RESEARCH ARTICLE

Exploring Western Ghats microbial diversity for antagonistic microorganisms against fungal phytopathogens of pepper and chickpea

B.N. Ramkumar [1]
K.M. Nampoothiri [1]
U. Sheeba [2]
P. Jayachandran [1]
N.S. Sreeshma [1]
S.M. Sneha [1]
K.S. Meenakumari [2]
P. Sivaprasad [2]

Authors' addresses:
[1] Biotechnology Division, CSIR - National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum-695 019, Kerala, India.
[2] Kerala Agricultural University, Trivandrum-695 019, Kerala, India.

Correspondence:
K.M. Nampoothiri
Biotechnology Division, CSIR - National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum-695 019, Kerala, India.
Tel.: +91-471-2515366
e-mail: madhavan85@hotmail.com

Article info:
Received: 2 January 2015
Accepted: 6 July 2015

ABSTRACT

Newly isolated microbial cultures from Western Ghat soil samples of Kerala region in India were screened for antagonistic activity by well diffusion and dual culture plating against *Phytophthora capsici* and *Rhizoctonia solani*, infecting pepper and chickpea, respectively. Bioactive samples were made by varying solvent extraction of the culture broths of the potent isolates belongs to *Actinomycetes*, *Pseudomonas*, *Bacillus* and *Trichoderma*. The efficacy of the isolates to produce other potent antifungal metabolites such as cell wall degrading enzymes, HCN and volatile compounds were also checked. Treatment with antagonistic isolates *in vivo* under greenhouse conditions revealed significant reduction of the disease intensity of foot rot disease of black pepper and collar rot of chick pea.

Key words: Western Ghats**,** *Phytophthora capsici, Rhizoctonia solani*, antagonism, microbial bioactive, foot rot, collar rot

Introduction

Biocontrol agents such as soil borne microbes showing antagonism towards disease-causing plant pathogens that cause severe economic losses in commercial crop production were getting more attention. Antifungal metabolites isolated from them appears to be promising as viable supplements or alternatives to plant disease control, compared to synthetic chemicals (Bloemberg & Lugtenberg, 2001; Vessey, 2003). Biological control of plant diseases would help in preventing increase of pathogen population and also to check health hazards caused due to excessive use of chemicals (Kennedy, 2005; Shashidhara et al., 2008).

Recently, various microorganisms have been using against a vast array of disease-causing plant pathogens. Root-colonizing fluorescent pseudomonads suppress many plant diseases caused by soil borne pathogens in greenhouse experiments and in field trials by niche exclusion, by production of antibiotics and siderophores, or inducing systemic resistance (Haas & De´fago, 2005; Lemanceau et al., 2006; Amkraz et al., 2010). They also stimulate plant growth by facilitating either uptake of nutrients from soil (De Weger et al., 1986) or by producing plant growth promoting substances (Ryu et al., 2005; Spaepen et al., 2007). Similarly, *Bacillus* species were reported to be effective biocontrol agents (Kloepper et al., 2004; Von-Der-Weid et al., 2005) and are reported as producers of antibiotics inhibiting various phytopathogens (Jacobsen et al., 2004). Various actinomycetes species also possess antibacterial or antifungal activity and suppress the growth of phytopathogens (Hamby & Crawford, 2000; El-Tarabily & Sivasithamparam, 2006; De-Vasconcellos & Cardoso, 2009). Examples of commercial biocontrol products are Mycostop (*Streptomyces griseoviridis* K61), Actinovate (*Streptomyces lydicus*), and Nogall

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

(*Agrobacterium radiobacter* Strain K1026) (Crawford et al., 2005).

The disease suppressing capability of these microbes depends on their ability to secrete several growth inhibiting bioactives against pathogenic fungi, such as antibiotics and small peptides (Andersen et al., 2003; Bolwerk et al., 2003; Zhang & Fernando, 2004; Velusamy et al., 2006) and secretion of cell wall degrading enzymes such as chitinase (El-Tarabily et al., 2000; Jung et al., 2003; Kamensky et al., 2003; Kishore et al., 2005), glucanase (Palumbo et al., 2005; El-Tarabily, 2006), cellulolytic enzymes (Schmidt et al., 2001) and protease (Dunne et al., 1998), which differ in their specificities based on the macroscopic features of the substrates.

The two dreadful pathogens addressed in this work are *Phytophthora capsici*, the causative agent of foot rot in pepper and *Rhizoctonia solani*, causing collar rot in chickpea. Foot rot of black pepper is a devastating disease in the nursery and in field, causing serious economic loss in the pepper growing countries such as India, Indonesia, Malaysia and Brazil (Jiang et al., 2006; Truong et al., 2010; Robles-Yerena et al., 2010). Similarly, collar rot caused by *Rhizoctonia solani* is the most destructive disease of chickpea and accounts to 5-60 % of crop loss. The basidiospores produced by *R. solani* in soil are an important means of its survival and makes it very difficult to control (Huang et al., 2012; Plodpai et al., 2013). These species evolve a strong association with other soil microorganisms in its ability to survive and grow through the soil effectively under a wide range of conditions. *Phytophthora* belongs to oomycetes fungi with cellulose as major cell wall constituent while for *Rhizoctonia* the cell wall is made mainly of chitin (Huang et al., 2012) making them a potential plant pathogen that are difficult to control. Various studies have been carried out to develop potent bio-control agents that are screened from various sites.

The Western Ghats of India, covering an area of 180,000 km^2, or 6% of the land area of India, is rich in its biodiversity. Being the "gene pool", harbouring millions of species of animals, plants and microbes, the heritage unit of UNESCO granted a section of Western Ghats lying in the states of Kerala, Karnataka, Maharashtra and Gujarat with a "heritage tag". There is a vast array of novel and unidentified microbes in this area which could be explored for potential applications (Jalaja et al., 2011; Balachandran et al., 2012; Mohandas et al., 2012; Nampoothiri et al., 2013). Hence, in the present study, attempts were made to screen for novel antagonistic microorganisms against the selected phytopthogens from this unique and rich biodiversity of Western Ghats of Kerala region.

Materials and Methods

Phytopathogens and isolation of antagonistic microorganisms

The test fungi, *Phytophthora capsici* and *Rhizoctonia solani* are from Kerala Agriculture University and were maintained on Potato Dextrose Agar (PDA) slants for routine use. They were isolated from infected plants showing the typical symptoms of the corresponding diseases such as foot rot of black pepper and collar rot of chick pea and were grown as pure cultures. It is further verified that the cultured pathogens caused the disease when inoculated in to a healthy plant. Thus, pathogenicity of both the test fungi was proved through Koch postulates. Microbes of different groups were isolated from varying rhizosphere soil samples collected from Western Ghat by serial dilution and spread plate method (Johnson & Curl, 1972) using specific culture media such as Nutrient Agar for bacteria (g^{-1}; beef extract 3.0, peptone 5.0, agar 15.0 pH 6.8 + 0.2), Potato Dextrose Agar for fungi (g^{-1}; potato infusion 4.0 (from 200 g), dextrose 20, agar 15.0 pH 5.6+0.2), Kings B for *Pseudomonas* (g^{-1}; Proteose peptone 20.0, K_2HPO_4 1.5, $MgSO_4.7H_20$ 1.5, glycerol, 10 ml, agar 15, 7.2±0.2) and ISP2 (g^{-1}; yeast extract 4.0, malt extract 10.0, dextrose 4.0, agar 20.0. pH 7.2 ± 0.2) for actinomycetes and a code number was provided to all pure isolates.

