J. Agr. Sci. Tech. (2015) Vol. 17: 1365-1377

Antiphytopathogenic and Plant Growth Promoting Attributes of *Bacillus* Strains Isolated from Rhizospheric Soil of Chickpea

S. Patil[1], C. T. Shivannavar[1]*, M. C. Bheemaraddi[1], and S. M. Gaddad[1]

ABSTRACT

In the present study, we aimed to screen indigenous rhizospheric *Bacillus* strains, capable of producing antiphytopathogenic and plant growth promoting traits. Isolate CTS-B19 and CTS-G24 exhibited quite noticeable antagonistic activity initially against *Fusarium oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola*, and, later, against a panel of phytopathogens. Partial 16S rRNA sequence analysis showed that the isolate CTS-B19 and CTS-G24 exhibited 99% homology with *Paenibacillus polymyxa* and *Bacillus subtilis* and the sequences were deposited in GenBank with accession numbers KF322038 and KF322037, respectively. *In vitro* detection for fungal wall degrading enzymes revealed that both isolates produced chitinases, *β*-1,3-glucanases, proteases and cellulases. While siderophores and catalase activities were observed only in *Bacillus subtilis* (CTS-G24), both strains exhibited a positive result for *in vitro* ammonia production. Besides, the strain CTS-B19 could also solubilize phosphate. Lytic enzymes and siderophore produced by *Bacillus subtilis* can be considered as potential antiphytopathogenic traits involved in the inhibition of fungal growth. Both strains exhibited either no or negligible antagonistic activity against other plant growth promoting bacteria. Additional to antagonism, plant growth promoting traits exhibited by these natural biocontrol agents may suppress plant diseases and might be applied in agriculture as an alternative to chemical pesticides and fertilizers.

Keywords: Cell wall lytic enzymes, Lytic enzymes, *Paenibacillus* spp., Siderophore,

INTRODUCTION

In most agricultural ecosystems, soil-borne plant pathogens can be a major limitation to the production of marketable yields. They are also more recalcitrant to management and control compared to pathogens that attack the above-ground portions of the plant (Bruehl, 1987). Microorganisms that adversely affect plant growth and health are the pathogenic fungi, bacteria, and nematodes. Most of the soil borne pathogens are adapted to grow and survive in the bulk soil, but the rhizosphere is the playground and infection court where the pathogen establishes a parasitic relationship with the plant (Raaijmakers *et al.,* 2009). Biocontrol methods utilizing antagonistic microorganisms associated with the plant rhizosphere have great potential for control of soil borne plant pathogens (Prashar *et al.,* 2013).

The rapidly multiplying population is exerting immense pressure on agricultural lands for more crop yields, which results in more and more extensive use of chemical fertilizers and pesticides (Karlidag *et al.,* 2007). The long residual action and toxicity of chemical pesticides have brought about serious environmental problems such as mammalian toxicity, and accumulation of pesticide residues in the food chain (El-Bendary, 2006). Non-pathogenic soil bacteria, having the ability to antagonize fungal phytopathogens, have emerged as an important alternative in managing soil-borne

[1] Department of Microbiology, Gulbarga University, Gulbarga-585106, Karnataka, India.
* Corresponding author; e-mail: ctshiv@gmail.com

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

plant diseases. Several rhizobacteria, also recognized as Plant Growth-Promoting Rhizobacteria (PGPR), exert their beneficial effect on plants by various mechanisms *viz*. siderophore production, Hydrogen Cyanide (HCN), antibiotics, lytic enzymes, competing for colonization sites, nutrients, and by inducing systemic resistance (Lugtenberg and Kamilova, 2009; Raaijmakers *et al.,* 2009, Jordan *et al.,* 2013). PGPRs may, in addition, stimulate host-plant growth directly by increasing nutrient and water uptake through the production of phytohormones e.g., Indole-3-Acetic Acid (IAA), organic acids involved in phosphorus solubilization, or the fixation of atmospheric N_2 (Pattern and Glick, 1996; Lugtenberg and Kamilova, 2009).

Among the first successful biocontrol agents used against insects and pathogens were members of the genus *Bacillus* (Powell and Jutsum, 1993). *Bacillus* strains are considered as safe biological control agents (El-Bendary, 2006). Intrinsic properties of *Bacillus* such as formation of oval endospores that remain dormant for a long period under unfavourable environmental conditions make them potential colonizers (Fritze, 2004).

The chances for selecting effective strains from the rhizosphere area as biocontrol agents are likely to improve when the microorganisms are isolated from the same geographical area where they would ultimately be required to act (Whipps, 1997). The inoculated bacteria cannot, sometimes, survive in the soil because they must compete with the better-adapted indigenous microflora (Bashan, 1998). Efficiency of the biocontrol agents against soil-borne pathogens to suppress plant diseases is important to achieve successful control activity due to the complex and dynamic environmental conditions in the rhizosphere (Jo and Stabb, 1996). Isolation of indigenous strains adapted to the environment and characterization of PGP traits may contribute in formulating potential inoculants that can be used for better promotion of the region crops. Therefore, this study was aimed to isolate indigenous strains from rhizospheric soils of chickpea (cultivated in north Karnataka region) capable of producing antiphytopathogenic and PGP traits.

MATERIALS AND METHODS

Test Microorganisms

The following microorganism, i.e. *Alternaria alternata*-7202, *A. solani*-2101, *Aspergillus flavus*-277, *A. niger*-282, *Bacillus subtilus*-441, *Botrytis cinerea*-2350, *Collectotrichum acutatum*-1037, *C. capsici*-3414, *Erwinia sp.*-6720, *Fusarium graminearum*-2089, *F. oxysporum*-2480, *Pseudomonas syringae*-1604, *Rhizoctonia solani*-4634, *Verticillum dahliae*-6954 and *Xanthomonas compestris*-2286 were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Agricultural Research Station (ARS) at Gulbarga, Karnataka, India, generously provided *Rhizobium* sp. (ARS), *Bacillus thuringiensis* (ARS), *Azotobacter* sp. (ARS) (which were used as approved bioinoculants) and pathogenic strains of *F. oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola* previously isolated from infected chickpea plants showing the symptoms of wilt and dry root rot, respectively. After retrieving fungal and bacterial strains, they were maintained on Potato Dextrose Agar (PDA) and Nutrient Agar (NA) medium for further studies.

