

Bacillus megaterium (AUM72)-mediated induction of defense related enzymes to enhance the resistance of turmeric (*Curcuma longa* L.) to *Pythium aphanidermatum* causing rhizome rot

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ABSTRACT

Bacillus megaterium (AUM72),a plant growth promoting rhizobacteria (PGPR) isolated from the rhizosphere soil were evaluated for their ability to control rhizome rot in turmeric(*Curcuma longa* L).Under in vitro condition, *B.megaterium* (AUM72), showed maximum inhibition of mycelia growth of *Pythium aphanidermatum*, were found effective in reducing rhizome rot of turmeric both under greenhouse and field conditions and increased the plant growth and rhizome yield. This isolate was further tested for its ability to induce production of defense related enzymes and chemicals in plants. Increased activities of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase,chitinase and β-1,3 –glucanase were observed in *B. megaterium* (AUM72) pre-treated turmeric plants challenged with *P.aphanidermatum*. Higher accumulation of phenolics was noticed in plants pre-treated with B.megaterium (AUM72) against *P.aphanidermatum*. Thus the present study shows that in addition to direct antagonism and plant growth promotion, induction of defense –related enzymes involved in the phenyl propanoid pathway collectively contributed to enhance resistance against invasion of *Pythium* in turmeric.

Key words: *Bacillus megaterium*, *Pythium aphanidermatum*, induced resistance, peroxidase

INTRODUCTION

India, "the land of spices" is the largest producer, consumer and exporter in the world. Turmeric (*Curcuma longa* L.) stands next to black pepper and cardamom. Turmeric is susceptible to diseases, such as leaf blight, anthracnose and rhizome rot. Among the various diseases, rhizome rot caused by *Pythium* sp. is a major problem in all turmeric-growing areas of India (Rathiah 1987, Nageshwar Rao 1994, Ramarethinam and Rajagopal 1999). The increased reflection on environmental concern over pesticide use has been instrumental in a large upsurge of biological disease control. Development of fungicide resistance among the pathogens, ground water, foodstuff pollution and the development of oncogenic risks have further encouraged the exploitation of potential antagonistic microflora in disease management. Although fungicides have shown promising results in controlling the damping-off disease, phytotoxicity and fungicide residues are major problems leading to environmental pollution and human health hazards. Development of fungicide resistance by *Pythium* sp. further discourages its use for disease control (Whipps and Lumsden 1991).

Bacillus species are the most common bacteria isolated from the soil, which accounted up to 36% of the bacterial population. *B. subtilis* is bestowed with the ability to endure stress, which is an advantage over other root colonizing bacteria. This bacterium also has the ability to produce antifungal metabolites and antibiotics to exploited for the control of many plant pathogens. Huang et al. (1999) reported that *B. licheniformis* and *Trichoderma* spp. effectively controlled Cucumber seedling damping off caused by *P. aphanidermatum* in soil less culture. *Bacillus* sp. effectively controlled *P. aphanidermatum* causing seedling disease of Okra (Anitha and Tripathi 2001). Seed bacterisation of Tomato with *B. subtilis* FZB 44 significantly reduced the yield loss caused by *P. aphanidermatum* (Maloupa and Gerasopoulos 2001). Individual strain of *B. subtilis* varied significantly with the fungal species and host crop. In Tomato, the highest efficacy against *P. debaryanum* was obtained with Bs 30.Induced systemic resistance (ISR) by definition refers to protection of the plants systemically by enhancement of plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate inducing agent upon infection by a pathogen. Induction of systemic resistance by PGPR against various diseases considered as the most desirable approach in crop protection. ISR once

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expressed activates multiple potential defense mechanisms that include increased activity of chitinases, β-1,3-glucanases and peroxidases (Dalisay and Kuc 1995, Xue et al. 1998) and accumulation of antimicrobial low molecular weight substances phytoalexins (Van Peer et al. 1991, Van Peer and Schippers 1992) and formation of protective biopolymers, e.g. lignin, callose and hydroxy proline-rich glycoprotein (Hammerschmidt et al. 1982).