Extraction of bioactive

Individual cultures were inoculated into 50 ml respective media in 250 ml Erlen Meyer flaks and were incubated at 30°C at 180 rpm in an orbital shaker for 3-4 days. The cell free supernatant was collected by centrifugation and transferred to conical flask with 4 g of Diaion HP 20 (Sigma, USA), an adsorbent resin and kept for incubation in rotary shaker at 30°C at 200 rpm for 45 min. The bioactive bounded resin was packed into a 20 ml syringe column, washed with one column distilled water and finally eluted slowly with 20 ml methanol. The filtrate was dried in evaporator at 60°C, dissolved in 2 ml of dimethyl sulphoxide (DMSO) and subsequently stored at -20°C and was later used for agar well diffusion assay against the test pathogens. In an alternate approach, different organic solvent extracts were also made from individual cell free supernatant of the culture filtrate. Different solvents such as

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

chloroform, acetonitrile and hexane were used for this purpose and the extracts were subsequently tested for its antifungal activity against the test pathogen.

In vitro evaluation of antagonistic microorganisms

Antagonistic property of the morphologically distinct isolates was further evaluated by dual culture assay using the isolate and test pathogen and agar well diffusion assay with bioactive fraction of the isolates on test pathogens using standard protocols (Zivkovic et al., 2010). In well diffusion assay, the crude culture supernatant, the resin absorbed fraction and the varying solvent extracted fractions were subjected for the antifungal assay. 100 µl of the above samples were added to the wells (5 mm diameter) made on PDA which was previously spread plated with 1×10^4 spores of test pathogens and kept at 30°C for four days. An inhibition of fungal growth more than 5 mm diameter was looked further for detailed studies. In dual culture assay, pathogen and isolate were streaked on opposite sides of the plate and the measure of inhibition of fungal growth was recorded every 24 h. The percentage of inhibition of positive cultures against test fungi after six days of incubation was calculated according to the equation (Rc-Ri)/Rc x 100 where, Rc - radial mycelial growth of test fungi in control plate and Ri is the radial mycelial growth of test fungi towards antagonistic organism.

Characterization of antifungal metabolite

Stability of the antifungal metabolite was tested by subjecting the purified bioactive fraction to different physico-chemical conditions viz., varying temperatures (37°C, 50°C, 70°C, and 90°C for 1 h and 121°C for 15 min) and pH 4-13. Tolerance to enzymatic actions was also tested by adding 50 µg ml^{-1} final concentration of lysozyme, proteinase K and pepsin to the reaction mixture containing the bioactive and incubating for 2 h at room temperature. Various detergents viz., SDS, urea, EDTA, Triton X-100 and Tween 80 at a final concentration of 0.01 g ml^{-1} were also used to determine their effect on the stability of the bioactive.

Enzyme profile of the selected isolates

The selected antagonist strains, which showed growth inhibition of both the pathogens were tested for their efficacy to produce cell wall degrading enzyme such as chitinase and cellulase. The cellulase activity was checked in carboxy methyl cellulose (CMC) agar plates (Gupta et al., 2012) and diameter of clearance zone obtained around the colonies after staining the plates with Congo red solution was measured. Chitinolytic activity was tested in plates with colloidal chitin (Haggag & Abdallh, 2012).

In vitro assay for volatile metabolites and HCN production

Selected antagonist isolates were also checked for the production of volatile metabolites and HCN production by Dual bottom plates experiment. PDA plate with the mycelial plug of test pathogen, *P. capsici* was placed upside down on the plate containing the selected microbial isolate. The plates were sealed with parafilm and kept for incubation at 28°C for 72 h. Radial growth of the pathogen was compared with that of control (Kloepper et al., 1991). Filter paper soaked in 2.5 g picric acid and 12.5 g Na_2CO_3 (gL^{-1}) was placed on the lid of the Petri dish, sealed with parafilm and kept for 72 h incubation. Change in colour of the filter paper from yellow to brown indicates the production of HCN.

In vivo evaluation of selected isolates and extracted bioactive for disease suppression

The effect of selected isolates on disease suppression was studied under greenhouse conditions with black pepper and chickpea plants, raised after exposure with the test pathogenic fungi. The plants were drenched and sprayed with selected antagonistic isolates prior to inoculation with pathogen and subsequent drenching and spraying of selected isolates was repeated in a thirty days interval time. The initial viable count of the antagonistic inoculum used for spraying vary from species to species and is of approximately 18×10^9 cfu/ml for pseudomonas, $25x10^6$ cfu/ml for trichoderma and $15x10^9$ for actinomycetes . In the case of *Trichoderma*, organic manure enriched with the respective culture was used for raising the plants. The disease intensity was measured and scored periodically. The disease score chart for *Rhizoctonia solani* **(**Mayolo et al., 1993) and *Phytophthora capsici* was prepared based on 0-9 scale and it helped to grade the disease by manual morphological observation.

Similarly, ten days before inoculating with the pathogens, the plants were drenched and sprayed with 5% concentration of metabolites of selected antagonist isolates. Fifteen days after pathogen inoculation, drenching and spraying of metabolites was repeated. Observations on growth characteristics (height and number of leaves) and disease intensity were recorded for a period of two months. A score chart for disease suppression was prepared for monitoring the disease intensity.

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Results

Isolation of antagonistic cultures

Soil samples were collected from 72 locations of Western Ghats's forest tract covering 720 sampling spots. About 147 pure cultures were isolated, purified and made glycerol stock for further study. Based on inhibition zone diameter against test pathogens, three bacilli (B4, B5, B12), two *Pseudomonas* (P1 and P66) and three *Streptomyces* (98(1), E7 and N4) cultures were shortlisted for detailed studies (Table 1).

The *Streptomyces* culture coded as 98.1 showed the maximum inhibition of 89 % against *R. solani* and 63 % against *P. capsici* (Table 1). Figure 1 shows the dual culture assay with growth inhibition of test pathogens by *Streptomyces* and *Pseudomonas* isolates. Selected *Trichoderma* cultures always showed over growth and covered the pathogen fully (Figure 2). The growth of the pathogen is slow compared to the spreading *Trichoderma* cultures.