Isolation of Rhizobacteria

Rhizospheric soil samples were collected from healthy chickpea and pigeon pea plants cultivated in various agricultural fields of North Karnataka regions, India. Obtained samples were serially diluted in 0.9% NaCl solution and 0.1 mL of different dilutions were spread inoculated on NA and Trypticase Soya Agar supplemented with 0.05% Cyclohexamide. After incubating for

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

three days at 28°C, diverse bacterial colonies were selected, checked for their purity by quadrant streaking on fresh NA plates, and maintained on NA slants for further studies. Morphological characterization was performed as per the standard methods described by Cappuccino and Sherman (2010). An Eclipse E200 (NIKON Corporation, Tokyo, Japan) microscope was used for recording the observations of Grams staining, endospore staining, and motility tests (Cappuccino and Sherman, 2010).

In vitro Assay for Antagonism

Only Gram-positive, spore forming, rhizobacterial strains were subjected for screening of antagonistic activity initially against phytopathogenic strains of *F. oxysporum* f. sp. *ciceri* (ARS) and *Rhizoctonia bataticola* (ARS) using dual culturing technique as described by Foldes *et al.* (2000) with some modification. The isolated strains of rhizobacteria were depth cultivated in nutrient broth for antagonistic test. The fungus was point inoculated at the centre of Petri-dishes with PDA (pH adjusted to 6 to facilitate the growth of both Pathogen and test strains) and cultivated at 28°C for three days. Approximately, 4 cm from the actively growing mycelium of mould, test strains were inoculated with a single streak. Petri dishes were incubated at 28°C for seven days and checked periodically for inhibition of pathogenic growth. Formation of a clear zone indicated a positive result. The *Bacillus* strains which most vigorously inhibited the growth of the phytopathogenetic fungi were subjected to further studies.

Assay of Supernatant Filtrates of Cultures

The antibacterial activity was determined by agar well method (Perez *et al.,* 1990). At first, *Xanthomonas compestris* MTCC-2286 was cultivated in nutrient broth to obtain 0.5 McFarland standard turbidity and swab inoculated on Muller Hinton agar plates to produce lawn of pathogens. Wells were bored with sterile cork borer and 100 µL of culture free filtrates (obtained after filter sterilizing using 0.22 µ Axiva Sichem Biotech syringe filters) of each rhizobacteria was filled up to the rim. The plates were incubated at 28°C for 18-24 hours and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter and recorded.

Based on the pronounced activity of antagonism with *F. oxysporum* f. sp. *ciceri* (ARS) *Rhizoctonia bataticola* (ARS) and *X. compestris*-2286, two strains of *Bacillus*, designated as CTS-B19 and CTS-G24 were selected for testing antagonistic activity against all other phytopathogens and PGPRs indicated above. Qualitative screening for direct and indirect PGP traits of selected antagonistic *Bacillus* strains was checked.

16S rDNA Sequencing and Phylogenetic Analysis of Prominent Antagonistic Strains

Two potential isolates were selected and preliminarily characterized based on their morphological and biochemical properties (Holt *et al.,* 2004). Furthermore, various sugar utilization tests were also performed using HiCarbo kit (Hi-Media, India).

Molecular identification of isolates CTS-G24 and CTS-B19 was carried out as per the standard procedures followed at Scigenom Pvt. Ltd, Kochi. Universal primers 16S-F Bacterial *5'CGC CGT TTT ATC AAA AAC AT3'* 16S-R Bacterial *5'CCG GTC TGA ACT CAG ATC ACG T3'* was applied for both strains. Sequenced products were resolved on an Applied BioSystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The sequences obtained were analyzed using online databases NCBI BLAST (www.ncbi.nlm.nih.gov/blast) to find the closest match of the contig sequence. Phylogenetic and molecular evolutionary

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

analyses were conducted using software included in MEGA version 5.0 (Tamura *et al.,* 2011) package. The 16S rDNA sequence of strains CTS-B19 and CTS-G24 were aligned using the CLUSTAL W program (Thompson *et al.,* 1994) against corresponding nucleotide sequences of representatives of the genus *Bacillus* sp. and *Paenibacillus* sp. retrieved from GenBank. Based on maximum identity score, the first few sequences were selected and aligned using multiple sequence alignment software CLUSTALW. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1,000 resampling of the neighbor-joining dataset.

Detection of Cell Wall Degrading Enzymes

Production of chitinase and β-1,3-glucanase were determined by following the methods of Cattelan *et al.,* (1990). Proteolytic activity was detected by inoculating test isolates on Skim Milk Agar (SMA) prepared as reported by Smibert and Krieg (1994). Detection of cellulase activity was performed by streaking the test strains and incubating overnight on Carboxy Methyl Cellulose (CMC) agar prepared as per Teather and Wood (1982).

Detection of Siderophore

Siderophore production was observed by the formation of orange halos surrounding bacterial colonies on blue agar plates of Chrome Azurol S agar (CAS) medium after 48 hours of incubation at 30°C (Schwyn and Neilands, 1987).

HCN and Catalase Production

Cyanide production was detected qualitatively as described by Lorck (1948). Briefly, the test strains were streaked on nutrient broth supplemented with 4.4 g glycine L^{-1}. A Whatman filter paper no.1 soaked in 2% sodium carbonate solution and 0.5% picric acid solution was placed on the top of the plates. Plates were sealed with parafilm and incubated at 30°C for four days. Development of orange to red color indicated HCN production. The catalase production was detected by mixing cultures with an appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen (Cappuccino and Sherman, 2010).