The objectives of the present study are (1) To evaluate the efficiency of *B. megaterium* AUM72 against *P. aphanidermatum* under in vitro conditions and against rhizome rot under greenhouse and field conditions. (2) To study the induction of various defense-related enzymatic activities implicated in response to infection by *P. aphanidermatum* and by treatment with *B.megaterium* (AUM72).

MATERIALS AND METHODS

Cultures used

The plant growth promoting rhizobacteria *Bacillus megaterium* AUM72 was obtained from the Department of Microbiology, Faculty of Agriculture, Annamalai University. The isolate has been maintained in NA slants at 30± 2°C with monthly transfer. The test pathogen *p. aphanidermatum* from the department of Microbiology, Faculty of Agriculture, Annamalai University, this isolate was maintained in Potato Dextrose Agar.

Field experiment

Efficacy of *B. megaterium* AUM72 against rhizome rot disease

Two field experiments were conducted in two villages, viz. Trial I at Azichigudi village in Cuddalore District and Trial II at Oruthur village in Cuddalore District, Tamil Nadu, India, where the rhizome rot disease is endemic. Rhizomes were treated with 10 g of *B. megaterium* AUM72 formulation ($x10^{-6}$) per turmeric seed kg. As a standard treatment, rhizomes were treated with 6 g of Ridomil per turmeric seed kg. Seeds treated with distilled water served as control. The talc-based formulations were applied to soil after third and fifth MAP to enhance the population of antagonistic bacteria in the soil. The experiment was laid out with randomized block design with four replications. The plant height, rhizome rot incidence and rhizome yield were recorded at the time of harvest.

In vitro experiment

Induction of defense mechanism and experimental design

The following treatments took place in a greenhouse. The humidity in the greenhouse was maintained at around RH 80%. The temperature was adjusted to 26°C (day)/20°C (night). 3 replicants were maintained in each treatment. Each replicant was composed by 8 pots. The experiments were conducted using randomized block design on a greenhouse bench. (1) rhizomes treated with *B. megaterium* AUM72; (2) rhizomes treated with *B. megaterium* AUM72 and challenge with *P. aphanidermatum* 30 days after planting (50 g sand–maize medium containing10^3 cfu g^{-1} medium in each pot); (3) plants inoculated with the pathogen 30 days after sowing; and (4) non-treated plants. Rhizomes were sown in earthen pots filled with sterilized potting soil at rate of three rhizomes per pot. Plants were carefully uprooted without causing any damage to rhizome tissues at different time intervals (0, 2, 4, 6, 8 and 10 days after the pathogen inoculation). Three plants were sampled from each replication of the treatment separately and were maintained separately for biochemical analysis. Fresh rhizomes were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized rhizome tissues were stored at -70°C.

Table 1: *Bacillus megaterium* AUM72 to efficacy on inhibition of *P. aphanidermatum* under *in vitro* condition

Treatment	Mycelial growth(cm)	Germination (%)	Vigour index
Control	00.00	35.00	630.00
B.megaterium	36.11	85.66	1950.10

The percentage data were arcsine transformed prior to the analysis. In a column, means followed by a common letter are not significantly different (p=0.05) by Duncan' Multiple Range Test.

Assay of peroxidase activity

One gram of fresh plant tissue was ground in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar The homogenate was centrifuged at 15,000 x g at 48°C for 15 min. The supernatant was used as enzyme source. 0.1 ml from the obtained enzyme extract was added to 1.5 ml of 0.05 M pyrogallol and 0.5 ml of 1% H_2O_2 to obtained the reaction mixture. The change in absorbance of the reaction mixture was recorded at 420 nm at 30-s interval for 3 min at room temperature (28°C)**.** The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in absorbance of the reaction mixture per minute per gram of rhizome (Hammerschmidt et al. 1982).

Assay of polyphenol oxidase

One gram of fresh plant sample was ground in 1 ml of 0.1 M sodium phosphate buffer (pH = 6.5). The homogenate was centrifuged at 15,000 x g for 15 min at 48°C and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 and 0.1 ml of the enzyme extract. The reaction

was initiated by the addition of 0.2 ml of catechol (0.01 M). The activity was expressed as change in absorbance at 495 nm at 30-s interval for 3 min. The enzyme activity was expressed as change in absorbance per minute per gram of rhizome (Mayer et al. 1965).