Antifungal metabolite

Well diffusion assay with the extracted metabolites revealed that ethyl acetate extract of the *Streptomyces sp.* coded as 98(1) and E7 showed maximum zone of inhibition against the test pathogens (Figure 3).

Table 1. *Antagonistic activity of selected isolates.*

Isolates	Zone of inhibition in diameter (mm)		Effect value (%)	
	P. capsici	***R. solani***	***P. capsici***	***R. solani***
Streptomyces sp. 98(1)	30.4 ±2.1	24.4 ± 1.9	63	89
Streptomyces sp. E7	22.0±1.1	12.8±0.9	53	74
Streptomyces sp. N4	22.2±1.1	14.6±1.2	54	55
Bacillus sp. B4	17±1.4	13.8±1.2	52	42
Bacillus sp. B5	21.2±0.9	19.4±0.8	58	56
Bacillus sp. B12	11.4±1.4	8±1.3	39	34
Pseudomonas fluorescence P66	18.6±1.3	9±1.2	52	38
Pseudomonas fluorescence P1	24.4±2.1	13.4±1.7	58	42
Trichoderma sp.[*]	ND	ND		

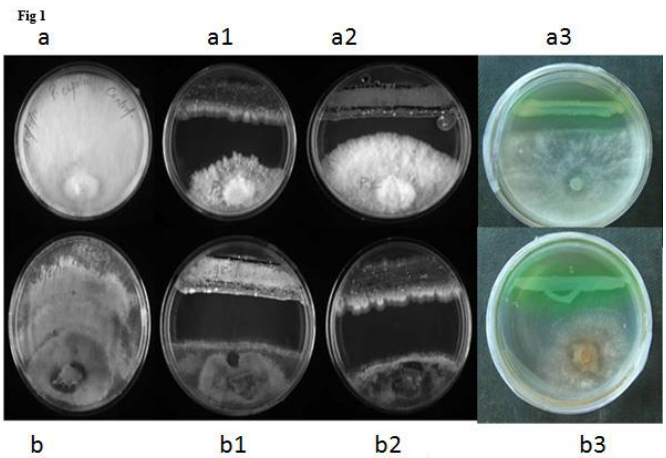

Figure 1. *Antagonistic isolates against plant pathogens. a- Phytophthora capscisi (Control); a1- Streptomyces 98(1); a2- Streptomyces (E7); a3- Pseudomonas (P66); b- Rhizoctonia solani (Control); b1- Streptomyces 98(1); b2- Streptomyces (E7); b3- Pseudomonas (P66).*

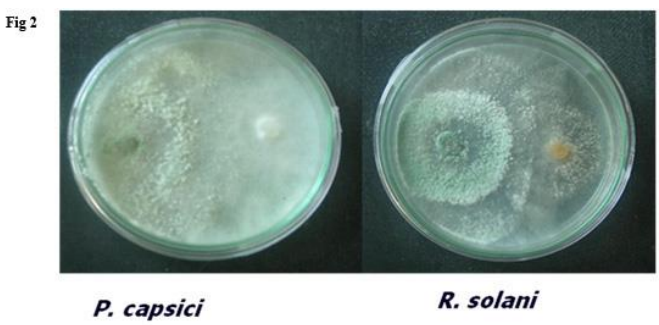

Figure 2. *Antagonistic Trichoderma isolates against plant pathogens. Isolate T 66 against P. capsici and T30 against R. solani. The isolates showed over growth on the test pathogens.*

Figure 4 shows production of the antifungal metabolite by *Streptomyces* sp. 98(1) culture. ISP2 medium was inoculated with the culture and incubated over a period of nine days to

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

get the maximum antifungal activity of the cell free supernatant against *P. capsici* and *R. solani*. Figure 5 shows the effect of metabolites on the morphology of fungal mycelia of the test pathogens, showing the cell wall degradation and condensation of cytoplasm.

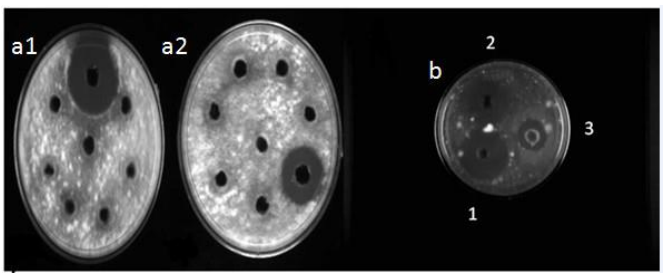

Figure 3. *Well Diffusion assay using the extract of antagonistic cultures showing the growth inhibition of Phytopathogens. a1 and a2 - clearing zone against Phytophthora capscisi by 100 µl extract of Streptomyces (98(1) and E (7) respectively. (Other wells are with varying volumes and mild zones are visible around them as well); b – Clearing zone Against Rhizoctonia solani by 100 µl extract of 1- Streptomyces 98(1), 2- Streptomyces (E7) and 3- Pseudomonas (P66).*

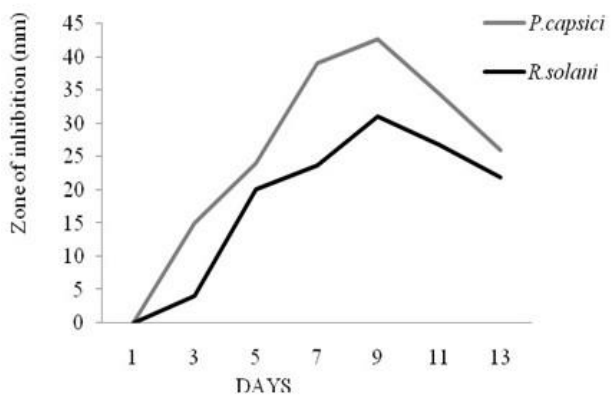

Figure 4. *Production of antifungal metabolite by Strptomyces isolates 98(1).*

Apart from the well-known siderphore production capability *(*data not included*), Pseudomonas fluorescens* was found to produce HCN which could be also responsible for its antifungal activity (Figure 6A). The colour of the filter paper was changed from yellow to yellowish red after 6 days of incubation, which indicated the production of HCN. Cyanide producing *P. fluorescens* strain is superior with respect to disease suppression in plants when compared with non-cyanogenic strains. Similarly, as shown in Figure 6B, the mycelial growth of *P. capsici* was completely inhibited by certain volatile metabolite as well produced by *P. fluorescens*.

