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Original Article

Nutrient Competition Mediated Antagonism of Microbes Against *Rhizoctonia solani*

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Abstract

Plant growth-promoting (PGP) microorganisms are beneficial soil micro creatures which may facilitate plant growth by direct or indirect ways. *Bacillus amyloliquefaciens* MB101 (*BA*), *Streptomyces atrovirens* N23 (*SA*) and *Hypocrea lixii* NAIMCC-F-01760 (*HL*) were earlier reported to have the ability to manage the tomato root rot disease caused by *Rhizoctonia solani* (*RS*) at some extent. In the present study, effect of nutrient supplementation like potato dextrose broth (PDB) and tomato root extract (TRE) on antagonism of these three microbes was characterized under the soil microcosm in order to understand the role of nutrient in microbe-microbe interaction. A moderate influence on the population of all three antagonists was resulted by PDB and TRE with *RS*. However, TRE and PDB were causing a significant impact on cell wall degrading enzymes and antifungal activity in the presence of *RS*. Moreover, hyphal degradation of *RS* was proved by scanning electron micrographs in the absence of substrates. Nutrient competition enhanced the call wall degrading enzyme production. Therefore, the present study concluded the role of substrate in the mycoparasitism and also sustain the potential of the hereby methodology (soil microcosm) for screening of other soil-inhibiting organism in the future.

Keywords: antifungal activity; chitinase; glucanase; potato dextrose broth; tomato root extract

Introduction

Among soil-borne pathogens, *Rhizoctonia solani* is one of the economically important soil-inhabiting fungus that can survive saprophytically on dead organic matter in soil for a long time, either a resting structure 'sclerotia' or an actively growing mycelium (Solanki *et al.*, 2011, 2014a; Patil and Solanki, 2016b). Biocontrol using antagonistic microbes has been considered as an alternative strategy to agrochemicals that are harmful to human health and environment. Numerous earlier reports describe the use of biocontrol agents (BCAs) such as *Bacillus* spp. (Solanki *et al.*, 2012a, b), *Trichoderma/Hypocrea* spp. (Harman *et al.*, 2004; Almeida *et al.*, 2007; Solanki *et al.*, 2011) and *Streptomyces* spp. (Boukaew *et al.*, 2011; Malviya *et al.*, 2014; Yandigeri *et al.*, 2015). There are several mechanisms by plant growth promoting microbes (PGPM) that restrain disease incidence or severity, including antibiosis, antagonism for nutrients and space, as well as the production of fungal growth inhibitory molecules like cyanide and siderophores (Doornbos *et al.*, 2012; Solanki *et al.*, 2014b; Patil and Solanki, 2016a).

Utilization of microbial inoculates for protection of the root from infection of pathogens was targeted for eco-safe management of numerous crops, and rhizospheric microbes play a substantial role in the management of plant pathogens (Bais *et al.*, 2006; Compant *et al.*, 2010; Patil and Solanki, 2016a). All microbes (bacteria and fungi) found in nature associated with the root and rhizhosphere have diverse ability to utilize (catabolise) all naturally occurring compounds as their sources of carbon for energy production (Yu *et al.*, 2002; Hartmann *et al.*, 2009; Malviya *et al.*, 2014; Finkel *et al.*, 2017). In comparison to bulk soil, the rhizosphere is rich in nutrients because of rhizodeposition (Bais *et al.*, 2004). Rhizodeposits consist of the total carbon transferred from the plant root to the soil. During the host-microbes interaction plants root plays key role by releasing

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carbon-containing metabolites, proteins, phenols, organic acids and amino acids into the soil matrix (Bais *et al*., 2006; Arora *et al*., 2008).

A wide variety of plant beneficial microbes act as mycoparasites and produce a number of cell wall degrading enzymes (CWDEs), including cellulases, chitinases, glucanases and proteases, which helps them to extract nutrients from others fungal pathogens (Cao *et al*., 2008; Huang *et al*., 2016). In our previous studies, it was found that CWDEs such as chitinases and β-1, 3-glucanases have been playing a prominent role in mycoparasitic interaction and management of *R. solani* by *Bacillus* spp. and *Trichoderma* spp. in tomato plants (Solanki *et al*., 2011, 2012b).