Assay of phenylalanine ammonia lyase

One gram of rhizome was homogenized in 5 ml of cold 25 mM borate HCl buffer (pH = 8.8) containing 5 mM mercaptoethanol. The homogenate was centrifuged at 15,000 x g for 15 min and the supernatant was used as enzyme source. The assay mixture consists of 0.2 ml of enzyme extract, 1.3 ml water and 0.5 ml borate buffer. The reaction was initiated by the addition of 1 ml of 12 mM L-phenylalanine. The reaction mixture was incubated for 1 h at 32°C. The reaction was stopped by the addition of 0.5 ml of 2 N HCl. A blank was run in which phenylalanine was added after adding 2 N HCl. The absorbance was measured at 290 nm. The enzyme activity was expressed as µmol of cinnamic acid/min/g of rhizome (Dickerson et al. 1984).

Estimation of phenols

One gram of the rhizome sample was ground in a pestle and mortar in 10 ml of 80% methanol. The homogenate was centrifuged at 10,000 x g for 20 min. The supernatant as evaporated to dryness and the residue was dissolved in 5 ml of distilled water. From this, 0.2 ml was taken and the volume was made up to 3 ml with distilled water. To that 0.25 ml of Folin-Ciocalteau reagent (1 N) was added. After 3 min, 1 ml of 20% sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed in mg of catechol per gram of plant tissue (Meena and Mathur 2003).

Assay of chitinase

Rhizome samples (1 g) were homogenized in 2 ml of 0.1 M sodium citrate buffer (pH = 5.0). The homogenate was centrifuged at 16,000 x g for 15 min at 48°C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 10 ml of 1 M sodium acetate buffer (pH = 4.0), 0.4 ml of enzyme extract and 0.1 ml of colloidal chitin (10 mg). After 2 h incubation at 37°C, the reaction was stopped by centrifugation at 8000 x g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 ml of 1 M potassium phosphate buffer (pH = 7.1) and incubated with 20 ml desalted snail gut enzyme (Helicase). Finally, the mixture was incubated with 2 ml of dimethyl amino benzaldehyde for 20 min at 37°C and the absorbance was measured at 585 nm. The enzyme activity was expressed as nmol GlcNAc equivalents per minute per gram Rhizosphere (Bakkar et al. 2003).

Assay of β-1,3-glucanase

β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan et al. 1991). Rhizome samples (1 g) were extracted with 2 ml of 0.05 M sodium acetate buffer (pH = 5.0) and centrifuged at 16,000 x g for 15 min at 48°C. The supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 ml of4% laminarin and 62.5 ml of enzyme extract. The reaction was carried out at 40°Cfor 10 min. The reaction was then stopped by adding 375 ml of dinitrosalicylic acid and heating for 5 min on boiling water, vortexed and its absorbance was measured at500 nm. The enzyme activity was expressed as mg glucose released minute per gram rhizome (Pieterse et.al 2001).

Statistical analysis

All the experiments were repeated once with similar results. The data were statistically analyzed (Gomez and Gomez 1984) and Duncan's multiple range test compared treatment means. The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

RESULTS

The recent survey in different turmeric growing areas of Tamil Nadu, recorded rhizome rot incidence ranging from 20 to 50% incidence. The pathogens responsible for causing rhizome rot were isolated and identified as *P. aphanidermatum*, *R. solani* and *Fusarium oxysporum*. Pathogenicity studies on the involvement of pathogen in causing disease were studied. It was observed that *P. aphanidermatum* (49.33%), *R. solani* (14.33%) and *F. oxysporum* (9.33%) were found to be associated with rhizome rot of turmeric. Among them, *P. aphanidermatum* was found to be more virulent than *R. solani* and *F. oxysporum* in causing rhizome rot of turmeric.

Based on the in vitro studies on the inhibition of mycelial growth of *P. aphanidermatum*, isolate such as *B. megaterium* AUM72 was able to inhibit the mycelial growth of *P.aphanidermatum* under in vitro conditions. Under greenhouse condition *B.megaterium* AUM72 recorded 30.20% rhizome rot incidence followed by control inoculated with *P. aphanidermatum* recorded a rhizome rot incidence of 72.66%. The efficacy of *B. megaterium* is comparable with that of chemical Ridomil (Table 2).