Detection of IAA Production

Bacteria were cultured overnight in Luria-Bertani broth in the dark at 30°C for 72 hours. Bacterial biomass was removed by centrifugation at 4,000 rpm for 15 minutes and the supernatant was used for detection of IAA according to the method of Brick *et al.* (1991). Briefly, Supernatant (1mL) was vigorously mixed with two drops of orthophosphoric acid and 2 mL of Salkowski's reagent. After 30 minutes of incubation, development of pink color indicated IAA production (Brick *et al.,* 1991).

Detection of Phosphate Solubilization

To detect the phosphate solubilizing ability, the strains were streaked on Pikovaskya's agar medium (Pikovaskya, 1948). The formation of transparent halos surrounding the bacterial colony was considered as positive.

Detection of Ammonia Production

Bacterial isolates were grown in 10 mL peptone water into each tube and incubated for 48-72 hours at 30°C. Nessler's reagent (0.5 mL) was added into each tube. Development of brown to yellow color indicates a positive result for ammonia

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

production (Cappuccino and Sherman, 2010).

RESULTS

In vitro Evaluation of the Antagonistic Activity of Bacterial Strains

A total of 75 bacterial strains were isolated from rhizospheric soil samples of healthy chickpea plants and cultivated in the fields of North Karnataka region. The selective isolated rhizobacteria were checked for their purity, and morphologically characterized as Gram–positive rods and spore–forming bacteria.

In a dual culture assay for fungicidal activity against *F. oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola,* four Gram-positive isolates *viz*. CTS–B19, CTS–G24, CTS–C44 and CTS–R21 exhibited fungicidal activity against *Rhizoctonia bataticola*, amongst which only two strains (CTS–B19 and CTS–G24) exhibited extended antagonism against *F. oxysporum* f. sp. *ciceri* (Figures 1a and 1c). After the additional incubation period of 10 days, the pathogenic mycelia did not cover the surface of the inhibition ring, which indicated strong antagonism (Lee *et al.,* 2008). The results of further antagonistic tests showed that strains CTS–B19 and CTS–G24 had the sterilizing or inhibitory effect on a panel of phytopathogens. Thus, selection was made from the antagonism test, where the confluent growth of bacteria inhibited the development of a panel of fungal and bacterial phytopathogens (Figure 1 and Table 1).

Identification and Characterization of Antagonistic Bacteria

The selected bacterial antagonists were identified to species level based on morphological, biochemical, and molecular characterization. The 16S rRNA gene sequences of isolate CTS–B19 and CTS–G24 were found closely affiliated to *Paenibacillus polymyxa* and *Bacillus subtilis,* respectively. Their phylogenetic allocation and 16S rRNA gene sequence identities are presented in Figure 2. The sequence of strain CTS–B19 and CTS–G24 indicated that these isolates showed high identity (99 and 99%) and were in clustering with *Paenibacillus* sp. and *Bacillus* sp., respectively.

In vitro Detection of Antagonistic Traits

Proteolytic and chitinolytic activities were detected in both of the bacterial strains exhibiting *in vitro* antagonism against plant pathogens. This was indicated by the appearance of clear halo around the colonies contrasting with opaque appearance of medium unaffected (Figure 3-a). Detection of β–1,3–glucanase and cellulase activity was confirmed after the translucent zones were observed in contrast with the surrounding dark brown and pink medium, respectively, suggesting the positivity of results (Figures 3-b and -c).

B. subtilis CTS–G24 formed an orange halo surrounding bacterial colonies on CAS agar. This change occurred due to removal of iron from the blue CAS–Fe (III) complex by siderophore (Figure 3-d). Test strains were negative for cynogenic activity.

In vitro Detection of Plant Growth Promoting Traits

The selected strains, *B. subtilis* CTS–G24 and *P. polymyxa* CTS–B19 were screened for their ability to produce ammonia, IAA–like substances, and to solubilize phosphate. Both of the indigenous antagonistic strains produced ammonia in the medium, but only *P. polymyxa* CTS–B19 could solubilize phosphate. PGPR activities and antagonistic traits produced by both potential strains are summarized in Table 2.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