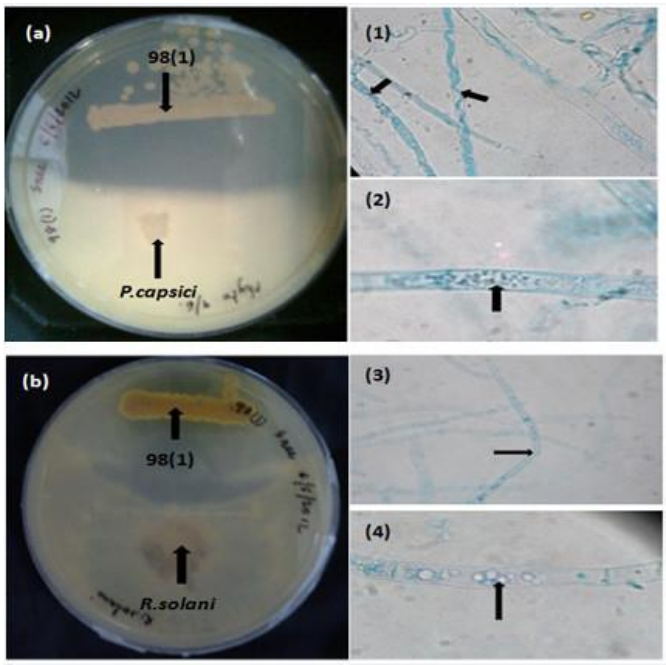

Figure 5. *Antagonism and microscopic view showing the fungal hyphae damage casused by the action of Streptomyces isolate (98(1)). (a) Against P.capsici: 1-2 hyphae of P.capsici; (b) Against R.solani: 3-4 hyphae of R. solni.*

None of the enzyme treatments showed any significant reduction in antifungal activity of the metabolite from *P. fluorescens*. In the case of *Streptomyces sp.* 98(1), the antifungal activity was reduced on addition of the enzyme lysozyme, but remains unaffected in presence of proteinase K and pepsin. The antifungal metabolites from *Pseudomonas* and *Streptomyces sp.* 98(1) were heat stable at different temperatures from 37-90°C when treated for 1 h and it loses its antifungal activity upon autoclaving at 121°C for 15 min. Both the cultures of *Pseudomonas* showed no cellulase or chitinase activity while the two bacilli isolate showed cellulase activity. Interestingly, *Streptomyces sp.* 98(1) and *Bacillus* (B4) showed both the activity (Table 2, Figure 7).

In vivo evaluation of selected isolates and metabolites for disease suppression under greenhouse study

There was a significant reduction of the disease intensity of foot rot disease of black pepper and collar rot of chick pea due to the treatment with antagonistic isolates, although

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

disease was incited by artificial inoculation. All the potential antagonists treated pepper plants invariably showed better growth in terms of height and number of leaves than control plants (Figure 8).

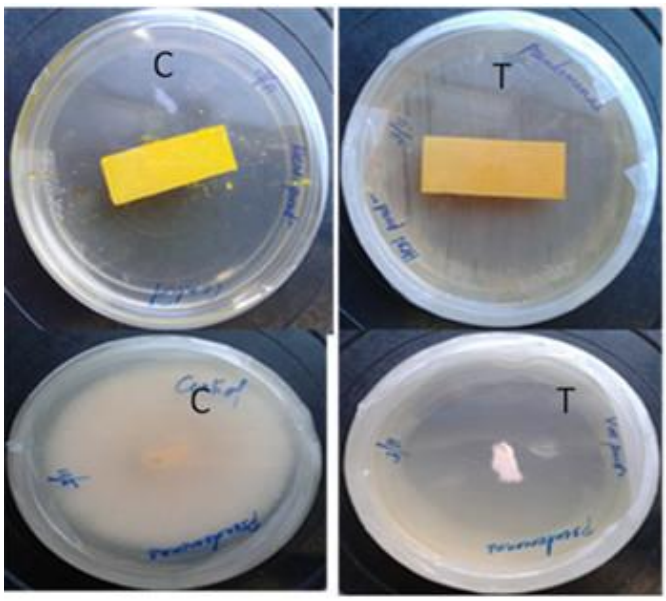

Figure 6. *HCN and Volatile compound production by Pseudomonas isolate. a- HCN Production. C-control; T-test; b- Volatile compound formation and growth inhibition of P. capsici; C-control; T-test.*

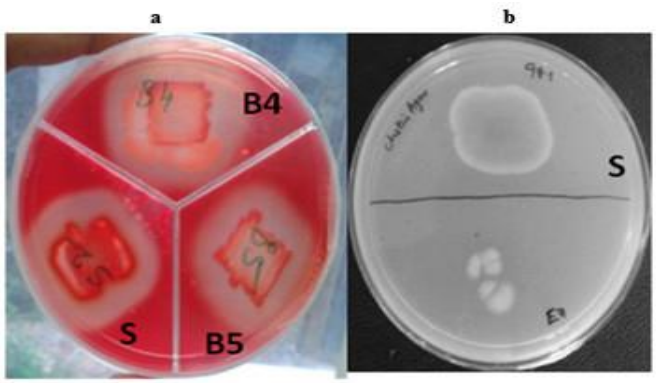

Figure 7. *Plate assay for cellulase and chitinase activity. a- Cellulase assay. S-Streptomyces isolate (98(1)); B4 and B5 – Bacillus cultures; b- Chitinase assay. S-Streptomyces isolate (98(1)).*

The maximum average height of the antagonist treated plant was recorded to be 125.80 cm after 60 days with *Pseudomonas* followed by B*acillus* (108.50 cm), compared to control which recorded only 70.20 cm (Table 3).

Table 2. *Enzyme profile of the isolates.*

Isolates	Cellulase activity	Chitinase activity
Bacillus sp. (B4)	+	+
Bacillus sp. (B5)	+	-
Bacillus sp. (B12)	+	-
Pseudomonas fluorescence (P66)	-	-
Pseudomonas fluorescence (P1)	-	-
Streptomyces sp. (98.1)	+	+
Streptomyces sp. (E7)	+	-
Streptomyces sp. (N4)	+	-

Figure 8. *Reduction in foot rot disease intensity in black pepper as influenced by selected isolates. Pepper plants infected with P. capsici. The control plants shows the symptoms of foot rot and the one treated with the antagonistic isolates shows better growth and less disease symptoms.*

The number of leaves was also maximum with *Pseudomonas* (45.75) followed by *Actinomycetes* which recorded 29.80 compared to control which is recorded 21.80 (Table 3).

Similarly, the results obtained from growth of chickpea plants also showed interesting degree of disease suppression (Figure 9) with the maximum height of pathogen infected plant after 60 days was recorded to be 49.98 cm upon treatment with the antagonist pseudomonas followed by actinomycetes which recorded 43.80 cm, compared to control which recorded 35.50 cm (Table 4). The number of leaves was maximum with *Actinomycetes*, which recorded 27 followed by *Pseudomonas* (25) compared to control treatment which recorded 20.50 (Table 4).

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Figure 9. *Reduction in collar rots disease intensity in chickpea as influenced by selected isolates. Chickpea infected with R. solani. The control plants shows the symptoms of foot rot and the one treated with the antagonistic isolates shows better growth and less disease symptoms.*

The trials with spraying the metabolites also showed potential reduction in the disease intensity of both pepper and chickpea plants (Tables 5 and 6). The disease score was considerably reduced in both the cases and was zero with the metabolites of pseudomonas after 90 days of growth. Tables 7 and 8 show the criterion of disease score for pepper and chickpea, respectively.

