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Bacillus megaterium mediated growth promotion and biological control of crown rot disease of *Oncidium* incited by *Sclerotium rolfsii* Sacc.

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Abstract

Crown rot disease of orchid caused by *Sclerotium rolfsii* is a major constraint in orchid cultivation especially during prolonged hot, humid weather. The disease causes the death of the infected plants, spreading infection to the nearby plants and destroying the whole plantation. Despite its aggressive nature, a number of microorganisms have been found to limit the growth. *Bacillus megaterium* de Bary TRS-3 isolated from rhizosphere of tea plants has been used as a biological control agent in the present study in controlling the growth of *S. rolfsii* in one of the most widely cultivated orchid genus-*Oncidium* sp. and to determine the role of defense enzymes in induction of systemic resistance and disease suppression. The bacterium suppressed the mycelial growth of fungal pathogen as well as sclerotial germination. *In vitro*, it produced volatiles which could inhibit the growth of the pathogen. *B. megaterium* induced resistance to fungal pathogen by eliciting the increase in activity of both chitinase and β-1,3- glucanase as well as peroxidase and ascorbate peroxidase and reduction in the activity of catalase of *B. megaterium* TRS-3 treated orchids plants. Bacterium treated plants also showed increase in content of total chlorophyll as well as both chlorophyll a and b. The present study proved the potentiality of *B. megaterium* TRS-3 as a biological control. .

Keywords: *Sclerotium rolfsii, Bacillus megaterium, orchid, stem rot*

Orchids are the top selling herbaceous perennials worldwide and cultivated in a large scale. However, the cultivation of orchid is often plagued by a disease called crown rot or stem rot that damages many plants in various stages of growth. This fungal disease, caused by *Sclerotium rolfsii*, begins its life cycle with germination of sclerotia and can severely damage established host within a week, and is difficult to eradicate. Symptoms begin to appear on host after prolonged hot, humid weather. The lower leaves begin to turn yellow, and then brown, and wilt from the margins back toward the base. The base of these damaged petioles shows a brown discoloration and mushy texture. Fluffy white threads (mycelium) of the crown rot fungus are often found to be present on the rotted tissue and surrounding soil (Fig. 1). The leaves begin to collapse and soon the plant dies rapidly spreading infection to the nearby plants and destroying the whole plantation.

Microorganisms that grow in the rhizosphere are ideal for use as biological control agents against soil borne pathogens since the rhizosphere provides the front line defense for roots against attack by pathogens. A number of microorganisms have been found to limit the growth, including *Bacillus* sp. *Pseudomonas* sp. *Gliocladium virens*, *Penicillium*, *Trichoderma harzianum* and *Trichoderma viride* (Kokalis-Burelle *et al.* 2006, Murphy *et al.* 2000, Zhang *et al.* 2002 and van Peer *et al.* 1991). *Bacillus megaterium* isolated from rhizosphere of tea plants has been used as a biological control agent in the present study because of its innate ability to form endospore which makes it easier to formulate the commercial biocontrol agent.

The present investigation was undertaken to test the ability of *B. megaterium* as potent biocontrol agent in controlling the growth of *S. rolfsii* in one of the most widely cultivated orchid genus like *Oncidium* sp. and to determine the role of defense enzymes in providing resistance and disease suppression to the plant.

Materials and methods

Antagonistic tests

Bacillus megaterium isolated from the rhizosphere of healthy tea plants growing at Darjeeling hills were used in the present study. The antagonism was determined *in vitro* by dual culture test against *S. rolfsii*. Observations were recorded from 72 h of growth onwards in petri plates and diameter of growth of the pathogen (fungal growth) was measured and compared to control growth without the bacterium. Each experiment was run in triplicate. Results were expressed as mean percent inhibition of the growth of *S. rolfsii* in presence of *B. megaterium* de Bary TRS-3.

Percent inhibition was calculated using the formula:
% inhibition=(1-fungal growth/control growth)X100

Volatile production

Inhibition of pathogen by volatile compound released by *B. megaterium* was also tested. For this purpose , *B. megaterium* TRS-3 was streaked on the one half of the petri plate containing PDA and 7mm disk of the freshly growing *S. rolfsii* was placed at the center of another petri plate. Both the half plates were sealed to prevent

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Table 1: Effect of *B. megaterium* on crown rot disease in *Oncidium* plants caused by *Sclerotium rolfsii*

Treatment	Percent disease incidence* *Days after inoculation*		
	15	30	45
S rolfsii	36	87	92
S rolfsii+B megaterium	12	23	42

* PDI was calculated as the number of diseased plants out of 25 inoculated plants in each treatment

the loss of volatiles formed. The plates were incubated at 28°C. The growth of the pathogen was measured and compared to control developed in the absence of the bioantagonist. Each experiment was performed in triplicate.

In vivo application

For application of bacteria in nursery grown plants, fresh bacterial culture was centrifuged at 10,000 rpm and pellets were collected and suspended in sterile distilled water at a concentration of $3x10^8$ cfu. The bacterial suspension thus obtained was sprayed on the plants. For assessment of disease control the plants were initially treated with *B. megaterium* followed by challenge inoculation with *S. rolfsii*. The percentage of disease incidence was calculated after 15, 30 and 45 days of inoculation as the number of diseased plants over the total number of inoculated plants.