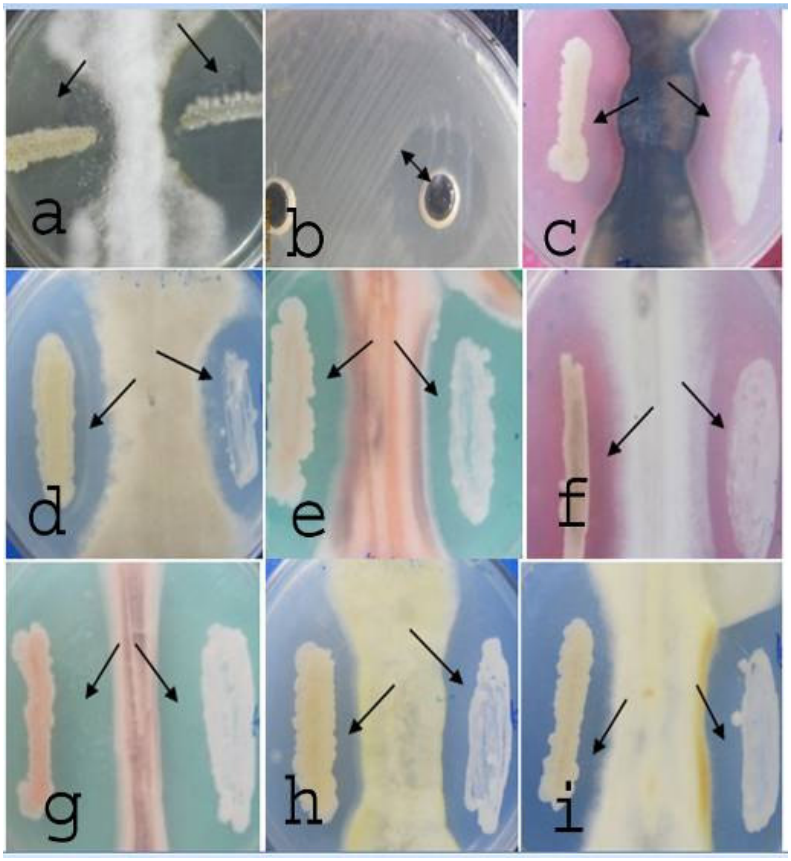

Figure 1. Antagonist activity of strain CTS-B19 (Right) and CTS-G24 (Left) against phytopathogens: (a) *Fusarium oxysporum* f. sp. *ciceri;* (b) *Xanthomonas compestris;* (c) *Alternaria alternate;* (d) *Botrytis cinerea;* (e) *Colletotrichum acutatum;* (f) *Fusarium oxysporum* f. sp. *Pisi;* (g) *Verticillium dahlia;* (h) *Aspergillus flavus,* (i) *Fusarium graminearum*. Arrows indicate the zones of inhibition.

DISCUSSION

The rhizosphere is a hot spot of microbial interactions, as exudates released by plant roots are a main food source for microorganisms. Many amongst rhizospheric microorganisms have a neutral effect on the plant, but also attract organisms that exert deleterious or beneficial effects on the plant (Raaijmakers *et al.,* 2009). An effective biological control strain isolated from one region may not perform in the same way in other soil and climatic conditions (Capper and Higgins, 1993; Duffy *et al.,* 1997). Therefore, considering this factor, screening for indigenous strains with various PGP traits was conducted by sampling rhizospheric soils of chickpea which is one of the major pulse grown in north Karnataka region.

Production of chickpea in India and other countries in Asia is severely affected by many plant pathogenic fungi, bacteria, virus, and nematodes which cause diseases such as *Fusarium* wilt, dry root rot, ascochyta blight, collar rot, bacterial blight, filiform virus and dirty root nematode (www.icrisat.org). A survey was conducted during 2010-2011 rabi cropping season to obtain information on the distribution and incidence of chickpea diseases in respect to soil type, cultivar used, and seed treatment in central and southern parts of India. Incidence of wilt and black root rot disease ranged from 9.7-13.8 and 6.6-7.4%, respectively. Among the several constraints affecting the productivity of chickpea, 8-14% loss in the yields are due to

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

Table 1. *In-vitro* antagonistic activity of strains CTS–B19 and CTS–G24 against phytopathogens and Plant Growth Promoting Rhizobacteria.

Test microorganism (MTCC: Microbial Type Culture Collection) (ARS: Agriculture Research Station)	Microbicidal effect of CTS-B19[a]	Microbicidal effect of CTS–G24[a]
Fungi		
Alternaria alternate MTCC No-7202	+++	+++
Alternaria solani MTCC No-2101	+++	+++
Aspergillus flavus MTCC No-277	+++	+++
Aspergillus niger MTCC No-282	+	+
Botrytis cinerea MTCC No-2350	+++	+++
Collectotrichum acutatum MTCC No-1037	+++	+++
Collectotrichum capsici MTCC No-3414	+++	+++
Fusarium graminearum MTCC No-2089	+++	+++
Fusarium oxysporum f. sp ciceri (ARS)	+++	+++
Fusarium oxysporum MTCC No-2480	+++	+++
Macrophomina phaseolina (ARS)	++	++
Rhizoctonia solani MTCC No-4634	+++	++
Verticillum dahliae MTCC No-6954	+++	+++
Bacteria		
Azotobacter sp. (ARS)	< 5 mm	NZI
Bacillus subtilus MTCC No-441	NZI	NZI
Bacillus thurengenesis (ARS)	NZI	NZI
Erwinia sp. MTCC No-6720	20 mm	18 mm
Pseudomonas syringae MTCC No-1604	18 mm	11 mm
Rhizobium sp. (ARS)	< 5 mm	NZI
Xanthomonas compestris MTCC No-2286	19 mm	11 mm

[a] +++: Strongly antagonistic; ++: Moderately antagonistic; +: Weakly antagonistic, NZI: No Zone of Inhibition. The number with millimeter (mm) as units indicates the zone of inhibition in diameter.

Table 2. Plant growth promoting characteristics of the two potential strains, CTS-B19. and CTS-G24.

PGP characteristics	*P. polymyxa* (CTS-B19)[a]	*B. subtilis* (CTS-G24)[a]
Proteolytic activity	++	+
Chitinolytic activity	++	++
β–1,3–glucanase activity	++	++
Cellulase activity	++	++
Siderophore production	–	+
HCN production	–	–
Catalase production	–	+
IAA production	–	–
Phosphate solubilization	+	–
Ammonia production	+	+

[a] +: Positive; –: Negative; ++: Good zone of hydrolysis, +: Moderate zone of hydrolysis.

wilt and dry root rot diseases caused by *F. oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola* even after the seed was treated with fungicide (Raju *et al.,*2013). Wilt and dry root rot are currently highly distributed in all surveyed chickpea growing areas of central and southern parts of India (Raju *et al.,* 2013).

