Identification of plant growth promoting *Rhizosphere Bacillus* sp. WG4 antagonistic to *Pythium myriotylum* and its enhanced antifungal effect in association with *Trichoderma*

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Abstract

Among the various rhizospheric bacterial isolates screened from Westren Ghat regions of Kerala, a *Bacillus* sp. with both antagonistic activity to *Pythium myriotylum* and plant growth promoting properties was identified. Scanning electron microscopic analysis revealed significant ultrastructural changes of hyphae and spores of *P. myriotylum* upon treatment with *Bacillus* sp. WG4. For the structural characterization of antagonistic compound in the crude extract, GC MS analysis was carried out and this has resulted in the identification of presence of pyrrolo [1,2- a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) as basis of antifungal activity. The *in vivo* antifungal activity of *Bacillus* strain was analysed by pre-inoculation of this to ginger rhizome. Here the presence of *Bacillus* strain was found to provide effective protection to ginger rhizome upon inoculation with *Pythium myriotylum*. Very interestingly, when the *Bacillus* strain was checked for its synergistic antifungal activity with commercially available biocontrol agent *Trichoderma*, the combination was found to have increased antifungal activity. Due to its antifungal and growth enhancement effects, the isolated strain can be of promising application as antifungal plant probiotic agent alone or in combination with other agents like *Trichoderma*.

Keywords: *Bacillus* sp., WG4, biocontrol, rhizosphere, plant growth promoting activity, *Pythium myriotylum*.

1. Introduction

Although several bacterial species are known for their antifungal activity, *Bacillus* sp., are highly impressive due to their endospore formation (Schaeffer 1969), extreme tolerance to heat and desiccation (Handelsman *et al.*, 1990) and also due to their potential to produce a wide range of antibiotics (Katz and Demain 1977), toxins, hydrolases and lipopeptides (Lamsal *et al.*, 2012). They have also been iden-

tified to have a wide range of distribution as soil-inhabiting, epiphytic, endophytic (Mora *et al.*, 2014) or rhizosphere organisms and due to this they have been reported as highly efficient biocontrol agents for seed treatment applications (Soylu *et al.*, 2005). Their safety and ability to produce compounds beneficial for agronomical purpose makes *Bacillus* sp. a highly studied organism (Earl *et al.*, 2008).

Phytopathogenic fungi including oomycetes are of global concern to food security, as they are difficult to control due to their genetic plasticity and population variability. In addition to this, they can have severe economic impact as in the case of crops like ginger, where the biotic stress due to *P. myriotylum* causes significant reduction in yield and thereby annual export. Evolution of pesticide resistant pathogens, environmental toxicity of pesticides and the difficulty in identifying disease resistant plant varieties makes the common procedures ineffective on a large scale. All these make it highly demanding to investigate rhizobacteria from various environments for plant protective properties to explore its agricultural applications. Fungal biological control agents like *Trichoderma* are among the most successful tools applied against plant pathogenic fungi, especially oomycetes. They are also reported to play an important role in sustainable agriculture because of its additional role in increase of plant growth and yield (Seidl *et al.*, 2005). So identifying promising rhizobacteria will pave the way to enhance the effect of these organisms by forming microbial consortia.

Current study was targetted towards identification of *Bacillus* sp. with strong activity to inhibit the growth of *P. myriotylum*. *Bacillus* sp. which was isolated and screened for antifungal properties was characterized by 16S rDNA sequence analysis. The isolated organism was found to have property to alter fungal morphology as confirmed by SEM analysis. Its enhanced activity in association with *Trichoderma* and the presence of additional plant growth enhancement features provide opportunities to develop compatible biocontrol formulations to explore its field applications.

2. Materials and Methods

2.1. *In vitro* antagonistic studies

Among the various rhizobacteria screened from Western Ghat regions, one isolate from *Curculigo orchioideis*

was found to have *in vitro* antagonistic activity towards *Pythium myriotylum*. For *in vitro* antagonistic studies, the rhizobacteria was streaked on the edge of the PDA in a petridish, and a mycelial plug (6 mm diameter) of the pathogen was inoculated at the centre. Growth inhibition was evaluated by analysing the inhibition zones from the bacterial colony to the edge of the spreading fungal mycelium after incubation for 5 days at 25 °C as per previously described methods (Chen *et al.*, 2008). For optimal production of the antifungal compound, the selected organism was cultured in various media like Nutrient broth, *Sabouraud's dextrose* broth, Potato Dextrose broth, Sucrose broth and Solid Rice media. For this the organism was incubated in 30 mL seed medium for 12 h with 200 rpm, and from this 10% inoculum was added to 100 mL fermentation medium followed by incubation at 30 °C for 10 days. The culture supernatant was extracted with ethyl acetate and the extract was concentrated in a rotary vacuum evaporator.

