢ |          |
| P74      | 27.75 ± 0.₃₅ᵀ                | 40.5 ± 2.₅₆ᵗᵉ               | + +             | 50.7 ± 0.₇⁸ | 35.4 ± 0.₃ᵇ |          |
| P80      | 32.5 ± 0.₇₁¹                  | s ± 1.₄ᵉʳ                   | + +             | 63.1 ± 0.₆ᵇ | 63.85 ± 0.₅₅ᵈ |          |
| P144     | 43.5 ± 0.₇₁ᵀ                | 48.5 ± 1.₅ᵈ                  | + +             | 50.7 ± 0.₇⁸ | 76 ± 0.₄ |          |
| P166     | 60.115 ± 0.₁₆ᶜ              | 40 ± 3.₅₆ᵀ                 | + +             | 73.1 ± 0.₆ᵉ | 73 ± 0.₈ |          |
| P174     | 55.5 ± 0.₇₁ᵈ                | 51 ± 1.₈ᶜ                  | + +             | 51 ± 1.₇⁸ | 82.95 ± 0.₇₅ᵇ |          |
| Max.     | 80.15                      | 64.5                        |                | 73.1 | 87.3 |          |
| Min.     | 22.365                      | 11.5                        |                | 10.8 | 17.1 |          |
| CV       | 2.352                       | 5.22                        |                | 6.625 | 3.645 |          |
| CD (0.01) | 2.962                      | 6.33                        |                | 6.876 | 5.12 |          |
| CD (0.05) | 2.198                      | 4.697                        |                | 5.102 | 3.799 |          |
tested positive for all siderophore tests. Carboxylate type of siderophore was determined by spectrophotometric method at 630 nm and the percentage of siderophore unit ranged from 12.23% (isolate P130) to 70.60% (isolate P43) (Table 1).

**Table 1 continued**

| Isolates | Quantitative % siderophore units | Qualitative siderophore test | HCN production | % inhibition |
|----------|---------------------------------|-----------------------------|----------------|-------------|
|          | CAS Arnow's FeCl₃ Tetrazolium HQ | R. solani S. rolfsii        |                |             |
| F cal    | 527.375** 53.39**                | 90.948** 247.998**          |                |             |

Values are average of three replications; values after ± represent standard deviation
As per Duncan’s grouping means with the same letter are not significantly different
CV coefficient of variance, CD critical difference, HQ hydroxyquinoline test
+++ Luxuriant/high growth
++ Medium growth
+ Low growth
– No growth
** Values are significant at 1 and 5% levels

Table 2 Efficacy of selected fluorescent *Pseudomonas* isolates against collar rot and yield of chickpea

| Isolate no. | No. of plants | Plant vigour | Collar rot incidence | Bundle weight (kg) | Yield (gm) |
|-------------|---------------|--------------|----------------------|--------------------|------------|
|             |               | % poor vigour | % high vigour | % wilted plants |            |            |
| Control     | 256           | 92.820± 0.63 | 7.965± 0.155 | 35.015± 0.635 | 2.070 ± 0.25 | 598.000± 25 |
| P66         | 289           | 17.425± 0.465 | 93.585± 0.545 | 16.130± 0.21 | 3.670± 0.45 | 1361.500± 59.5 |
| P141        | 303           | 5.130± 0.51  | 96.070± 0.69 | 11.105± 0.545 | 4.890± 0.43 | 1732.500± 10.5 |
| P200        | 350           | 21.625± 0.485 | 79.353± 0.675 | 7.275± 0.705 | 3.785± 0.425 | 1085.000± 36 |
| P229        | 305           | 92.955± 0.165 | 7.820± 0.61 | 2.930± 0.31 | 2.735± 0.355 | 574.500± 14.5 |
| P260        | 367           | 7.7620± 0.51 | 14.000± 0.65 | 2.645± 0.465 | 1.425± 0.225 | 277.000± 34 |
| CV          | 1.331         | 1.715        | 5.750     | 16.755    | 5.126     |
| Fcal        | 7145.771**    | 5242.546**   | 570.035** | 11.890** | 262.364** |
| CD (0.01)   | 5.529         | 3.062        | 2.668     | 1.923    | 178.267   |
| CD (0.05)   | 1.669         | 2.021        | 1.761     | 1.269    | 117.675   |

Values after ± represent standard deviation
Superscript values indicate Duncan’s grouping means with the same letter are not significantly different
** Values are significant at 1 and 5% levels

tested positive for all siderophore tests. Carboxylate type of siderophore was determined by spectrophotometric method at 630 nm and the percentage of siderophore unit ranged from 12.23 (isolate P130) to 70.60% (isolate P43) (Table 1).