Amongst isolated rhizobacterial strains, only two isolates, namely, CTS-B19 and CTS-G24 (Identified as *Paenibacillus polymyxa* and *Bacillus subtilis,* respectively) exhibited clear antagonistic activity when initially checked against wilt and dry root rot caused by two fungi.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

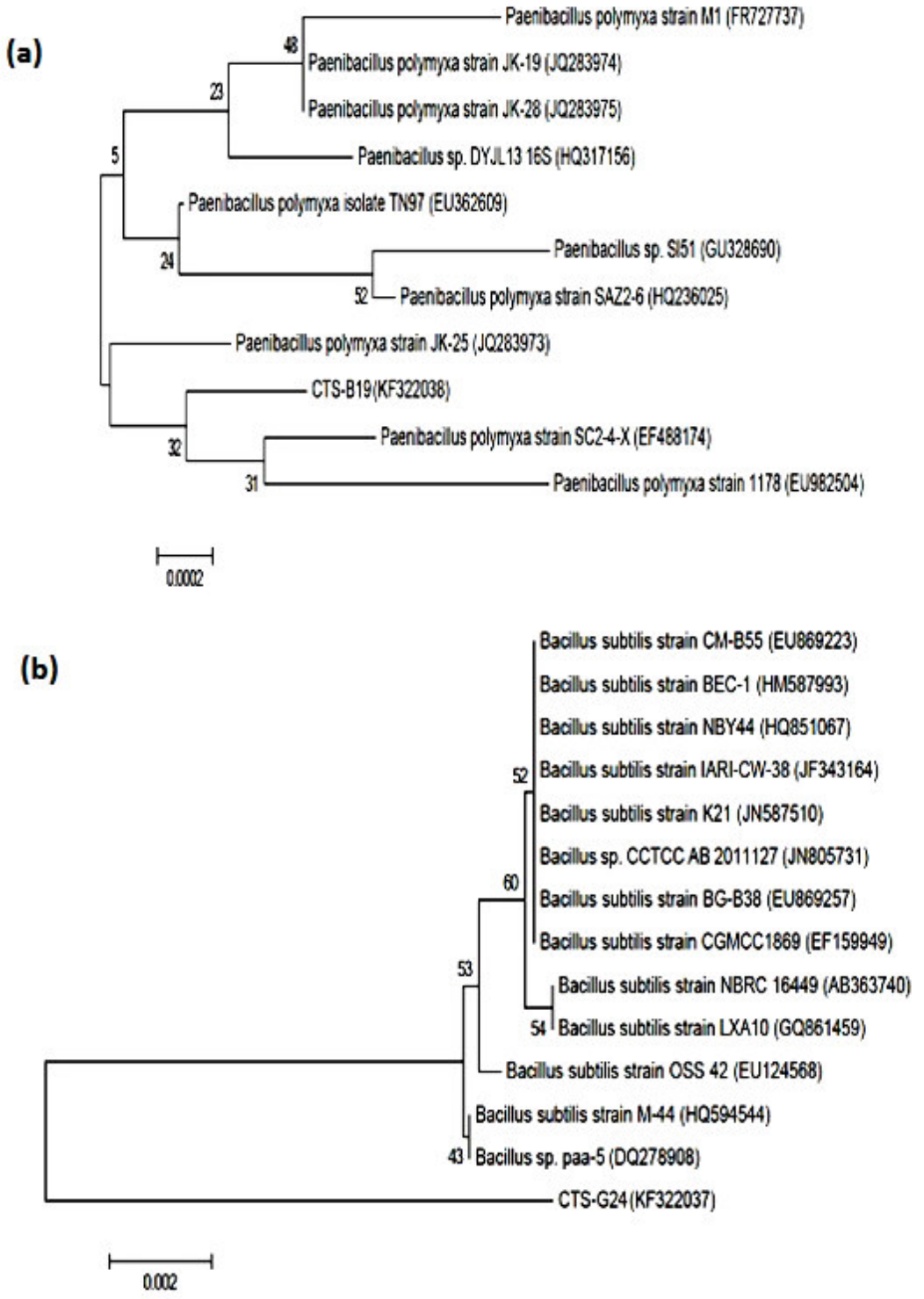

Figure 2. 16 S rDNA-based phylogenetic tree of antagonistic bacteria constructed by MEGA version 5: (a) *Paenibacillus polymyxa* CTS–B19 and, (b) *Bacillus subtilis* CTS–G24.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

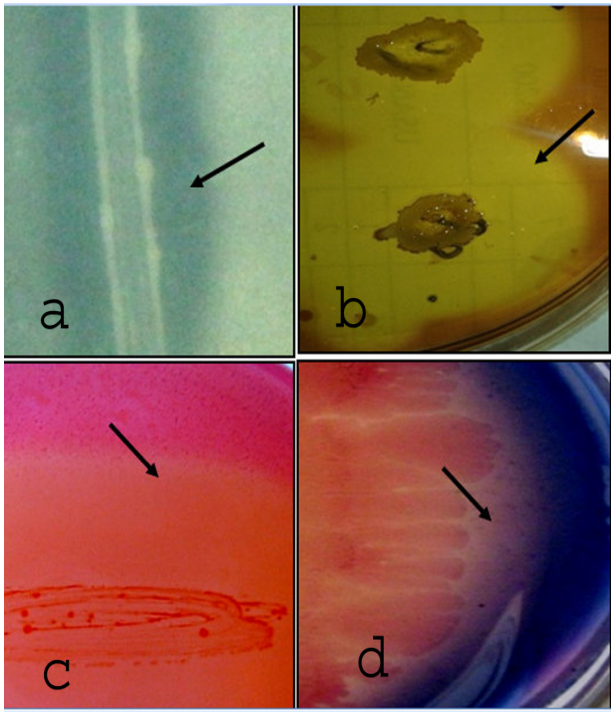

Figure 3- Detection of antagonistic traits **(a)** Proteolytic activity on SM agar, **b)** β–1,3–glucanase activity on MM9 agar supplemented with Laminarin, **c)** Cellulase activity on CMC agar and., **d)** Siderophore production on CAS agar; Arrows indicate the zones of hydrolysis **(a-c)** and formation of orange halo around blue CAS-Fe (III) complex **(d)**.