Discussion

Foot rot disease caused by *P. capsici* initiates on leaves as water soaked lesions and expands into large dark brown spots. It becomes uniformly dark with grayish centre. In stems, certain blackish brown marks are observed at ground level or slightly above, later they cause rot and stem disintegrates (Dastur, 1935).The cultured test pathogen also caused the infection in the same manner.

Table 3. *Effect of selected isolates of antagonists on growth characteristics of pepper plants under greenhouse condition (Values are the average of two independent trails).*

Isolates	Growth characteristics at different intervals					
	10 days		30 days		60 days	
	Height (cm)	Number of leaves	Height (cm)	Number of leaves	Height (cm)	Number of leaves
Pseudomonas	16.00	8.44	38.50	18.25	49.98	25.00
Bacillus	19.78	9.67	28.00	15.25	39.80	23.00
Actinomycetes	16.50	7.80	32.40	14.00	43.80	27.00
Trichoderma	19.0	8.53	34.20	16.30	40.00	24.50
Control	15.77	10.22	28.80	12.25	35.50	20.50

Table 4. *Effect of selected isolates of antagonists on growth characteristics of chick pea plants under greenhouse condition (Values are the average of two independent trails).*

Isolates	Growth characteristics at different intervals					
	10 days		30 days		60 days	
	Height (cm)	Number of leaves	Height (cm)	Number of leaves	Height (cm)	Number of leaves
Pseudomonas	16.00	8.44	38.50	18.25	49.98	25.00
Bacillus	19.78	9.67	28.00	15.25	39.80	23.00
Actinomycetes	16.50	7.80	32.40	14.00	43.80	27.00
Trichoderma	19.0	8.53	34.20	16.30	40.00	24.50
Control (infected ones without any treatment)	15.77	10.22	28.80	12.25	35.50	20.50

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Table 5. *Effect of metabolites of selected isolates on foot rot disease of black pepper under greenhouse conditions.*

Type of antagonistic culture	Disease score					
	Days after inoculation with pathogen					
	3	10	20	30	60	90
Pseudomonas	0.50	1.00	1.58	2.10	1.85	**0.00**
Bacillus	1.00	1.95	2.50	2.70	2.00	1.33
Actinomycetes	0.76	1.82	2.35	2.65	1.95	0.66
Trichoderma	0.65	1.73	2.85	2.61	1.90	0.66
Control (without metabolite)	2.58	3.30	4.65	5.65	5.90	6.00

Table 6. *Effect of metabolites of selected isolates on collar rot disease of chickpea plants under greenhouse conditions.*

Isolates	Disease score					
	Days after inoculation with pathogen					
	3	10	20	30	60	90
pseudomonas	1.10	1.43	2.30	2.00	1.00	**0.00**
Bacillus	1.20	1.50	2.85	3.90	2.85	2.00
Actinomycetes	1.20	1.65	2.80	3.65	2.60	1.90
Trichoderma	1.33	1.75	2.90	3.95	2.95	2.00
Control (without metabolite)	3.00	4.33	5.67	5.80	5.92	6.00

Table 7. *Disease scoring chart for Foot rot of pepper caused by Phytophthora capsici.*

Disease scale	% of infection	Description of the disease development
0	0	No infection
1	1-10%	Water soaked lesion at pinprick along with initiation of blackening
2	11-25%	Water soaked lesion size increase along with blackening
3	26-50%	Spreading of water soaked lesion along with covering of major portion of leaf lamina
4	51-75%	Defoliation of leaves
5	76-90%	Black coloured lesions spreading to the stem
6	91-100%	Stem darkening. Wilting and death of the plant

Table 8. *Disease scoring chart for collar rot of chick pea caused by Rhizoctonia solani.*

Disease scale	% of infection	Description of the disease development
0	0	No infection
1	1-10%	Lesion at pinprick
2	11-25%	Lesion at pinprick along with initiation of browning
3	26-50%	Lesion size increases along with browning
4	51-75%	Drying of lesions, development of ash colour, further spread
5	76-90%	Yellowing, lesion progress defoliation
6	91-100%	Yellowing, lesions spreading to all leaves, death of the plant

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Rhizoctonia solani considered as a basidiomycete and the pathogen had light brown to brownish mycelium which produced a few, light brown, small round sclerotia of size 0.3-1.5 mm, which never showed conglomeration. Hyphal thickness ranged from 6.8-8 µm with 4-6 numbers of nuclei per vegetative hyphal compartment. Based on these microscopic examinations of the morphological characters the pathogen was identified as *R. solani*.

The forest soils of Western Ghats are proved to be a rich source of potential antagonists against the selected phytopathogens. The study could effectively identify few *Streptomyces*, *Pseudomonas*, *Bacillus* and *Trichoderma* strains that are capable of producing anti- phytopathogenic metabolites. Nguyen et al. (2012) isolated an actinomycete strain H7602 from rhizosphere soil, identified it as *Streptomyces griseus* and demonstrated its antifungal activity against various plant pathogens including *P. capsici* with 47.35% of root mortality and enhanced also growth of pepper plants for 56.37% in fresh root and 17.56% g in fresh shoot as compared to control, resulting in greater protection to pepper plants against *P. capsici* infestation. The *Streptomyces sp*. (98.1) isolated in the present study showed a better inhibition of *P. capsici* and it is active against *R. solani* as well. The treatment of fungal pathogens with antifungal metabolite of the isolated *Streptomyces sp.* showed significant change in the morphology of fungal mycelia of the test pathogens, that includes the cell wall degradation and condensation of cytoplasm. Antifungal activity of Antifungalmycin 702 isolated by Xiong et al. (2012) made similar observations with *Streptomyces padanus* against *R. solani*. Similarly, thirty-four endophytic actinomycetes were isolated by Goudjal et al. (2014) from the roots of native plants of the Algerian Sahara which were identified to be *Streptomyces* genus and showed *in vitro* antifungal activity against *Rhizoctonia solani* and some of the positive antagonist strains were identified to be related to *Streptomyces mutabilis* and *Streptomyces cyaneofuscatus*. In a research program carried out by Lee et al. (2005), Paromomycin, one of the antibiotic depsipetides isolated from *Streptomyces* sp. AMG-P1, was found to be used as one of the biocontrol agents to suppress the soil borne diseases caused by two important pathogens, *Phytophthora* and *Pythium* species and also to evaluate the potential of the antibiotics against *Phytophthora* late blight on plants under greenhouse conditions. During the screening program for fungicides conducted by Yang et al. (2010), actinomycete strain, identified as *Streptomyces diastaticus* was isolated with the potential activity against the fungal pathogen and the macrolide antibiotics, similar to oligomycins A and C, (the macrolide antibiotics produced by *Streptomyces diastatochromogenes)* exhibited a strong activity against *Aspergillus niger, Alternaria alternata*, *Botrytis cinerea* and *Phytophthora capsici*.