2.2. Extraction of culture supernatants

Extracts were prepared from 3L culture of cell free supernatant with ethyl acetate followed by evaporation to dryness. The residue was dissolved in methanol and agar well diffusion assay was used for determination of antagonistic activity of the extract. For this, mycelial mat (5 mm in diameter) of *Pythium myriotylum* was placed at the center of the plate containing PDA medium and a well of 5 mm diameter was made on the agar with a sterile steel borer at the edge of the plate and 60 µL of the extract was poured into the well followed by incubation for 72 hr at 27 °C.

2.3. Screening of rhizosphere bacteria for plant growth promoting properties

The selected isolate was screened for 1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity by

using DF minimal media. For indole-3-acetic acid (IAA) production analysis, the bacteria was cultured in nutrient broth with 0.2% tryptophan and IAA was analysed by Salkowski method. Phosphate (P) solubilization assay was carried out by inoculating on Pikovskaya medium and siderophore production was analysed using modified blue agar CAS medium containing chrome azurol S (CAS) and hexa decyl trimethyl ammonium bromide (HDTMA) as indicators (Jasim et al., 2013). Ammonia production was determined as described by (Ahmad et al., 2008) and HCN production was analysed as described by (Chakraborty et al., 2009) and nitrogen fixation was checked on Jensen's media (Jimtha et al., 2014).

2.4. Identification of the bacterial isolate

Identification of the selected bacterial isolate was done by biochemical and 16S rDNA based sequencing based method. Genomic DNA was isolated from the bacterial isolate and was used as template for PCR. Primers used for the amplification of part of 16S rDNA were 16SF (50-AgA gTTTgA TCM Tgg CTC-30) and 16SR (50-AAg gAg gTg WTCCAR CC-30) and were selected based on the previous reports of Chun and Goodfellow (Chun and Goodfellow 1995) (t>580@). PCR was carried out in a 50 µL reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 µM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a MycyclerTM (Bio-Rad, USA) with the initial denaturation at 94 °C for 3 min, cyclic denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 2 min with a final extension of 7 min at 72 °C. The PCR product was checked by agarose gel electrophoresis, purified and was further subjected to sequencing in Scigenome, Ernakulam, Kerala, India. The sequence data was checked by BLAST analysis

(Zhang et al., 2000). The phylogenetic analysis of the 16SrDNA sequences of the isolates obtained in the study was conducted with MEGA 5 using neighbor-joining method with 1,000 bootstrap replicates (Tamura et al., 2011).

2.5. Morphological changes of *Pythium myriotylum* induced by the selected rhizobacteria

For the detection of morphological and ultra structural changes of *Pythium myriotylum* due to the effect of the bacterial isolate, dual culture method was used as explained above. Both the Light Microscopy (Chaurasia et al., 2005) and SEM analysis were carried out for *Pythium myriotylum* from the dual culture plate along with *Pythium myriotylum* alone from PDA as control. Sample preparation and SEM observation were done as described by previous methods (Furlaneto et al., 2012). Hyphal samples of *Pythium myriotylum* were excised and were fixed in 2.5% glutaraldehyde at 4 °C for 2 h, washed in phosphate buffered saline (PBS) for 4 times (15 min each), dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100%) (10 min each) air dried and viewed under SEM.

2.6. Enhanced antiungal effect of selected isolate in association with *Trichoderma*

The selected bacterial isolate and commercially available *Trichoderma* strains were analysed for their combined activity against *Pythium myriotylum*. For this, the extracts of these strains were made using the same methodology described above. Then the effect of individual applications (60 µL) and the combined applications (30 µL each) of extract were evaluated by well diffusion method with incubation at 24 °C in dark for 4 days. The *Pythium myriotylum* which was simultaneously grown was used as control (Ruano-Rosa et al., 2013).