Later, these two isolates showed potential inhibitory effects against other fungal pathogens tested on PDA medium (Figure 1 and Table 1). Similarly, Dijksterhuis *et al*. (1999) reported clear antagonistic activity of *Paenibacillus polymyxa,* against *Fusarium oxysporum* in *in vitro* experiments. *Bacillus subtilis*, which is ubiquitous in soil, are amongst beneficial rhizobacterium that can promote plant growth and protect against fungal pathogen attack (Emmert and Handelsman, 1999).

So far, reports on natural biological agents against phytopathogens have revealed that *Bacillus* spp. have emerged as potential antiphytopathogenic and PGP agent. From this study, it could be concluded that, both the *Bacillus* strains CTS-B19 and CTS-G24 are positive for chitinase, *β*-1,3-glucanase, protease and cellulase activities. Singh *et al*. (2013) reported that chitinase producing strain *Lysinibacillus fusiformis* B-CM18, isolated from chickpea rhizosphere, exhibited *in vitro* antifungal activity against a wide range of fungal plant pathogens. Strain B-CM18 was also found to produce several PGP activities (Singh *et al*., 2013). Another enzyme, i.e. *β*-1,3-glucanase, significantly contributed to biocontrol activities of *Lysobacter enzymogenes* C3 (Palumbo *et al*., 2005). The fungal wall components such as chitin, *β*-1,3-glucan, mannan, cellulose, and proteins (Adams, 2004), may induce the lytic enzymes, thus showing antagonistic activities. The lysis of cell walls leads to leakage of cell contents and collapse. Dijksterhuis *et al*. (1999) showed that *P. polymyxa* antagonized the plant pathogenic fungus *F. oxysporum* in liquid medium by means of an interaction process in which the presence of living bacteria is a prerequisite for continuous suppression of fungal growth. Interaction of *F. oxysporum* and *P. polymyxa* starts with polar attachment of bacteria to the fungal hyphae followed by the formation of a large

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

cluster of non-motile cells embedded in an extracellular matrix in which the bacteria develop endospores. Chitinases may act synergistically with enzymes such as proteinases and *β*-1,3-glucanases, Both isolates, CTS-B19 and CTS-G24 produced chitinase and β-1, 3- glucanase (Enzymes strictly involved in fungal cell wall lysis) which may act synergistically in degrading fungal cell wall thus achieving biocontrol of pathogenic fungi. *In vivo,* chitinases and *β*-1,3-glucanases degrade fungal cell wall and insect peritrophic membranes, leading to cell fluid loss following osmotic unbalance and killing by dehydration (Bishop *et al.,* 2000). This mode of action reveals the enormous biotechnological potential of these enzymes in agribusiness.

In this study, antibiotic production was not evaluated, however, the cell free filters, when examined initially against *Xanthomonas compestris,* indicated the possibility of an antibiotic mechanism of action (Figure 1). *P. polymyxa* CTS-B19 and *B. subtilis* CTS-G24 showed higher inhibitory activity against phytopathogenic Gram-negative bacteria than non-pathogenic Gram-positive and N_2 fixing bacteria (Table 1). Especially *B. subtilis* CTS-G24 was not antagonistic against N_2 fixing bacteria. This character of rhizobacterial strains, in future, may help in formulating the strains along other PGPRs. Siddiqui *et al.* (2007) found that the inoculation of *P. polymyxa* alone or together with *Rhizobium* increased lentil plant growth both in *Meloidogyne javanica*-inoculated and uninoculated plants.

Bacillus subtilis CTS-G24 was siderophorogenic bacteria isolated from rhizospheric soil sampled from Gulbarga region. Siderophores absorb and accumulate the soluble iron available in the rhizosphere which creates depletion in micronutrients for the pathogenic microorganisms. PGPRs produce siderophores with stronger Fe^{+3} affinity uptake systems than the siderophores produced by deleterious rhizosphere microorganisms, which in turn leads to iron unavailability for the latter microorganisms (Schwyn and Neilands, 1987). It has earlier been observed that slow growing cultures make smaller haloes on siderophore agar plates than fast growing cultures (Schwyn and Neilands, 1987).

The strain *P. polymyxa* CTS-B19 showed positive for the solubilization of tri-calcium phosphate (Ca_3 $(PO_4)_2$) by formation of visible dissolution halo on Pikovaskya's agar. Phosphorous deficiency, a major constraint to crop production, is present in the soil as insoluble forms that cannot be absorbed readily by plants. Microorganisms have the ability to solubilize the insoluble phosphates and maintain the soil health and quality (Richardson *et al.,* 2001). The production of ammonia by both tested strains was another important PGP trait. The molecular nitrogen (N_2) is transformed to ammonium by bacteria in some plant cells and transfer ammonium from the bacteria to the plant cytoplasm (Lugtenberg and Kamilova, 2009).

Only *Bacillus subtilis* CTS-G24 can produce catalase, which is considered as very important enzyme in protecting the cell from oxidative damage by reactive oxygen species. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical, and chemical stress, thus, enabling the organism to survive and adapt even under stress conditions. Our findings, based on survey of literatures, reveal that the multiple PGP activities by native strains of India are less commonly explored.

CONCLUSIONS

Effective biocontrol of plant disease requires an intricate array of interactions. Present study revealed that the antagonistic action exerted due to lytic enzymes, siderophores and certain secondary metabolites produced by *Bacillus subtilis* CTS-G24 seem to have a good cumulative effect. However*, Paenibacillus polymyxa* CTS-B19 showed antiphytopathogenic characteristics and additional PGP traits such as phosphate solubilization. Further research

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

by using consortia of both antagonistic rhizobacterial isolates as biocontrol agents instead of a single one, may lead to development of a rational biocontrol agent for sustainable agriculture.

ACKNOWLEDGEMENTS

Authors would like to acknowledge, Dr. S. K. Jaya Laxmi, Faculty at College of Agriculture, Gulbarga, for kindly providing pure cultures of bioinoculants and fungal phytopathogens. Authors are also thankful to Department of Microbiology, Gulbarga University, Gulbarga, for providing the facilities to carry out the present investigation.