Active principle extraction and testing

The active principle was extracted from bacterial lawn using acetone and partially purified. Extracts were then tested in vitro against growth of S.rolfsii.

Extraction and assay of enzyme activity

The enzyme activity of the plant was studied after 72 hrs of bacterial application and compared to that of the untreated control plants. For this the plant tissues were weighed and ground to powder in liquid nitrogen and extracted with 0.05 M Na_2HPO_4/ NaH_2PO_4 buffer (pH 6.9) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulphonylfluoride (PMSF) and 20mg of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 4°C for 20 minutes at 15,000 g. The supernatant was used for the activity assay of peroxidase, ascorbate peroxidase and catalase.

Peroxidase activity was assayed spectrophotometrically as increase in absorbance at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.* 1993).

Ascorbate Peroxidase activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm (Asada, 1994).One unit of activity was defined as 1 µM of ascorbate oxidized by the enzyme in 1 minute.

Catalase activity was measured according to Chance and Maehly (1955) by monitoring the disappearance of H_2O_2 at 240 nm in a UV-VIS Spectrophotometer and enzyme activity was expressed as enzyme units/mg protein.

Chitinase was extracted activity was assayed using 0.1 M sodium citrate buffer (pH 5) following the method described by Boller and Mauch (1998).

Extraction and assay of activity of β-1, 3- glucanase was done following the method described by Pan *et al.* (1991).

Results and discussion

In vitro antagonism of B.megaterium

Antagonism of *B. megaterium* TRS-3 against *S. rolfsii* was investigated by dual culture assay in solid medium. *B. megaterium* TRS-3inhibited the hyphal growth by 55.5% compared to control. The zone of inhibition was found to be 2.3 cm after 7 days of incubation. Several strains of *Pseudomonas* sp. and *Bacillus* sp. have been reported to suppress soil borne diseases caused by fungal pathogens (Powell,1992). *B. megaterium* TRS-3 released some volatile compound which inhibited the mycelial growth of fungal pathogen *in vitro*. The compound extracted from bacterial lawn of *B. megaterium* TRS-3 was found to be highly active and inhibited the growth of fungal pathogen completely when added to the media in 1:5 ratio. It also inhibited sclerotial germination. None of the treated sclerotia germinated whereas the water treated sclerotia showed full germination.

In the present study it is evident that *B. megaterium is* antagonistic to *S. rolfsii* and also produced some volatile component which significantly inhibited the growth of pathogen.

In vivo tests

When applied in field *B. megaterium* TRS-3 was effective in reducing crown rot disease incidence. The percentage of disease incidence after 45 days of inoculation was 92% in untreated *Oncidium* plants which reduced to 42% on pretreatment of plants with *B. megaterium* TRS-3 (Table 1). In several cases, disease suppression has been attributed to the ability of the bacteria to produce antibiotic compounds (Homma and Suzui, 1989; Howell and Stipanovic, 1979; Dahiya *et al.* 1988).

Table 2: Changes in enzyme activities in *Oncidium* induced by *B megaterium* and *S.rolfsii*

Treatment	Enzyme activities				
	Peroxidase[1]	Ascorbate[2] peroxidase	Catalase[3]	Chitinase[4]	β-1,3 glucanase[5]
Control	0.18	0.76	2.08	3.93	11.20
B megaterium	0.58	2.07	1.81	6.16	13.00
S. rolfsii	0.28	0.91	2.32	4.58	12.50
B megaterium + S rolfsii	0.39	1.38	1.93	4.67	12.25

1=ΔA_{460} g^{-1} fresh wt. tissue; 2=ΔA_{290} nm mg^{-1} fresh weight min^{-1}; 3=Enzyme unit/mg protein/min;
4=µg GlcNAc g^{-1} tissue; 5=mg glucose g^{-1} tissue min^{-1}

Fig 1: Healthy plant of Oncidium (A&B); Sclerotium rolfsii Sacc. Infected plants showing the leaf yellowing typical of stem rot (C&D); Sclerotium rolfsii Sacc. Mycelia showing mustard like sclerotia.

Effect of B.megaterium on enzyme activities

Changes in the activities of different enzymes were tested in the plants treated with *B. megaterium* TRS-3 alone, *B. megaterium* TRS-3 treated plants challenged with *S. rolfsii* and *S. rolfsii* inoculated plants and compared their activity with that of control plants.

Assay of chitinase and β-1,3-glucanase was used to evaluate whether *B. megaterium* could induce resistance to fungal pathogen. *B. megaterium* elicited increase in activity of both chitinase and β-1,3- glucanase in *B. megaterium* treated *Oncidium* plants compared to untreated control and pathogen treated plants. *B. megaterium* TRS-3 also elicited significantly greater peroxidase and ascorbate peroxidase activities in leaves of treated *Oncidium* plants compared to untreated and pathogen inoculated plants. Catalase activity on the other hand was found to be significantly reduced in *B. megaterium* TRS-3 treated plants with respect to untreated control plants (Table 2).

Results of the present finding therefore, indicate that *B. megaterium* TRS-3 is a potential biological control and can be utilized for the control of *S. rolfsii* in orchid plantations successfully after field trial.

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