2.7. GC-MS analysis of crude cell extract

Identification of the antimicrobial metabolites was done by Gas Chromatography-Mass Spectrometry (GC-MS) analysis with Agilent Technologies-7890 GC System, 5975 C inert MSD. 1 μ L of sample solution was injected into the GC system provided with 30 m X 250 μ m X 0.25 μ m Agilent 190913-433 column at 325 $^{\circ}$ C and Helium was used as a carrier gas at a flow rate of 1.1 mL/min. For GC-MS the GC oven was held at 100 $^{\circ}$ C and then ramped from 100 $^{\circ}$ C to 250 $^{\circ}$ C at 5 $^{\circ}$ C/min. The chromatogram and mass spectra were recorded and analyzed. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum of NIST (National Institute for Standards and Technology) library of the corresponding organic compound.

2.8. Control of *Pythium myriotylum* infection on Ginger rhizome using *Bacillus* sp.

To analyze the effect of *Bacillus* sp. on ginger rhizome, surface sterilized ginger was treated with sterile nutrient

broth (T1), distilled water (T2) and overnight culture of *Bacillus* sp. (T3). Each set contained three pieces of the ginger taken in a petri dish which was soaked in T1, T2 and T3 for 30 minutes and inoculated with *P. myriotylum*. These rhizomes were observed for *Pythium myriotylum* infection for 7 days.

3. Results

3.1. Isolation and characterization of potential bacterial antagonists

Among the various rhizosphere isolates purified from Western Ghat, the isolate from *Curculigo orchoides* produced prominent zone of inhibition against *Pythium myriotylum* (Figure 1).

The zones of inhibition against *Pythium myriotylum* were particularly well developed with about 44% inhibition. Extracts prepared from 3 L of nutrient broth culture was used to check the antifungal activity. Among the five media used, extract from nutrient broth showed excellent growth inhibition towards *Pythium myriotylum*.

Figure 1. Antagonistic effect of *Bacillus* sp. WG4 against *Pythium myriotylum* along with its control, (a) Dual culture assay, (b) activity of crude extract.

3.2. Identification of the Bacterial Isolate and Screening of multiple plant growth promoting potentials

Plant growth promoting properties like Indole acetic acid, ACC Deaminase, Phosphate solubilization and HCN were found to be positive for the selected

isolate (Figure 2). The selected isolate was identified biochemically and by molecular analysis (Figure 3) as *Bacillus* sp. and it showed 99% identity with *Bacillus cereus* in BLAST analysis (Figure 4). The sequences were deposited to NCBI (1913842).

Figure 2. Plant growth promoting properties exhibited by the selected isolate. A: Phosphate solubilization, B: ACC Deaminase production, C: IAA production, D: HCN production along with its uninoculated control.

Figure 3. PCR amplification of 16S rDNA-(Lane 1)- Lambda DNA/EcoR1/Hind III Double digest marker (Lane 2)- Negative Control, (Lane 3) Rhizosphere isolate.

3.3. Effect of crude extract on mycelium growth of *Pythium myriotylum*

Dual culture showed inhibitory effect of isolated organism on growth and morphology of *P. myriotylum* as it could not extend as normal and were distorted and granulated, whereas the mycelia from the control

sample were normal and healthy. A crescent inhibition line was formed around the extract containing sample (Figure 5), while the mycelia close to the control sample covered the well. Disintegration of mycelia and hyphae, lysis of hyphae and suppression of formation of new oogonia were also observed in light and scanning electron microscopic images.

Figure 4 Phylogenetic tree showing the relationship of the isolate to closely related bacteria. The tree was obtained using the neighbour-joining method. The numbers at branching points refer to bootstrap values, based on 1000 replicates.

Figure 5.1. Light Microscopic images of *Pythium myriotylum* mycelia showing morphological distortion in dual culture. (a) Normal mycelial morphology of *Pythium myriotylum*; (b) Altered mycelial morphology of *Pythium myriotylum* in presence of antagonistic *Bacillus* sp. WG4.