The results from the present study suggest that many of the antagonistic cultures possess enzymes like cellulase or chitinase. Sophereth et al. (2013) had isolated similar chitinolytic bacterial strains having strong antifungal activity against *Phytophthora capsici*. The isolate was identified as *Burkholderia cepacia* MPC-7 and produced benzoic acid (BA) and phenylacetic acid (PA) which is responsible for its antifungal activity. Ghasemi et al. (2010) isolated a *Bacillus pumilus* strain from high salinity ecosystem in Iran that produced a bifunctional lysozyme and chitinase enzyme that made the strain potentially active against the fungal pathogens such as *Fusarium graminearum*, *Rhizoctonia solani*, *Magnaporthe grisea*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Bipolaris sp*. Chitinolytic strain *Streptomyces albidoflavus* was isolated from soil of the central region of Poland by Brzezinska et al. (2013) and it inhibited the growth of the fungal pathogens, *Alternaria alternata, Fusarium culmorum, F. oxysporum and B. cinerea.*

Mei et al. (2010) isolated a total of 98 isolates from the rhizosphere soil of healthy pepper plants in the fields seriously infected by *P. capsici*. The isolates *Paenibacillus polymyxa* and *Bacillus pumilus* showed maximum antagonistic activity against *Phytophthora capsici*, *Verticillium dahliae, Fusarium oxysporum*, *Rhizoctonia solani*, *Phtophthora parasitica*, and *Ralstonia solanacearum.* In a similar study made by Lee et al. (2013) an antifungal compound Fusaricidin, a cyclic depsipeptide isolated from *Paenibacillus polymyxa* E681 (E681), was demonstrated to control *Phytophthora* blight infection caused by *Phytophthora capsici* in red-pepper. Application of different concentrations of the fungus *Trichoderma asperellum* strain T34 isolated by Segarra et al. (2013) was found to suppress the incidence of disease caused by *P. capsici* in pepper and hence revealed the potential use of trichoderma strains as a useful biological alternative to chemicals for the control of *P. capsici* in pepper. A bioformulation using *Trichoderma harzianum*, *Trichoderma asperellum*, and *Talaromyces flavus* and some organic and inorganic carriers that showed potential antagonism against *Rhizoctonia solani*, causing sugar beet seedling damping-off disease, was developed by Kakvan et al. (2013). Thus, these studies showed that

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Trichoderma sp. can be potential biocontrol agents for *R. solani* and *P. capsici.*

The *Pseudomonas sp*. isolated in the present study is a potential producer of HCN and volatile metabolites and showed potential inhibition of the test pathogens. *Pseudomonas* strains of bacterium are found to generally possess antifungal activity and are considered as the bio control agents against a wide range of fungal phytopathogens. Similar observations to what made in the present study were published from the work by Zhou et al. (2012) with the isolation of a bacterial strain, *Pseudomonas brassicacearum* that produce 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide (HCN), siderophore (s) and protease and strongly inhibit the growth of phytopathogenic bacteria *Ralstonia solanacearum*. Cordero et al. (2012) reported that *Pseudomonas* strains producing antimicrobial secondary metabolites play an important role in the biocontrol of phytopathogenic fungi and several new strains of *Pseudomonas* spp. were isolated from agricultural soils, river silt, sea mud and from rhizosphere soils and herbarium specimens (Kim et al., 2012; Mavrodi et al., 2012; Hammami et al., 2013).

Successful management of *P. capsici* by *P. fluorescence* has been reported earlier as well (Anadaraj, 1997; Sivaprasad et al., 2003). Similar studies by Pande & Choube (2003) also indicated the enhanced plant growth in terms of leaf number, length and weight of shoot and root of cow pea by *P. fluorescens*. The success of any microbial inoculants used as biocontrol agent depends upon its inherent antagonistic property and ability for multiplication, persistence and activity in the given ecosystem. Such efficient organisms are to be identified from the unexplored diversity available in nature through extensive isolation and testing.

Conclusion

The study revealed the presence of potential microbial biodiversity in the Western Ghats forest soil which can act as a rich resource for isolating antagonistic microbial cultures against phytopathogens. However, the success will greatly depended on developing a biocontrol agent (single or consortium) which can withstand real field conditions over a period of time. Metabolite focused studies such as purification and structure elucidation aspects were also equally important in developing natural antifungal agents which can later acts as back bones for several derived antifungal compounds as well.

Acknowledgement

This is a network project between CSIR- NIIST, Trivandrum and Kerala Agriculture University financially supported by the Department of Biotechnology (DBT), New Delhi. The support from CSIR Network Project, NAPAHA (CSC0130) for microbial bioactive studies was also greatly acknowledged .The authors also thank the Forest department of the Kerala state, India for permitting soil sample collection from restricted areas in the Western Ghat.

References

Amkraz N, Boudyach EH, Boubaker H, Bouizgarne B, Ait Ben Aoumar A. 2010. Screening for fluorescent pseudomonades, isolated from the rhizosphere of tomato, for antagonistic activity toward *Clavibacter michiganensis* subsp. michiganensis. World J. Microbiol. Biotechnol., 26(6): 1059–1065.

Anandaraj M. 1997. *Pseudomonas fluorescens* for disease management and growth enhancement of crop plants In: P.Sivaprasad and K.S.Meenakumari (eds) Microbial inoculants technology for sustainable farming, Kalyan Publishers, New Delhi pp. 26.

Andersen JB, Koch B, Nielsen TH, Sorensen D, Hansen M, Nybroe O, Christophersen C, Sorensen J, Molin S, Givskov M. 2003. Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum*. Microbiology, 149: 1147–1156.

Balachandran C, Duraipandiyan V, Ignacimuthu S. 2012. Cytotoxic (A549) and antimicrobial effects of *Methylobacterium* sp. isolate (ERI-135) from Nilgiris forest soil, India. Asian Pacific Journal of Tropical Biomedicine, 2: 712-716.

Bloemberg GV, Lugtenberg BJJ. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol., 4(4): 343–350.

Bolwerk A, Lagopodi AL, Wijfjes AHM, Lamers GEM, Chin-A-Woeng TFC, Lugtenberg BJJ, Bloemberg GV. 2003. Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* sp. radicis-lycopersici. Mol. Plant Microbe In, 11: 983–993.

Brzezinska MS, Jankiewicz U, Burkowska A. 2013. Purification and characterization of *Streptomyces albidoflavus* antifungal components. Appl. Biochem. Microbiol., 49(5): 451–457.