**Screening for hydrogen cyanide production**

To identify cynogenic fluorescent *Pseudomonas*, isolates were inoculated on KMB plates supplemented with 4.4 g/l glycine (precursor molecule of HCN) and incubated for 7 days at 28 ± 2°C. Development of brown colour on filter paper strips soaked in saturated picric acid solution indicated –ve for HCN production. Out of the 29 fluorescent *Pseudomonas* tested, 12 isolates (P7, P29, P45, P59, P132, P144, P150, P166, P174, P187, P191 and P192) tested positive for HCN producing ability. We observed a correlation between inhibitory effects observed following confrontation assays and the ability to produce HCN by fluorescent *Pseudomonas*. Cynogenic (HCN producers) isolates P132, P7, P192, P191, P59, P29, P187 exerted strong antagonism against *S. rolfsii*, where as a non-cynogenic isolate P74 exerted strong inhibitory effects against *R. solani*. On the contrary, a noncynogenic (P80) and three cyanogenic (P144, P166, P174) fluorescent *Pseudomonas* exerted strong inhibitory effects against both the soilborne fungal pathogens *R. solani* and *S. rolfsii* (Table 1).

**In vitro antagonistic activity of *Pseudomonas* isolates against *R. solani* and *S. rolfsii***

There were differences in the antagonistic abilities of fluorescent *Pseudomonas* isolates against both (*R. solani* and *S. rolfsii*) pathogens (Fig. 1; Table 1). All of the 29 isolates of fluorescent *Pseudomonas* showed different degree of growth inhibitions of *R. solani* and *S. rolfsii*, ranging from 10.8 to 73.1% and 17.1 to 87.3%, respectively.
Confrontation assays revealed P166 and P29 as potential antagonists against *R. solani* and *S. rolfsii*, respectively, while isolates P56 and P3 were ineffective. Fluorescent *Pseudomonas* isolates P29, P187, P191, P192 and P207 exerted strong inhibitory effects on the mycelia growth of *S. rolfsii* whereas isolates P66, P141, P144, P166 and P174...
expressed strong inhibitory effects on both *R. solani* and *S. rolfsii*.

**Efficacy of selected fluorescent *Pseudomonas* isolates against root rot and yield of chickpea**

Through confrontation assay, isolates P66 and P141 was identified to be having very strong antagonistic activities against *R. solani* and *S. rolfsii*, whereas P200 was antagonistic only against *R. solani*; other two isolates P229 and P260 were ineffective against both the pathogens. For field trials these five fluorescent *Pseudomonas* isolates were selected for their efficacy against collar rot disease and yield of chickpea. Frequency of infected plants was very high in untreated (control) plot, whereas seed treatment with bacterial isolates had lower incidence of collar rot (Table 2; Fig. 2). Vigorous plant growth was observed following seed bacterization with P141, P200, P240 (95.38, 83.04 and 78.86%, respectively), whereas poor vigour was observed with P229 (7.21%) and P260 (13.35%) (Table 2; Fig. 2). Bundle weight and yield indicated significant differences between control and treated plots. In the order of increasing bundle weight and yield per plot, the bioefficacy of the isolates were P260 < P229 < P66 < P200 < P141 (Table 2).

**Discussion**

Understanding of the mechanisms involved in the antagonist interactions between bacteria, pathogen and host plant is important for efficient utilization of these natural resources in crop health management. Thomashow and Weller 1991). Siderophore production by strains of *Pseudomonas* spp., for plant disease control, is of great interest because of its possibilities in the substitution of chemical pesticides. Similarly, microbial cyanogenesis has been demonstrated in a few bacterial species (belonging to the genera *Pseudomonas, Chromobacterium, Rhizobium* and several cyanobacteria (Blumer and Haas 2000). Glycine has generally been used as a precursor of cyanide in fungi and bacteria (Brysk et al. 1969; Wissing 1974) and cyanogenesis is one of the mechanisms of antagonism and biocontrol properties (Haas and Défago 2005; Lanteigne et al. 2012). In the present study, we have compared the ability of several fluorescent Pseudomonads to produce siderophores, cyanogenesis and antagonism in plate assay. Our study revealed that the isolates vary in the mechanisms and ability to inhibit pathogens. During the study a simple confrontation assay technique was developed which was advantageous as compared to earlier reported techniques (Dennis and Webster 1971; Fokkema 1978; Santoyo et al. 2010), wherein bipartite interactions were performed on media plates by streaking bacterial bio-agents (forming quadrant) and placing mycelial plug of 4 mm in the centre. Our combined in vitro and field data show the potential of isolates P66, P141 and P200 to be developed as a commercial bioagent for the control of chickpea collar rot, a perennial problem in chickpea production compounded by the lack of host resistance against the pathogen *S. rolfsii*.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest in the publication.

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