In the present study, attempts were made to identify the role of artificial (potato dextrose broth) and natural (tomato root extract) substrates during the interaction of antagonist and plant pathogen. Therefore, three microbes, *Bacillus amyloliquefaciens*, *Streptomyces atrovirens* and *Hypocrea lixii* were evaluated against tomato root rot, causing pathogen *R. solani*, under an artificial soil microcosm, and antagonist population, antifungal activity and cell wall degrading enzymes (CWDEs) were estimated in the presence and absence of *R. solani*.

Materials and Methods

Microorganisms and antagonism assay

Active strains of *Rhizoctonia solani* (*RS*), *Bacillus amyloliquefaciens* MB101 (*BA*), *Streptomyces atrovirens* N23 (*SA*) and *Hypocrea lixii* NAIMCC-F-01760 (*HL*) were obtained from National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh, India and all strains were maintained as guideline of NAIMCC. *In-vitro* antagonistic activity of all three microbes (*BA*, *SA* and *HL*) was performed against *RS* by dual culture assay according to the procedure of Arora *et al.* (2008) and Morton and Stroube (1955). Each experiment performed in triplicates and pooled data were used for analysis.

Root exudates preparation

Seeds of *Lycopersicon esculentum* Mill. cv. 'Novratan' were the surface sterilized with ethanol (70% v/v) for 5 min, followed by sodium hypochlorite (1%) for 1 min and rinsed thrice with sterile distilled water, and germinated in autoclaved (20 min; 121 °C) perlite. After germination, plantlets were transferred in a steam sterilized (20 min; 121 °C) mixture of silicate sand, expanded clay and soil (1:1:1; v/v). Plants were grown in a growth chamber (day/night cycle: 16 h; 23 °C/8 h; 19 °C; relative humidity 50%) for five weeks; the root system of each tomato plant was washed under tap water and incubated in Erlenmeyer flasks filled with 100 mL sterilized Milli-Q water for 22 h. Solutions were sterilized by filtration through Whatman No. 4 and No. 42 filter papers and then Ø 0.22 mm nitrocellulose filters (Millipore) and lyophilized. Concentrations were adjusted to a ratio of 1 g of the root fresh weight equivalent to 10 mL of exudates solution with sterilized Milli-Q water. The pH was then adjusted to 6.0 before being sterilized again by filtration through Ø 0.22 mm nitrocellulose filters and lyophilised solution was kept at -20 °C until use.

Pathogen biomass preparation

The inoculum of *RS* was prepared in Erlenmeyer flasks (250 mL) containing pearl millet seeds (80 g), bean meal (20 g) and distilled water (30 mL) autoclaved for two consecutive days for 1 h at 121 °C. Three disks of *R. solani* (5 days old) were transferred into each flask and incubated at 28 ± 2 °C for 3 weeks and grown biomass (~10 g kg^{-1}) was mixed thoroughly in autoclaved soil to prepare pathogen infested microcosm and non-infested microcosm also infested with the same amount of autoclaved mycelial biomass.

Microbial inoculums preparation

The suspension of *BA* was prepared by inoculating 0.1 mL of actively growing culture in 250 mL of nutrient broth (NB, HiMedia, India) and incubated on a rotary shaker (120 rpm) at 28 ± 2 °C for 24 h. Bacterial cells were harvested by centrifugation 8,000 × *g* for 10 min (Sigma 3K30 centrifuge, Germany), resuspended and diluted in sterile distilled water up to ~10^7 cells mL^{-1}. For *SA*, a loopful culture was inoculated in ISP2 broth (250 mL) and incubated for ten days, at 32 ± 2 °C at 150 rpm. The cell suspension was centrifuged at 8,000×*g* and the pellet was washed with sterile distilled water and cell concentration maintained at ~10^7 cells mL^{-1}. *HL* was multiplied in a broth containing molasses (100 mL), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (0.2 g), sucrose (10 g) and distilled water (1L) (pH 5.8). After 10 days incubation, conidia were separated from mycelia by filtering though sterile glass wool and quantified by a haemocytometer. The conidial count was adjusted to ~10^7 conidia mL^{-1} and these microbial suspensions were used for the soil microcosm experiment.