Cordero P, Cavigliasso A, Príncipe A, Godino A, Jofré E, Mori G, Fischer S. 2012. Genetic diversity and antifungal activity of native Pseudomonas isolated from maize plants grown in a central region of Argentina. Systematic and Appl. Microbiol. Biot., 35(5): 342-351.

Crawford DL, Kowalski M, Roberts MA, Merrel G, Deobald LA. 2005. Discovery, development and commercialization of a microbial antifungal biocontrol agent *Streptomyces lydicus*

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

WYEC 108: history of a decade long endeavour. Society for Industrial Microbiology and Biotechnology News, 55: 88–95.

Dastur JF. 1935. Diseases of pan (*Piper betle*) in the Central Provinces. Proc. Indian Acad. Sci. B. 1: 778-815.

De-Vasconcellos RLF, Cardoso EJBN. 2009. Rhizospheric *Streptomycetes* as potential biocontrol agents of *Fusarium* and *Armillaria* pine rot and as PGPR for *Pinus taeda*. Biocontrol, 54: 807–816.

De Weger LA, Boxtel VR, Burg VDB, Gruters RA, Geels FP, Schippers B, Lugtenberg B. 1986. Siderophores and outer membrane proteins of antagonistic, plant- growth-stimulating, root colonizing *Pseudomonas* spp. J. Bacteriol., 165: 585–594.

Dunne C, Moe¨nne-Loccoz Y, McCarthy J, Higgins P, Powell J, Dowling DN, Gara F. 1998. Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of *Pythium*-mediated damping-off of sugar beet. Pathology, 47: 299–307.

El-Tarabily KA. 2006. Rhizosphere-competent isolates of streptomycete and non-streptomycete actinomycetes capable of producing cell-wall degrading enzymes to control *Pythium aphanidermatum* damping-off disease of cucumber. Can. J. Bot., 84: 211–222.

El-Tarabily KA, Sivasithamparam K. 2006. Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoter Soil Biol. Biochem., 38: 1505–1520.

El-Tarabily KA, Soliman MH, Nassar AH, Al-Hassani HA, Sivasithamparam K, McKenna F, Hardy GESJ. 2000. Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Plant Pathol., 49: 573–583.

Ghasemi S, Ahmadian G, Jelodar N B, Rahimian H, Ghandili S, Dehestani A, Shariati P. 2010. Antifungal chitinases from *Bacillus pumilus* SG2: preliminary report. World J. Microbiol. Biotechnol., 26: 1437–1443.

Goudjal Y, Toumatia O, Yekkour A, Sabaou N, Mathieu F, Zitouni A. 2014. Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara Microbiol. Res., 169(1): 59–65.

Gupta P, Samant K, Sahu A. 2012. Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. Int. J. Microbiol.,ID 578925 http://dx.doi.org/10.1155/2012/578925.

Haas D, De´fago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol., 3: 307–319.

Haggag WM, Abdallh EG. 2012. Purification and characterization of chitinase produced by endophytic *Streptomyces hygroscopicus* against some phytopathogens. J. Microbiol. Res., 2(5): 145-151.

Hamby MK, Crawford DL. 2000. The enhancement of plant growth by selected Streptomyces species. In *100th General meeting of American Society for Microbiology*, Los Angeles, CA. Abstract 567.

Hammami I, Hsouna AB, Hamdi N, Gdoura R, Triki MA. 2013. Isolation and characterization of rhizosphere bacteria for the biocontrol of the damping-off disease of tomatoes in Tunisia. Comptes Rendus Biologies, 336(11–12): 557–564.

Huang X, Zhang N, Yong X, Yang X, Shen Q. 2012. Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiol. Res., 167(3): 135–143.

Jacobsen BJ, Zidack NK, Larson BJ. 2004. The role of *Bacillus*-based biological control agents in integrated pest management systems plant diseases. Phytopathology, 94: 1272–1275.

Jalaja V, Sweta S, Kumar SS, Deepthy A , Kumar RS. 2011. Isolation and characterization of a novel α-amylase from a metagenomic library of Western Ghats of Kerala, India. Biologia, 66: 939-944.

Jiang Z, Guo Y, Li S, Qi H, Guo J. 2006. Evaluation of biocontrol efficiency of different *Bacillus* preparations and field application methods against *Phytophthora* blight of bell pepper. Biol. Control, 36(2): 216–223.

Johnson LF, Curl EA. 1972. Methods for research on ecology of soil borne plant pathogen. Burgess publishing Co. Minneapolis P. 247.

Jung WJ, An KN, Jin YL, Park RD, Lim KT, Kim KY, Kim TH. 2003. Biological control of damping off caused by *Rhizoctonia solani* using chitinase producing *Paenibacillus illinoisensis* KJA-424. Soil Biol. Biochem., 35: 1261–1264.

Kakvan N, Heydari A, Zamanizadeh HR, Rezaee S, Naraghi L. 2013. Development of new bioformulations using *Trichoderma* and *Talaromyces* fungal antagonists for biological control of sugar beet damping-off disease. Crop Protection, 53: 80–84.

Kamensky M, Ovadis M, Chet I, Chernin L. 2003. Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. Soil Biol. Biochem., 35: 323–331.

Kennedy AC. 2005. Rhizosphere. In D. M. Sylvia, J.J. Fuhrmann, P.G. Hartel, D.A. Zuberer (ed) *Principles and applications of soil microbiology* Pearson, New Joursey (pp. 242–262).

Kim H, Sang MK, Jung HW, Jeun Y, Myung I, Kim KD. 2012. Identification and characterization of *Chryseobacterium wanjuense* strain KJ9C8 as a biocontrol agent of Phytophthora blight of pepper. Crop Protection, 32: 129–137.

Kishore GK, Pande S,Podile AR. 2005. Biological control of late leaf spot of peanut (*Arachis hypogaea*) with chitinolytic bacteria. Phytopathology, 95: 1157–1165.

Kloepper JW, Ryu CM, Zhang S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology, 94: 1259–1266.

Kloepper JW, Rodrigo RK, McLroy JA ,Collins DJ. 1991. Analysis of populations and physiological characterization of microorganisms in rizhospheres of plant with antagonistic properties to phytopathogenic nematodes. Plant Soil, 136: 95-102.

Lee SH, Cho YE, Park S, Balaraju K, Park JW, Lee SW, Park K. 2013. An antibiotic fusaricidin: a cyclic depsipeptide from *Paenibacillus polymyxa* E681 induces systemic resistance against Phytophthora blight of red-pepper. Phytoparasitica, 41: 49-58.

Lee HB, Kim Y, Kim JC, Choi GJ, Park SH, Kim CJ, Jung HS. 2005. Activity of some aminoglycoside antibiotics against true fungi, Phytophthora and Pythium species. J. Appl. Microbiol., 99(4): 836-843.

Lemanceau P, Maurhofer M, De´fago G. 2006. Contribution of studies on suppressive soils to the identification of bacterial biocontrol agents and to the knowledge of their modes of action. In S.S.Gnanamanickam (Ed.), *Plant-associated bacteria* (pp. 231–267). New York: Springer.