REFERENCES

1. Adams, D. J. 2004. Fungal Cell Wall Chitinase and Glucanases: Mini-Review. *Microbiol.*, **150**: 2029-2035.
2. Bashan, Y. 1998. Inoculants of Plant Growth-promoting Bacteria for Use in Agriculture. *Biotechnol. Adv.*, **16**: 729-770.
3. Bishop, J. G., Dean, A. M. and Mitchell-Olds, T. 2000. Rapid Evolution in Plant Chitinases: Molecular Targets of Selection in Plant-pathogen Co-evolution. *Proc. Natl. Acad. Sci.* USA, **97**: 5322–5327.
4. Brick, J. M., Bostock, R. M. and Silverstone, S. E. 1991. Rapid *In situ* Assay for Indole Acetic Acid Production by Bacteria Immobilized on Nitrocellulose Membrane. *Appl. Environ. Microbio.*, **57**: 535–538.
5. Bruehl, G. W. 1987. *Soilborne Plant Pathogens*. Macmillan, New York.
6. Capper, A. L. and Higgins, K. P. 1993. Application of *Pseudomonas fluorescens* Isolates to Wheat as Potential Biological Control Agents against Take-all. *Plant Pathol.*, **42**: 560–567.
7. Cappuccino, J. C. and Sherman, N. 2010. *Microbiology*: *A Laboratory Manual*. Ninth Edition, Cummings Publishing, Benjamin, CA.
8. Cattelan, A. Z., Hartal, P. G. and Fuhrmann, J. J. 1990. Screening for Plant Growth-promoting Rhizobacteria to Promote Early Soybean Growth. *Soil Sc. Am. J.,* **43**: 1670–1680.
9. Dijksterhuis, J., Sanders, M., Gorris, L. G. M. and Smid, E. J. 1999. Antibiosis Plays a Role in the Context of Direct Interaction during Antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. *J. Appl. Microbiol.*, **86**: 13–21.
10. Duffy, B. K., Ownley, B. H. and Weller, D. M. 1997. Soil Chemical and Physical Properties Associated with Suppression of Take-all of Wheat by *Trichoderma koningii*. *Phytopathol.*, **87**: 1118–1124.
11. El-Bendary, M. A. 2006. *Bacillus thuringiensis* and *Bacillus sphaericus* Biopesticides Production. *J. Basic Microbiol.*, **46**: 158–170.
12. Emmert, E. A. B. and Handelsman, J. 1999. Biocontrol of Plant Disease: A Gram-positive Perspective. *FEMS Microbiol. Lett.*, **171**: 1–9.
13. Felsenstein, J. 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, **39**: 783-791.
14. Foldes, T., Banhegyi, I., Herpai, Z., Varga, L. and Szigeti, J. 2000. Isolation of *Bacillus* Strains from the Rhizosphere of Cereals and *In vitro* Screening for Antagonism against Phytopathogenic, Food-borne Pathogenic and Spoilage Microorganisms. *J. Appl. Microbiol.*, **89**: 840–846.
15. Fritze, D. 2004. Taxonomy of the Genus *Bacillus* and Related Genera: The Serobic Endospore-forming Bacteria. *Phytopathol.,* **94**: 1245-1248.
16. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. 1994. *Bergey's Manual of Determinative Bacteriology*. 9th Edition, Williams and Wi1kins, Ba1timore, MD.
17. Jo, H. and Stabb, E. V. 1996. Biocontrol of Soil-borne Plant Pathogens. *Plant Cell*, **8(10)**: 1855－1869.
18. Jordan, V., Guilhem, D., Marie, L. B., Bruno, T., Yvan, M. L., Daniel, M., Laurent L, Florence, W. D., and Claire P. C.,, 2013. Plant Growth-promoting Rhizobacteria and Root System Functioning. *Frontier. Plant Sci.,* **4**: 356.
19. Jukes, T. H. and Cantor, C. R. 1969. *Evolution of Protein Molecules: Mammalian Protein Metabolism*. Academic Press, New York, PP. 21-132.
20. Karlidag, H., Esitken, A., Turan, M., and Sahin, F. 2007. Effects of Root Inoculation of Plant Growth Promoting Rhizobacteria (PGPR) on Yield, Growth and Nutrient Element Contents of Leaves of Apple. *Sci. Horticult.*, **114**: 16-20.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

21. Lee, K. J., Kamala-Kannan, S., Sub, H. S., Seong, C. K. and Lee, G. W. 2008. Biological Control of Phytophthora Blight in Red Pepper (*Capsicum annuum* L.) Using *Bacillus subtilis*. *World J. Microbiol. Biotechnol*., **24**: 1139-1145.

22. Lorck, H. 1948. Production of Hydrocyanic Acid by Bacteria. *Physiol. Plant*, **1**: 142–146.

23. Lugtenberg, B. and Kamilova, F. 2009. Plant-growth-promoting Rhizobacteria. *Annu. Rev. Microbiol*., **63**: 541–556.

24. Pal, M. 1998. Diseases of Pulse Crop, Their Relative Importance and Management. *J. Mycol. Plant Pathol*., **28 (2)**: 114-122.

25. Palumbo, J. D., Yuen, G. Y., Jochum, C. C., Tatum, K. and Kobayashi, D. Y. 2005. Mutagenesis of *β-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. *Phytopathol.*, **95**: 701-707.

26. Pattern, C. L. and Glick, B. R. 1996. Bacterial Biosynthesis of Indole-3-acetic Acid. *Can. J. Microbiol*., **42**: 207–220.

27. Perez, C., Pauli, M. and Bazerque, P. 1990. An Antibacterial Assay by Agar Well Diffusion Method. *Acta Bio. Et. Med. Exp.,* **15**: 113-115.