Figure 5.2. Scanning Electron Microscopic images of *Pythium myriotylum* mycelia showing morphological distortion in dual culture

(a) Normal mycelial morphology of *Pythium myriotylum*; (b) Altered mycelial morphology of *Pythium myriotylum* in presence of antagonistic *Bacillus* sp. WG4

3.4. Effect of bacterial and Trichoderma extract on Pythium myriotylum growth

A morphological interaction such as contact inhibition has already been reported between *Pythium myriotylum* and commercial biocontrol agent *Tricho-*

derma. Interestingly, the *Bacillus* extract significantly reduced the *in vitro* growth of *Pythium myriotylum* alone as well as in association with *Trichoderma* extract. But the combination, significantly reduced *Pythium myriotylum* growth relative to their independent effect due to synergistic action (Figure 6).

Figure 6. Antiungal activity of crude extracts on the radial growth of *Pythium myriotylum*
(a) Effect of crude extract of *Trichoderma* sp., (b) Effect of crude extract of *Bacillus* sp. WG4
(c) combined effect of *Trichoderma* and *Bacillus* sp. WG4 extracts.

3.5. GC-MS analysis of crude cell extract

GC-MS analysis of crude extract of *Bacillus* sp. revealed the presence of pyrrolo [1,2- a] pyrazine-1,4-

dione, hexahydro-3-(phenylmethyl) with a molecular weight of 244.2 at a retention time of 30.358 min with 98% similarity with NIST library data (Figure 7).

Figure 7. GC-MS (a) chromatogram and (b) spectrum of pyrrolo [1, 2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) present in the crude extract of *Bacillus* sp. WG4

3.6. In vivo rhizome protective effect of *Bacillus* sp. on ginger rhizome

The *Bacillus* sp. was pre-inoculated on to ginger rhizome which was further challenged with *Pythium myriotylum* to study the inhibitory effect of the bacteria on colonization by fungal pathogen. Very inter-

estingly, after 96 h of incubation only those rhizome pieces which had been pre-treated with nutrient broth as control showed the presence of mycelial growth of *Pythium myriotylum*. At the same time, the rhizome pieces which had been pre-incubated with *Bacillus* sp. culture were found to have inhibition of growth of *Pythium myriotylum* even after 96 h. (Figure 8).

Figure 8. Rhizome inoculation study showing the difference in the infection of *Pythium myriotylum* when inoculated on rhizome pretreated with *Bacillus* sp. WG4 (T1-Nutrient broth control, T2-Distilled water control, T3- *Bacillus* sp. WG4 treated sample).

4. Discussion

Beneficial microorganisms are highly promising for more rational and safe crop management practices. *Bacillus* sp., are important biocontrol agents (Lee and Kim 2016) with potential antifungal activity against a number of plant pathogens (Zaccardelli et al., 2013). In addition to this they also have been reported to have remarkable impact on host plant by increasing the availability of nitrogen and phosphorous and thereby causing nutrient unavailability to phytopathogens (Malviya et al.,

2012). The first commercially available biopesticides prepared from strains of *B. subtilis*, branded as Quantum®, Kodiak® and Epic®, appeared in the US market in 1985. Their success in the control of soil-dwelling phytopathogenic microorganisms has laid the foundation for exploring the application of *Bacillus*-based biopreparations to commercially important crops (Dardanelli et al., 2010; Khabbaz et al., 2015). In the study, *Bacillus* sp., which was isolated from Western Ghat soil was found to have remarkable antagonistic activity against *Pythium myriotylum*.

The broad antifungal activity of *Bacillus* sp., against the selected pathogen was agriculturally important as these pathogens infect plants of great economic importance. *Pythium* species are best known for causing damping-off and seed rot disease that often occurs just after planting as young seedlings emerge. *Pythium* also causes root rots on newly emerged or more mature plants and can also cause soft rots of fleshy fruit. Damping-off disease affects seedlings worldwide. Often, young seedlings are completely destroyed by this pathogen with significant yield reduction. Older plants once emerged might not be significantly affected by *Pythium*, but do show symptoms of root rot. The ethyl acetate extract prepared from culture supernatant of *Bacillus* sp. was shown to retain the activity against *Pythium myriotylum* which confirmed the extracellular production of antifungal metabolites by the selected organism. From previous studies, *Bacillus* species has been reported to have the potential of producing wide array of antibiotics in strain specific manner (Shu-Mei 2012). In a study by Rao (Rao *et al.*, 2013) *Bacillus* strains EU07, QST713 and FZB24, were investigated for their inhibitory effect on *Fusarium* by production of lytic enzymes, cellulases, proteases, 1,4- β -glucanase and hydrolases. There are reports on the isolation and characterization of *Bacillus* sp. WG4 that protects alfalfa from seedling disease caused by *Pytophthora megasperma* (Handelsman *et al.*, 1990). Bipolaris stem rot caused by *B. cactivora* was shown to be controlled by antifungal properties of *Bacillus amyloliquefaciens* species (Bae *et al.*, 2013). In recent study a novel bacterium BH072 *Bacillus amyloliquefaciens* isolated from honey has showed strong antifungal activity against a lot of molds (Zhao *et al.*, 2013). All these support the result of the current study. The additional impressive features with *Bacillus* sp. WG4 isolated in the current study are the plant growth promoting properties.