Soil microcosm study

Rhizospheric soil with the following characters: sand 64%; silt 29.2%; clay 23.4%; bulk density 49.3 g/cm^3; water holding capacity 67.42%; pH 6.25; EC_e 1.52dS m^{-1}, was used in the study. Soils were freshly collected prior to the experiments and mixed thoroughly, sieved to 2 mm particle size and sterilized by three time's consecutive autoclaving (121 °C for 1 h). Two types of experiment were performed in soil microcosm (2L glass beaker). First *RS* infested (with pathogen) and second non-infested (without pathogen) microcosm and both microcosms were treated with 5 mL of microbial suspensions: *BA* (~10^7 cells mL^{-1}), *SA* (~10^7 cells mL^{-1}) and *HL* (~10^7 spores mL^{-1}) by pipetting. Then, all inoculated microcosms were supplemented every alternate day with PDB and TRE at a rate of 0.1 mL g^{-1} soil up to 21 days. Each supplement was applied with sterilize distilled water in a soluble form, and only distilled water used as control. After microbes and substrate addition, the whole bulk of soil in the microcosm was thoroughly mixed and all microcosms were covered with sterilized tin foil and transferred to a growth chamber (19 °C night and 26 °C day, relative humidity 65%) for 21 days, regularly checked

and adjusted to 40% of soil water holding capacity (WHC) with sterile distilled water. The microcosms were stirred every day and when correction of the moisture content was necessary. The experiment was performed with three individual replicates. After 21 days, soil samples were collected from each soil microcosm and analysis performed.

Population monitoring in microcosm

To monitor the population of the introduced biocontrol agents in the soil after 3 weeks (21 days), 10 g of soil from each microcosm was taken and assessed by dilution plating method. Different agar media were used for isolation and enumeration of *BA*, *SA* and *HL*, respectively. The population of *BA* was enumerated on trypticase soy medium (g L^{-1}: pancreatic digest of casein15.0; papaic digest of soybean 5.0; sodium chloride 5.0; pH 7.3) and *SA* on ISP2 medium (g L^{-1}: yeast extract 4.0; malt extract 10.0; glucose 4.0; pH 7.3) supplemented with an antifungal antibiotics nystatin (25 μg mL^{-1}) and actidione (50 μg mL^{-1}). Fungal culture *HL* was enumerated using potato dextrose agar (HiMedia, India) supplemented with antibacterial antibiotics streptomycin (50 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}). The identities of the cultures were confirmed by the compound microscopy on the basis of microscopic characteristics. The morphological characteristic of SA was confirmed based on sand yellow substrate and grey aerial mycelial colour on ISP2 medium. *BA* culture was identified on the basis of a rod shaped cells in microscopy studies and Gram positive reaction. Identity of *HL* was established on the basis of mycelia growth conidia and conidiophores morphology and mycelial growth and *RS* on the basis of septate mycelium.

Soil extract extraction

Soil extract was extracted after 21 days incubation by vigorously shaking of the microcosm soil of all treatments at a ratio of 1:2 (w/v) for 30 min in sterile phosphate buffer (NaCl- 80.0 g, KCl- 2.0 g, Na_2HPO_4-11.5 g, KH_2PO_4-2.0 g, H_2O-1 l and pH-7.2). An aliquot of the mixture (250 mL) was transferred to a sterile centrifuge tube and centrifuged at 1 000 × g for 10 min to remove large particles, then at 3,000 × g for 10 min to obtain the active supernatant and then sterilized by filtration through Ø 0.22 mm nitrocellulose filters and solutions were kept in -20 °C until use.