28. Pikovaskya, R. I. 1948. Mobilization of Phosphorus in Soil in Connection with the Vital Activity of Microbial Species. *Microbiol.*, **17**: 362–370.

29. Powell, K. A. and Jutsum, A. R. 1993. Technical and Commercial Aspects of Biological Control Products. *Pestic. Sci*., **37**: 315–321.

30. Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C. and Moenne-Loccoz, Y. 2009. The Rhizosphere: A Playground and Battlefield for Soilborne Pathogens and Beneficial Microorganisms. *Plant Soil*, **321**: 341–361.

31. Raju, G., Mamta, S., Rameshwar, T. and Suresh, P. 2013. Occurrence and Distribution of Chickpea Diseases in Central and Southern Parts of India. *Am. J. Plant Sci.*, **4**: 940-944.

32. Richardson, A. E., Hadobas, P. A., Hayes, J. E., O'Hara, J. E. and Simpson, R. J. 2001. Utilization of Phosphorus by Pasture Plants Supplied with Myoinositol Hexaphosphate Is Enhanced by the Presence of Soil Microorganisms. *Plant Soil*, **229**: 47-56.

33. Prashar, P., Kapoor, N., and Sachdeva, S. 2013. Isolation and Characterization of *Bacillus sp* with *In-vitro* Antagonistic Activity against *Fusarium oxysporum* from Rhizosphere of Tomato. *J. Agr. Sci. Tech.,* **15**: 1501-1512

34. Saitou, N. and Nei, M. 1987. The Neighbour-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.*, **4:** 406-425.

35. Schwyn, B. and Neilands, J. B. 1987. Universal Chemical Assay for the Detection and Determination of Siderophores. *Anal. Biochem*., **160(1)**: 47–56.

36. Siddiqui, Z. A., Baghel, G. and Akhtar, M. S. 2007. Biocontrol of *Meloidogyne javanica* by *Rhizobium* and Plant Growth-promoting Rhizobacteria on Lentil. *World J. Microbiol. Biotechnol*., **23**: 435–441.

37. Singh, R. K., Kumar, D. P., Solanki, M. K., Singh, P., Srivastva, A. K., Kumar S, Kashyap P. L., Saxena A. K., Singhal P. K., Arora D. K.,, 2013. Optimization of Media Components for Chitinase Production by Chickpea Rhizosphere Associated *Lysinibacillus fusiformis* B-CM18. *J. Basic Microbiol*., **53**: 451–460.

38. Smibert, R. M. and Krieg N. R. 1994 *Phenotypic Characterization. Methods for General and Molecular Biology*. American Society for Microbiology, Washington, DC, PP. 607–654.

39. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol*., **28**: 2731-2739.

40. Teather, R. M. and Wood, P. J. 1982. Use of Congo Red Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rumen. *Appl. Environ. Microbiol*., **43**: 777–780.

41. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-specific Gap Penalties and Weight Matrix Choice. *Nucleic Acid. Res*., **22**: 4673-4680.

42. Whipps, J. M. 1997. Ecological Considerations Involved in Commercial Development of Biological Control Agents for Soil-borne Diseases. Modern Soil Microbiology. Marcel Dekker, New York, PP. 525–546.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]

خاصیت های ضد بیماریزایی گیاهی و بهبود دهنده رشد گیاه در ریسه های باسیلوس(*Bacillus*) جدا شده از خاک ریزوسفر نخود

س. پتیل، س. ت. شیواناوار، م. س. بهمارادی، و س. م. گاداد

چکیده

هدف این پژوهش غربال کردن ریسه هایی بومی از باسیلوس در ریزوسفر بود که خاصیت های ضد بیماریزایی گیاهی و بهبود دهنده رشد گیاه داشته باشند. در اجرای پژوهش، جدایه های CTS-B19 وCTS-G24 در ابتدا فعالیت های آنتاگونیستی قابل توجهی علیه *Fusarium oxysporum* f. sp. *ciceri* و *Rhizoctonia bataticola*و سپس بر علیه گروهی از بیمارگرهای گیاهی بروز دادند. در تجزیه جزیی توالی *16 S rRNA* ،جدایه های CTS-B19 و CTS-G24 با*Paenibacillus polymyxa* و*Bacillus subtilis* ، ۹۹٪ همسانی نشان دادند و توالی ها در *GenBank* به ترتیب با شماره های *KF322038* و *KF322037*ثبت شدند. ردیابی درون شیشه ای آنزیم های تخریب کننده دیواره قارچ ها آشکار ساخت که هر دو جدایه مواد *chitinases, β-1,3-glucanases*، پروتئاز، و سلولاز تولید می کردند. همچنین، در حالی که فعالیت مواد آهن بر(سیدروفور) و کاتالیز(*catalase*) فقط در *Bacillus subtilis (CTS-G24)* مشاهده شد، هر دو ریسه نتیجه مثبتی در مورد تولید آمونیاک در درون شیشه نشان دادند. نیز، ریسه *CTS-B19* توانست فسفات ها را محلول کند. آنزیم های لیز کننده(*Lytic*) و سیدروفور های تولید شده توسط *Bacillus subtilis*را می توان مستعدا به عنوان خواص *ضد* بیماریزایی گیاهی که در جلوگیری از رشد قارچ ها دخالت دارند در نظر گرفت.اما هر دو ریسه فعالیت ناچیزی بر علیه دیگر باکتری های بهبود دهنده رشد گیاه نشان دادند. افزون بر فعالیت آنتاگونیستی، خواص بهبود دهنده رشد گیاه که توسط این عوامل کنترل بیولوژیکی بروز داده شد می تواند بازدارنده امراض گیاهی باشد و ممکن است در کشاورزی آنها را جایگزین کار برد آفتکش ها و کودهای شیمیایی کرد.

[DOR: 20.1001.1.16807073.2015.17.5.22.1]