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Selection of a compatible biocontrol strain mixture based on co-cultivation to control rhizome rot of ginger

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ABSTRACT

Rhizobacteria and *Trichoderma* spp. that were antagonistic against *Fusarium oxysporum* f.sp. *zingiberi* and *Pythium splendens* inciting rhizome rot disease of ginger were identified. *In vitro* assays identified positive interactions for growth, antifungal and chitinase gene expression between antagonistic TEPF-Sungal (*Burkholderia cepacia*) and S2BC-1 (*Bacillus subtilis*) with S17TH (*Trichoderma harzianum*), and *vice versa*. In comparison with the other strains and strain mixtures, one strain mixture, TEPF-Sungal + S17TH, recorded a maximum rhizome production of 84% efficiency with less incidences of yellows and rhizome rot at an 84.2 and 79.7% reduction over the pathogen control, respectively, in a polyhouse with a challenge inoculation with the pathogens. Inoculation of the mixture of biocontrol agents was associated with an increase in known defence gene products such as chitinase etc., so we speculate that these are involved in the mechanism of disease suppression. In field experiments, the strain mixture reduced yellows and rhizome rot to 45.9 and 49.3% over the untreated control, respectively, which is comparatively better than that produced with fungicide (44.3 and 45.3%, respectively). The treatment also produced an increased rhizome yield with an average increase of 60.0% relative to the control.

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1. Introduction

In the biological management of plant diseases, biocontrol agents are usually applied singly to combat a given pathogen. The application of a single biocontrol agent often results in inconsistent field performance because it is less likely to be active in different soil environments, and agricultural ecosystems (Raupach and Kloepper, 1998). The level and consistency of control are greatly enhanced by multiple modes of action, a more stable rhizosphere community and effectiveness over a wider range of environmental conditions (Larkin and Fravel, 1998). Positive and negative interactions between introduced biocontrol agents or between an introduced biocontrol agent and the indigenous microflora can influence their performance in the rhizosphere (de Boer et al., 1999). Combinations of biocontrol agents that utilize fungi and bacteria reduces the inconsistency from using a single biocontrol agent as the combination can protect the plants at different times or under different conditions and occupy different or complementary niches (Larkin and Fravel, 1998). Interestingly, such combinations also reduce the level of the disease-suppressing activity of individual antagonists, resulting in reduction in efficacy of disease control (Miller and May, 1991). The designation of effective strain mixtures is, therefore, essential to exploiting their potential by selecting strains that complement rather than interfere with the antagonistic activity of each other (Lutz et al., 2004).

Rhizome rot of ginger (*Zingiber officinale* Rosc.) is a complex disease often incited by mixed infections with soft rot and yellows caused by species of *Pythium* and *Fusarium*, respectively, and it adversely affects seed rhizomes and crop stands, causing significant yield loss and even complete failure of the crop under favorable environmental conditions. Considering the annual nature of these crops and the high organic matter status of the soil in which they are cultivated, biological control is an ideal option for managing rhizome rot pathogens. The biocontrol of soft rot (Shanmugam et al., 2000) or yellows (Rajan et al., 2002) by cell suspensions has been reported in pot experiments. However, the possible mechanisms of disease suppression have not been assessed, and no reports are available that address rhizome rot caused by mixed infections using carrier-based bio-formulations under field conditions. The success of the biological control of plant diseases

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depends on the availability of effective formulations of biocontrol agents, particularly the strain mixtures and their testing under field conditions. This problem has emphasized the need to select and evaluate carrier-based formulations of compatible strain mixtures of antagonistic microbes to include in the integrated control of rhizome rot of ginger. Combinations of antagonistic fungi and bacteria could be a better option with added advantages. The present study, therefore, aims to identify rhizobacteria and *Trichoderma* spp. that are highly antagonistic against the rhizome rot pathogens of ginger, and to design effective strain mixtures for rhizome rot control in polyhouse and field conditions based on *in vitro* interactions between them on growth, antagonism and chitinase activity, the attributes of a biocontrol agent.

2. Materials and methods

2.1. Fungal and bacterial strains

Fusarium oxysporum f.sp. *zingiberi* (FOZ) isolate GIFOZ-UHF (GenBank Acc. No. GQ121297) and *Pythium splendens* (PS) (Shanmugam et al., 2010) isolate GIPY-UHF A6 (GenBank Acc. No. GQ121298), which cause rhizome rot of ginger, were isolated from diseased rhizome tissues. These strains were characterized, and their pathogenicities were established. The rhizobacterial strains (Table 1) and *Trichoderma* isolates (Table 2) were obtained from the culture repository located in the Plant Pathology Laboratory at the Institute of Himalayan Bioresource Technology (IHBT, Palampur, India).