Poisoned food technique and hydrolytic enzyme estimation

Mycelial growth inhibition was evaluated by the poisoned food technique (Luo *et al*., 2005). PDA and PDB media were amended with aqueous soil extract (10 and 20%) and active *R. solani* mycelia discs inoculated on the PDA plate in center and in 100 mL potato dextrose broth and incubate at 28 ± 2 °C for 5 days. Moreover, CWDEs of soil extract also determined, chitinase (EC 3.2.1.14) activity of the extract was determined using the modified method of Trotta *et al.* (1996) and β-1, 3 Glucanase was assayed by a modified method using laminarin as substrate according to Lethbridge *et al.* (1978). Soil chitinase (EC 3.2.1.14) activity was assessed by measuring the amount of the reducing end group, N-acetyl glucosamine (NAG), produced from colloidal chitin (Wen *et al*., 2002). Soil extract (1mL) was mixed with 0.25 mL of toluene, 4 mL of 50 mM NaOAc buffer (pH 5.0) and 1 mL of 0.5% colloidal chitin in a test tube and kept at 37 °C for 2 hours. After this period, 1 mL 0.5M of $CaCl_2$ and 4 mL of 0.5M NaOH were added and mixed thoroughly. The mixture was centrifuged at 1,000 *g* for 20 min to yield a soil-free supernatant then filtered through Whatman No. 2 filter paper. A 1.0 mL quantity of Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide) was added to 0.75 mL of the filtrate, and then the reaction was stopped by heating in boiling water for 15 min. Chitinase activity was calculated by measuring NAG concentration at 420 nm, in conjunction with data from a NAG standard curve. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μg of NAG g^{-1} h^{-1} at 37 °C. Enzyme analysis was performed three replicates and all the data collected were pooled for statistical analysis. The β-1,3 Glucanase was assayed using laminarin as substrate (Lethbridge *et al*., 1978). The reaction mixture for laminarin contained 2 mL soil extract, 0.2 mL of 0.1 M citrate buffer (pH 4.6) and 1.6 mg soluble laminarin. The reaction mixture was incubated at 37 °C for 1 h with gentle agitation, and then was stopped in both tests by boiling for 20-30 min. The citrate buffer was prepared by dissolving separately 2.1 g of citric acid monohydrate in 100 mL distilled water and 2.941 g tri-sodium citrate dihydrate in an-other 100 mL distilled water, then 44.5 mL of the first were mixed with 55.5 mL of the second. One millilitre of the sample was added at 4 °C to 5 mL anthrone reagent, the sample–reagent mixture was immediately boiled for 10 min, and then the samples were kept in the dark at 4 °C for 20-30 min for colour development. Then, the absorbance of the reactant mixtures was measured at 620 nm by spectrophotometer. This enzyme analysis was performed using three replicates and all the data collected were pooled for statistical analysis. All experiments were performed with three individual replicates and data pooled before analysis.

Scanning electron microscopy

The samples of test fungi *RS* collected by sterile needle from all microcosms (*BA*, *SA* and *HL*) with or without substrates and all samples were evaluated by scanning electron microscopy (SEM). The visual hyphae of *RS* was taken after 21 days and fixed for 4-6 h in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) at room temperature. Samples were rinsed thoroughly for 1-2 h with 0.2 M phosphate buffer (pH 6.8) and then dehydrated in a graded acetone series (30, 50, 70, 80, 90 and 100%), each grade for 30 min and three times for 100% acetone. Fully dehydrated samples were dried in a critical point dryer (HCP-2, Hitachi), mounted on stubs and then coated with gold (200 nm thickness) in a sputter coater (JFC-1600, JEOL). The coated specimens were examined with an SEM (JSM63804, JEOL Ltd) at 10 kV.

Statistical analysis

Data were analyzed with the SPSS program version 16.0 by analysis of variance (ANOVA) and significant differences among treatments and control groups were determined by Duncan's Multiple Range Test (DMRT) ($P \leq 0.05$).

Results

Effect of nutrient supplementation on microbial propagation

In the dual culture assay, all three antagonistic isolates inhibited the growth of *RS* up to 55% on PDA medium. On the basis of *in-vitro* antagonism, these isolates were selected for the microcosm study. The effect of potato dextrose broth and tomato root exudates (TRE) on microbial population was studied in soil microcosm and the results were represented in Fig. 1. In the soil microcosm study *BA* alone showed less growth with *RS* and a maximum population of *BA* resulted with PDB treated microcosm without pathogen (Fig. 1a). However, it was slightly reduced in the treatment *BA*+*RS*+PDB. But in the presence of *RS*+TRE, *BA* population was enhanced significantly as compared to the TRE alone (Fig. 1a). In the next, maximum population of *SA* resulted with *RS*+PDB, and was followed by PDB, *RS*, *RS*+TRE and TRE. However, *SA* alone showed less population as compared to other treatments like with *RS*, PDA and TRE (Fig. 1b). Moreover, similar kind of growth pattern also resulted in the case of *HL* with *RS*+PDB treated microcosm, followed by *HL*+*RS*+TRE, but the rest of the treatments showed non-significant changes in *HL* population (Fig. 1c).