DOI: 10.69085/jbb20152207

RESEARCH ARTICLE

Mavrodi OV, Walter N, Elateek S, Taylor GC, Okubara PA. 2012. Suppression of *Rhizoctonia* and *Pythium* root rot of wheat by new strains of *Pseudomonas.* Biol. Control., 62(2): 93–102.

Mayolo NG, Lipps PE, Schmitthenner AF. 1993. Reactions of dry bean, lima bean and soy bean cultivars to Rhizoctonia rot and hypocotyls rot and web blight. Plant Disease, 77: 234-238.

Mei XL, Zhao QY, Tan SY, Xu YC, Shen B, Shen QR. 2010. Screening, identification, and biocontrol effect of antagonistic bacteria against *Phytophthora capsici*. Ying Yong Sheng Tai Xue Bao, 21(10): 2652-2658.

Mohandas SP, Ravikumar S, Menachery SJ, Suseelan G, Narayanan SS. 2012. Bioactives of microbes isolated from Western Ghat belt of Kerala show β-Lactamase inhibition along with wide spectrum antimicrobial activity. Appl. Biochem. Biotech., 167: 1753-1762.

Nampoothiri KM, Ramkumar B, Pandey A. 2013. Western Ghats of India: Rich source of microbial biodiversity. J. Sci. Ind. Res. India, 72: 617-623.

Nguyen X, Naing K, Lee Y, Tindwa H, Lee G, Jeong B, Ro H, Kim S, Jung W, Kim K. 2012. Biocontrol potential of *Streptomyces griseus* H7602 against root rot disease (*Phytophthora capsici*) in pepper**.** Plant Pathol. J., 28(3): 282-289.

Palumbo JD, Yuen GY, Jochum CC, Tatum K, Kobayashi DY. 2005. Mutagenesis of beta-1, 3-glucanase genes in *Lysobacter* enzymogenes strain C3 results in reduced biological control activity toward Bipolaris leaf spot of tall fescue and *Pythium* damping-off of sugar beet. Phytopathology, 95: 701–707.

Pande VS, Choube HS. 2003. Enhanced plant growth of chickpea and biocontrol of wilt caused by *Fusarium oxysporum* by native isolates of Pseudomonas fluorescence. In (Eds) Reddy, M.S., Anandraj, M., Eapen, S.J., Sarma, Y.R and Klopeer, J.W. (2003). Proceedings of the International workshop on plant growth promoting *Rhizobacteria* 5-10, October, IISR, Calicut, P. 202.

Plodpai P, Chuenchitt S, Petcharat V, Chakthong S, Voravuthikunchai PS. 2013. Anti-*Rhizoctonia solani* activity by *Desmos chinensis* extracts and its mechanism of action. Crop Protection, 43: 65–71.

Robles-Yerena L, Rodríguez-Villarreal RA, Ortega-Amaro MA, Fraire-Velázquez S, Simpson J, Rodríguez-Guerra R, Jiménez-Bremont JF (2010) Characterization of a new fungal antagonist of *Phytophthora capsici.* Scientia Horticulturae, 125(3): 248–255.

Ryu CM, Hu CH, Locy RD, Kloepper JW. 2005. Study of mechanisms for plant growth promotion elicited by rhizobacteria in Arabidopsis thaliana. Plant Soil, 268: 285–292.

Schmidt CS, Lorenz D, Wolf GA. 2001. Biological control of the grapevine dieback fungus *Eutypa lata* I: screening of bacterial antagonists. J. Phytopathol., 149: 427–435.

Segarra G, Avilés M, Casanova E, Borrero C, Trillas I. 2013. Effectiveness of biological control of *Phytophthora capsici* in pepper by *Trichoderma asperellum* strain T34. Phytopathol. Mediterr., 52(1): 77−83.

Shashidhara S, Lokesh MS, Lingaraju S, Palakshappa MG. 2008. In vitro evaluation of microbial antagonists, botanicals and fungicides against *Phytophthora capsici* Leon, the causal agent of foot rot of black pepper. Karnataka Journal of Agricultural Sciences, 21(4): 527-531.

Sivaprasad P, Sulochana KK, Kavitha MS, Joseph PJ, Meenakumari KS. 2003. Effect of fluorescent *pseudomonas* isolates on foot rot disease and growth of black pepper (*piper nigrum L*). Proceedings of 6th International PGPR workshop 5-10 October, Calicut, India pp. 68-74.

Sophearetha M, Naing KW, Lee YS, Hyun HN, Kim YC, Kim KY. 2013. Biocontrol of late blight (*Phytophthora capsici*) disease and growth promotion of pepper by *Burkholderia cepacia* MPC 7. The Plant Pathology J., 29(1): 67-76.

Spaepen S, Vanderleyden J, Remans R. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev., 31 :425–448.

Truong NV, Liew ECY, Burgess LW. 2010. Characterization of *Phytophthora capsici* isolates from black pepper in Vietnam. Fungal Biol., 114(2–3): 160–170.

Velusamy P, Immanuel JE, Gnanamanickam SS, Thomashow L. 2006. Biological control of rice bacterial blight by plant-associated bacteria producing 2, 4-diacetylphloroglucinol. Can. J. Microbiol., 52: 56–65.

Vessey JK. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil, 255: 571–586.

Von-Der-Weid I, Artursson V, Seldin L, Jansson JK. 2005. Antifungal and root surface colonization properties of GFP tagged *Paenibacillus brasilensis* PB177. World J. Microbiol. Biotechnol., 2(1): 1591–1597.

Xiong Z, Zhang Z, Li J, Wei S, Tu G. 2012. Characterization of *Streptomyces padanus* JAU4234, a producer of actinomycin X_2, fungichromin, and a new polyene macrolide antibiotic. Appl. Environ. Microbiol., 78(2): 589–592.

Yang PW, Li MG, Zhao JY, Zhu MZ , Shang H, Li JR, Cui XL, Huang R, Wen ML. 2010. Oligomycins A and C, major secondary metabolites isolated from the newly isolated strain *Streptomyces diastaticus*. Folia Microbiologica, 55(1): 10-16.

Zhang Y, Fernando WGD. 2004. Zwittermicin: A detection in *Bacillus* spp. controlling *Sclerotinia sclerotiorum* on canola. Phytopathology, 94: S116.

Zhou T, Chen D, Li C, Sun Q, Li L, Liu F, Shen Q, She B. 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiol. Res., 167(7): 388–394.

Zivkovic S, Stojanovic S, Ivanovic Z, Gavrilovic V, Popovic T, Balaz J. 2010. Screening of antagonistic activity of microorganisms against *Colletotrichum acutatum* and *Colletotrichum gloeosporioides.* Archiv. Biolog. Sci., 62(3): 611-623.

DOI: 10.69085/jbb20152207