Rhizosphere Bacillus subtilis was reported to cause structural deformities in pathogenic fungi under *in vitro* culture conditions due to the production of diffusible and volatile antifungal compounds (Chakraborty *et al.*, 2009). In current study, *in vitro* challenges of phytopathogenic fungi with the selected bacterium demonstrated efficient growth-inhibiting alterations in the morphology and structure of *P. myriotylum* by extracellular metabolites of *Bacillus* sp. WG4. Ultrastructural studies of the hyphae revealed the changes in the width and regeneration of its cell walls, hyphal constrictions in the region of the transversal septum and the induction of secondary branching in the fungal cell. The periodical swelling, torsion and formation of bulbs in hyphae from *P. myriotylum* have been previously reported to be linked to the action of antifungal metabolites (Ongena *et al.*, 2004). The results also showed inhibition of conidial germination of *Pythium myriotylum* and is important as inhibition of the pathogen at various stages can have efficient property to control the pathogen (Walker *et al.*, 1998). Synergistic effect of two antagonists combined into a single treatment can have more efficient biocontrol property (Khabbaz and Abbasi 2014). A microbial biological control agent may express contact inhibition against pathogens during their antagonistic activity by weakening or destroying the pathogen or by parasitizing it directly by producing antimicrobial compounds to compete for space and nutrients and by producing enzymes that attack the cell components of the pathogens. In this study, antagonistic effect expressed by the *Trichoderma* spp. in dual culture method might be due to one or combination of all the above mechanisms. Recent works suggest that the combination of several PGPRs could be more effective than individual strains as a horticultural product (Domenech *et al.*, 2006). So the synergistic effect observed in this study can have promising applications.

The antifungal activity of the bioactive fractions collected was checked, and was subjected to GC MS analysis. The MS of bioactive crude extract revealed a peak at a retention time of 30.358 min showing 98% similarity with pyrrolo [1,2- a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) with a molecular weight of 244.2. This novel pyrrolopyrazine alkaloid occurs in many plant-microbe relationships. These compounds have been isolated from the endophytes *Epichloe* species and the *Neotyphodium* sp. of grasses in cold regions and can protect plants against worms and phytopathogens (Faeth 2002; Leuchtmann and Schardl 2005; Malinowski et al., 2005; Zhang et al., 2012). This fungal metabolite consists of a lipophilic pyrrolopyrazine heterocyclic ring structure with a hydrophilic guanidinium side chain. It is related to other diketopiperazine-mycotoxins, such as aspergillic acid, and related pyrazine derivatives are produced by the *Aspergillus* and *Candida* species (Zhang et al., 2012).

Because of the broad biocontrol properties of *Bacillus* sp. WG4 isolated in the study, it was selected for studying its *in vivo* role to control rhizome rot in ginger. Rhizome rot is caused mainly by various species of *Pythium*. No feasible and ecofriendly method is available to protect the ginger plant from rhizome rot disease. Rhizospheric microorganisms capable of producing antimicrobial compounds could be a useful tool to prevent the disease. The *in vivo* antifungal activity of *Bacillus* sp. was analysed by pre-inoculation of this strain with ginger rhizome. Here the presence of *Bacillus* sp. WG4 was found to provide effective protection to ginger rhizome upon inoculation with *Pythium myriotylum*. This opens up novel approaches to exploit rhizospheric microorganisms for biocontrol applications.

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