Effect of nutrient supplementation on antifungal activity and CWDEs

Microbial soil microcosm study was performed to evaluate the effect of PDB and TRE on antagonism of three antagonistic microbes. Results in Table 1 had shown that antifungal activity of soil extract influenced by the substrate supplementation and the presence of the *RS*. *BA* alone and with *RS* showed maximum inhibition of *RS* growth and minimum inhibition resulted with extract of *BA*+*RS*+PDB at 10% concentration (Table 1). Likewise, *SA* alone showed more effect on mycelia growth, but *SA*+*RS*+PDB reduced the antifungal activity and *SA*+*RS* inhibited the growth up to 78% on PDA plates, while minimum mycelia dry wt. 0.21 g resulted (Table 1). Similarly, *HL*+*RS* showed maximum antifungal activity up to 75% (Table 1). After *in-vitro* antifungal activity, quantification of CWDEs was performed and chitinase and β-1, 3-glucanase enzyme resulted significantly higher with *RS* as compared to without *RS*, whereas it also significantly reduced by PDB and TRE supplementation as compared to only water. Maximum chitinase accumulation accrued with *BA*+*RS* and it was significantly different as compared to the *SA*+*RS* and *HL*+*RS* in the presence of water (Fig. 2a), and similar trend also followed by *HL*+*RS*. Likewise, β-1, 3-glucanase also resulted higher with soil extract of *BA*+*RS* treated microcosm without the nutrient supplements and it was significantly different as compared to control (Fig. 2b).

Scanning electron microscopy

After study of CWDE's and antifungal activity, the mycoparasitic behaviour of all antagonists conformed by SEM. Fig. 3a showed healthy mycelia of *RS* and damaged mycelia of *RS* by antagonist *BA* recovered from *BA*+*RS* treated microcosm without supplementation, and rod shaped colony of *BA* were attached on the damaged *RS* mycelia (Fig. 3b). Likewise, mycelia of *RS* clearly inhibited in the *SA*+*RS* treated microcosm in the absence of the nutrient (Fig. 3c); under microscope deformation and increased branching on the *RS* mycelia was observed (Fig. 3c). Likewise, microscopic observation of the *HL*+*RS* microcosm showed that the mycoparasitism of *HL* hyphae on *RS* mycelia induced in the absence of nutrient (Fig. 3d) and deformation of *RS* mycelia by coiling were clearly detected.

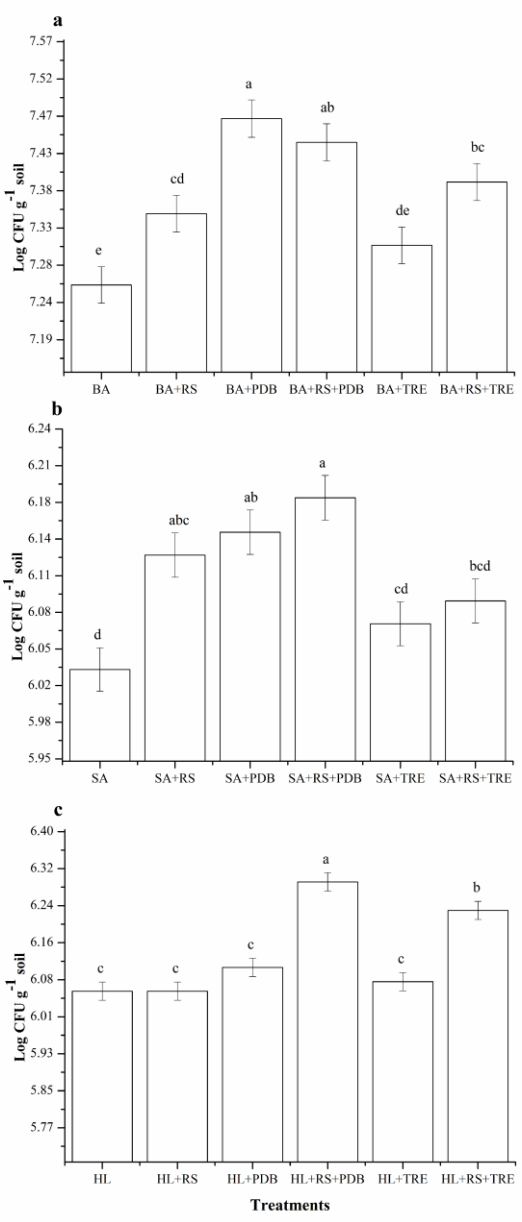

Fig. 1. Effect of nutrient supplements (potato dextrose broth-PDB and tomato root extract-TRE) on populations of antagonist with or without pathogen: **a** - *Bacillus amyloliquefaciens* MB101 (*BA*); **b** - *Streptomyces atrovirens* N23 (*SA*); **c** - *Hypocrea lixii* NAIMCC-F-01760 (*HL*), the vertical bars indicate the standard error of three replications (n = 3) and different letters on the error bars indicate significant difference ($P \leq 0.05$) analyzed by Duncan's Multiple Range Test (DMRT)