2.2. Evaluation of microbes for antagonism against rhizome rot pathogens

The rhizobacteria strains were tested *in vitro* for antagonism against the pathogens by dual culture (DC) assays on Potato Dextrose Agar (PDA), as described in Shanmugam et al., (2008a). Similarly, the *Trichoderma* spp. was tested for antagonism by DC and cell free culture (CFC) filtrate assays, as previously described (Shanmugam et al., 2008b). PDA plates inoculated with either pathogen alone were maintained as the control. The rhizobacterial strains and *Trichoderma* spp. showing the greatest inhibition were used for further studies.

2.3. Interactions of antagonistic microbes on their growth and antagonism

Rhizobacteria antagonistic to either of the pathogens were evaluated for their interactions with the antagonistic *Trichoderma* isolates, GITX-Panog (C) and S17TH, selected from the above studies by DC and CFC filtrate assays, as described in subsection 2.2. The CFC filtrate of the rhizobacteria was prepared as described (Vaidya et al., 2004) and evaluated on the growth of S17TH at a 10% v/v concentration. To test the effect of S17TH on the growth of rhizobacteria, the CFC filtrate was incorporated into a nutrient broth at 10% v/v and inoculated separately with bacterial cell suspensions grown for 24 h to a density of 1.5×10^8 cfu ml^{-1}. After an incubation period of 48 h in a rotary shaker, the population density was assessed by observing the optical density of the broth in a colorimeter at 600 nm. The cultures of the rhizobacteria without the filtrate served as the control. An increase or no change in the population density of the treatments over the control was considered a positive interaction. The effect of the CFC filtrates (10% v/v) of either of the microbes on the antifungal efficacy of the co-inoculant was assessed with a DC assay as described earlier.

2.4. Differential treatment of bacterial cells on the growth of S17TH

An inductive ferment broth was prepared as follows: cell suspensions of S2BC-1 and TEPF-Sungal were amended separately at 1.5×10^8 cells ml^{-1} (grown for 24 h) into 100 ml of minimal synthetic broth (MSB) (0.2 g of $MgSO_4 \cdot 7H_20$, 0.9 g of K_2HPO_4, 0.2 g of KCl, 1.0 g of NH_4NO_3, 2 mg of $FeSO_4 \cdot 7H_20$, 2 mg of $ZnSO_4 \cdot 7H_20$ and 2 mg of $MnCl_2 \cdot 7H_20$ supplemented with 0.1% glucose, pH 5.6) and incubated on a rotary shaker (200 rpm) for 48 h. For live cells, the optical density of the broth was adjusted to 0.7 at 600 nm (1.5×10^8 cells ml^{-1}) with sterile distilled water. The live cells were inoculated with 1 ml of conidial suspension (1×10^5 conidia ml^{-1}) prepared from 5-day-old S17TH and incubated at 28 ± 2 °C for 5 days in shake cultures. The mycelium was collected by centrifugation (5000 rpm, 15 min) and weighed on sterile Whatman no. 1 filter paper. For heat inactivated cells, the bacteria-amended MSB was sterilized, and the mycelium was collected as described above. The weighed mycelia were fine ground with liquid nitrogen and

Table 1
Antagonistic plant growth promoting rhizobacteria selected against rhizome rot pathogens.

Code	Species	GenBank accession no.	Zone of mycelial growth inhibition (mm) in dual culture assay for FOZ	Zone of mycelial growth inhibition (mm) in dual culture assay for PS
BBPF-Holta	*Burkholderia cepacia*	GU048850	13	0
TEPF-Sungal	*Burkholderia cepacia*	GU048848	16	7
XXPF-MDU2	*Burkholderia cepacia*	GU048852	19	0
XXPF-MDU1	*Pseudomonas fluorescens*	GU048851	13.5	0
PAPF-Nagrota	*Pseudomonas putida*	GU048846	12	0
ROPF-Chandpur	*Pseudomonas putida*	GU048849	13	0
BGPF-Nagrota	*Pseudomonas putida*	GU048847	0	0
LSD ($P = 0.05$)			1.6	0.6
XXBC-TN	*Bacillus subtilis*	AM275327	14.5	13
CRBC-Kohola (E)	*Bacillus pumilis*	AM265567	11	3
GIBC-Kohala (D)	*Bacillus thuringiensis*	AM286796	0	0
S2BC-1	*Bacillus subtilis*	AM268039	15	10
S2BC-2	*Bacillus atrophaeus*	AM268040	12.5	10
GIBC-Jamog	*Bacillus subtilis*	GU048875	18.5	9
GIBC-Kiari (1)	*Bacillus cereus*	GU048876	12	0
CABS-IHBT	*Bacillus subtilis*	AM265566	0	0
GIBC-Solan (1)	*Bacillus simplex*	GU048877	0	0
LSD ($P = 0.05$)			1.4	1.3

Data are mean of three replications; the inhibition zone was measured from the edge of pathogen mycelium to the bacterial streak, when the control plates showed full growth.

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