Table 1. Effect of soil extract (extracted from microcosm treated with different substrate and antagonistic microbes) on the growth of *Rhizoctonia solani* by poison food method

Treatments	Supplementation	Growth in mm (cm)		% Inhibition		Mycelia dry wt (g)		% Inhibition	
Concentrations (v/v)		10%	20%	10%	20%	10%	20%	10%	20%
BA	alone	2.97h	2.26g	66.96e	74.89f	0.41g	0.30gh	46.74i	65.57f
	PDB	3.96e	3.27d	55.96h	63.67i	0.45e	0.44b	41.26j	49.20l
	TRE	3.76f	3.07ef	58.19g	65.89gh	0.44ef	0.42c	45.50i	51.56k
BA+RS	alone	1.83kl	1.11k	79.63ab	87.67b	0.27l	0.19m	63.20cd	78.48a
	PDB	1.99jk	1.78h	77.89bc	80.19e	0.35i	0.23j	51.36h	73.50d
	TRE	1.97jk	1.49i	78.11bc	83.48d	0.33j	0.20lm	59.46e	76.95ab
SA	alone	3.66f	3.02f	59.30g	66.41g	0.36i	0.31fg	55.03fg	64.57fg
	PDB	4.95b	3.76c	44.96k	58.19j	0.49d	0.40d	38.71j	54.47j
	TRE	4.75c	3.17de	47.19j	64.78hi	0.43f	0.36e	44.38i	59.03h
SA+RS	alone	1.94jk	1.29j	78.44abc	85.70c	0.22m	0.21kl	69.28a	76.14bc
	PDB	3.27g	2.98f	63.67f	66.85g	0.29k	0.29h	66.03b	66.22f
	TRE	2.08ij	1.30j	76.89cd	85.59c	0.23m	0.25i	64.35bc	71.16e
HL	alone	3.27g	2.28g	63.67f	74.67f	0.33j	0.32f	56.20f	63.38g
	PDB	4.95b	1.09k	44.96k	87.89b	0.35i	0.39d	52.71gh	54.16j
	TRE	4.26d	3.96b	52.67i	55.96k	0.38h	0.37e	53.18gh	57.15i
HL+RS	alone	1.78l	0.96l	80.19a	89.33a	0.29k	0.21kl	60.96de	75.68bc
	PDB	2.18i	1.68h	75.78d	81.30e	0.32j	0.25i	56.24f	70.65e
	TRE	1.88kl	1.17k	79.07ab	87.00b	0.29k	0.22jk	64.40bc	74.83cd
RS	alone	9.00a	9.00a	0.00l	0.00l	0.85b	0.87a	0.00k	0.00m
	PDB	9.00a	9.00a	0.00l	0.00l	0.87a	0.88a	0.00k	0.00m
	TRE	9.00a	9.00a	0.00l	0.00l	0.80c	0.87a	0.00k	0.00m

Values are mean of three independent (n = 3) experiments and means followed by the same letter on superscript within a column are not significantly different (P≤0.05) according to Duncan's Multiple Range Test (DMRT). *Rhizoctonia solani* (*RS*), *Bacillus amyloliquefaciens* MB101 (*BA*), *Streptomyces atrovirens* N23 (*SA*) and *Hypocrea lixii* NAIMCC-F-01760 (*HL*); potato dextrose broth (PDB) and tomato root extract (TRE); alone only water treated.

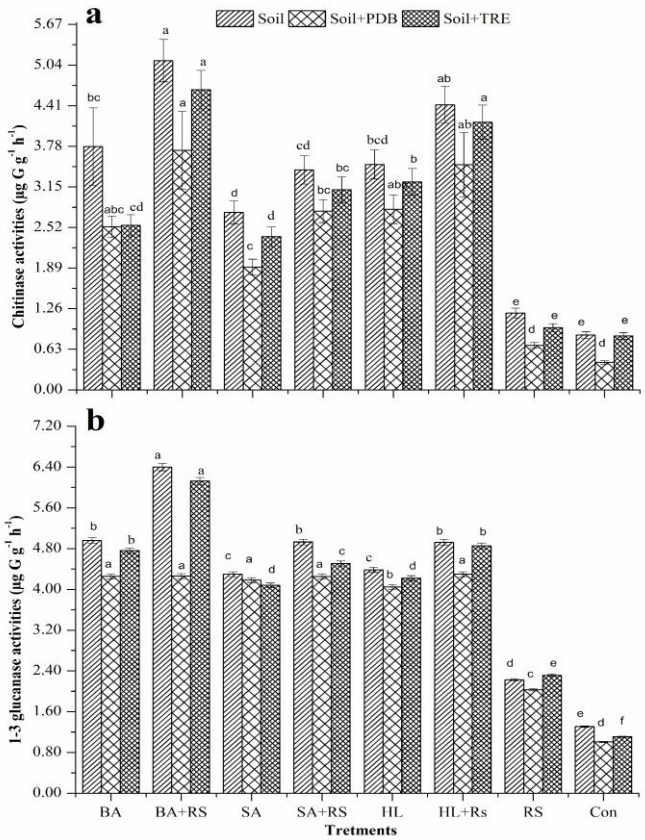

Fig. 2. Effect of nutrient supplements (potato dextrose broth-PDB and tomato root extract-TRE) on production of cell wall degrading enzymes: **a** - Chitinase and **b** - β-1,3 Glucanase; *Rhizoctonia solani* (*RS*), *Bacillus amyloliquefaciens* MB101 (*BA*), *Streptomyces atrovirens* N23 (*SA*) and *Hypocrea lixii* NAIMCC-F-01760 (*HL*), the vertical bars indicate the standard error of three replications (n = 3) and different letters on the error bars indicate significant difference ($P \leq 0.05$) analyzed by Duncan's Multiple Range Test (DMRT)

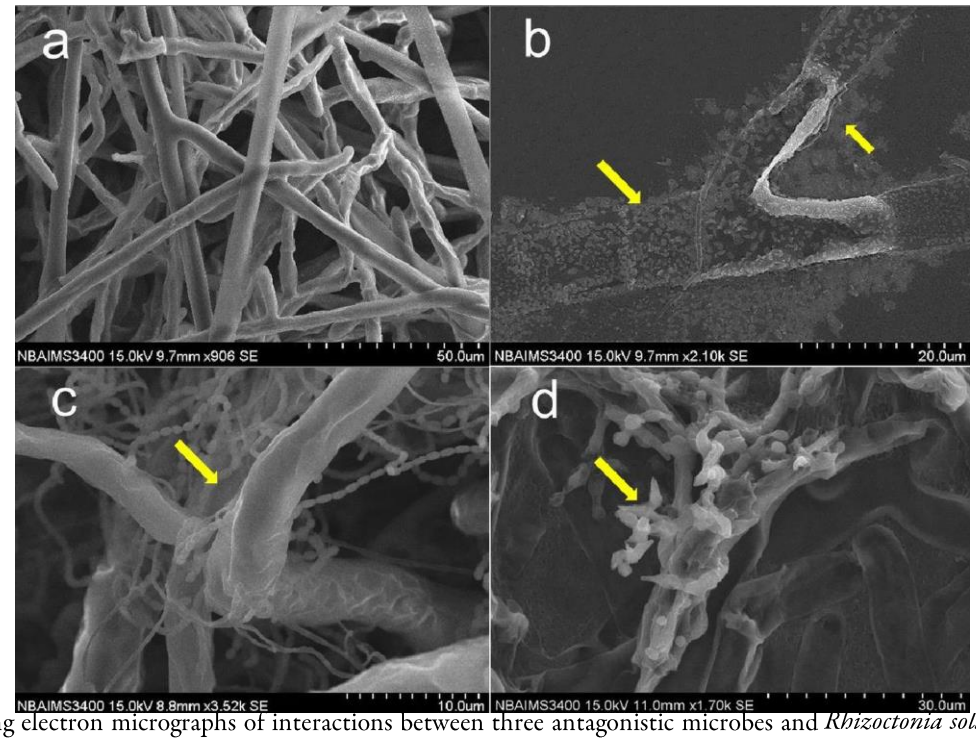

Fig. 3. Scanning electron micrographs of interactions between three antagonistic microbes and *Rhizoctonia solani* (RS) under a soil microcosm after 21 days: **a** - RS control adopted from Malviya *et al.* (2014) for comparison; **b** - *Bacillus amyloliquefaciens* MB101 (BA); **c** - *Streptomyces atrovirens* N23 (SA); **d** - *Hypocrea lixii* NAIMCC-F-01760 (HL), yellow arrows showing the attachment and coiling of antagonism on pathogens

Discussion

Mostly, plant microbe interaction can be classified as pathogenic, saprophytic and beneficial (Kundan and Pant, 2015; Patil and Solanki, 2016a). Biological control is the one of the most beneficial interaction, where micro-organisms manage the plant diseases caused by soil-borne plant pathogens and it has been considered to be a more natural and biologically acceptable alternative to the existing chemical treatment methods (Finkel *et al.*, 2017). Among all microbes, *Trichoderma/Hypocrea* spp., *Bacillus* spp. and *Streptomycetes* spp. have been identified as promising resources for biocontrol of plant diseases (Shoresh *et al.*, 2010; Solanki *et al.,* 2012b, 2016). In the current study, three microbes (*BA, SA* and *HL*) were used against a sclerotia producing pathogen *R. solani*. On the basis of *in-vitro* confrontation assay results, the experiment was conducted in soil microcosm in controlled conditions to understand the role of nutrient based antagonism. Nutrient competition is the key mechanism and mostly enhanced the parasitism and antibiosis activity of antagonistic microbes (Harman *et al.*, 2004). Concerning the competition for nutrients, in the hereby study an artificial rhizosphere system (soil microcosm) was organized with or without *R. solani* a virulent pathogen of tomato root rot. Artificial substrate (potato dextrose broth-PDB) and natural substrate (tomato root extract-TRE) were used as nutrient supplements in the soil microcosm with three important antagonistic microbes (*BA, SA* and *HL*), and after 21 days, soil extracts were collected and antifungal activity and CWDE's quantity were assessed. Results showed nutrient supplementation influence the antifungal activity of soil extract with the presence of *RS* and antagonistic microbes. Maximum antifungal activity thought growth inhibition resulted with *HL+RS* and was followed by *BA+RS* and *SA+RS* in the presence of *RS* without supplementation. However, only *BA* inhibited maximum mycelia growth of *RS* without substrate as compared to *HL* and *SA* and it was followed by the natural substrate (TRE). Different antifungal activity against *RS*, in the antagonist treated microcosm give an idea that CWDEs may play a critical role in the inhibition of *RS*.

Moreover, several reports also proved that chitinases and β-1, 3-glucanases have been playing a significant role in the mycoparasitic interaction of *Trichoderma* species against the different fungal pathogen (Almeida *et al.*, 2007; Solanki *et al.*, 2011). Because the major component of the cell wall of plant pathogen is made up by chitin and glucans (Gao *et al.*, 2005; Cao *et al.*, 2009). Therefore, the quantification of CWDEs with or without pathogen becomes essential for the present study. Thus, in the presence of *RS*, maximum chitinase accumulation accrued with *BA+RS* without supplementation and similar trends also followed by *HL+RS*. Similarly, β-1, 3-glucanase also resulted maximum with *BA+RS* in the absence of PDB and TRE and was followed by RS+TRE. In the presence of *RS*, all microbes produced significant amounts of chitinases and β-1, 3-glucanases and it is to be reduced when supplements were added, respectively. After antifungal activity and quantification of CWDE's, antagonist interaction accessed *via* SEM, and significant damaged mycelia of *RS* recovered with all antagonists treated microcosm without supplementation.

Conclusions

In conclusion, the present investigation stated that nutrient supplementation negatively affect the antagonism and the results had great significance in the mycoparasitism study. The present findings support the nutrient competition mechanism and prove that the presence of substrate can reduce the mycoparasitism and antibiosis of microbes; a natural substrate like tomato root extract is also the best choice to understand the biocontrol mechanism as compared to the artificial substrates. Soil microcosm technique can be used to characterize different kinds of antagonistic microbes in the future and it is quite similar like soil system. Moreover, chains of experiments are needed to understand the nutrient competition in the presence of physiological factor and abiotic stress conditions and it is our future mandate to explore all possible factors and their regulatory effect on the antagonism.

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