The efficacy of talc-based formulations of *B. megaterium* AUM72 against rhizome rot was tested under field conditions. Formulations of *B. megaterium* AUM72 were effective for the control of rhizome rot incidence and increased plant growth under field conditions studied in two locations. The efficacy was comparable with chemical treatments.

Table 2: Efficacy of *Bacillus megaterium* (AUBM29) against rhizome rot disease of turmeric under green house conditions

Treatments	Rhizome rot incidence (%)	Height (cm)	Yield (g/plant)
Bacillus megaterium	30.00	75.50	450.00
Pathogen inoculated	72.55	50.10	250.00
Bm+pathogen inoculated	32.20	74.20	430.00
Control (water)	0.00	54.00	320.00
Ridomil (chemical)	33.40	70.40	360.00

Effect of rhizobacteria on the induction of defense- related enzymes and phenolic compounds against rhizome rot of turmeric

The activity of peroxidase (PO) increased in *B. megaterium* AUM72 treated rhizome tissues challenged with the pathogen. The maximum PO activity was observed on the fourth day after challenge inoculation and the activity was maintained at higher levels throughout the experimental period. Plants inoculated with the pathogen alone had comparatively less PO activity. PO activity in rhizomes treated with *B. megaterium* AUM72 alone remained unchanged during the experimental period but compared with control, the activity was higher (Figure 1(a)).

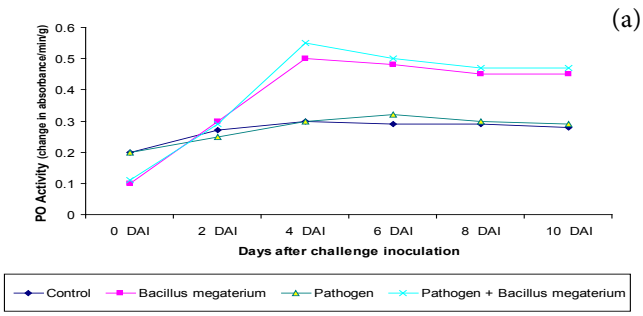
A similar pattern of increased activity of PPO was observed in bacterised turmeric plants challenged with the pathogen and the activity reached maximum on the fourth day after challenge inoculation (Figure 1(b)). The activities of chitinase (Figure 1(c)) and β-1,3-glucanase (Figure 1(d)) were maximum in bacterised rhizome challenged with the pathogen and higher activities were observed at sixth and fourth days after challenge inoculation, respectively.

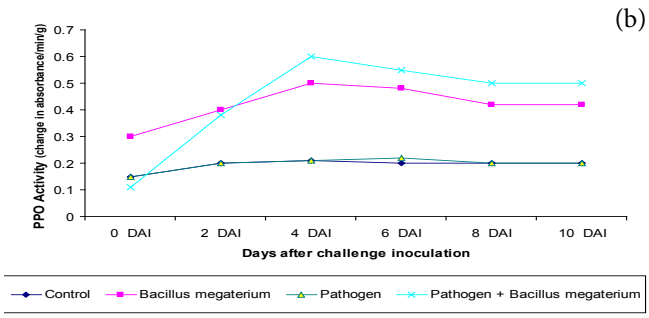
Enzyme activity

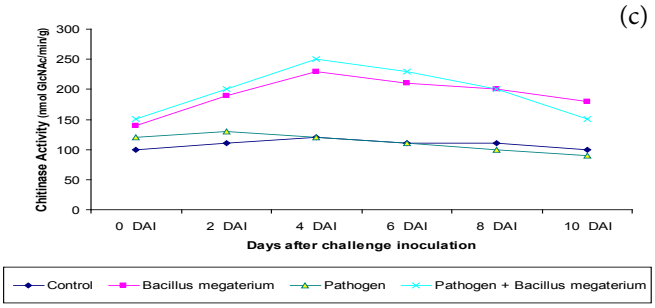
Generally, the enzyme activities were the maximum in bacterised rhizomes challenged with the pathogen. Induction of these enzyme activities was observed two days after challenge inoculation in bactericide plants. In plants inoculated with the pathogen alone, the enzyme activity increased initially at 4 day but later declined drastically at 8 day.

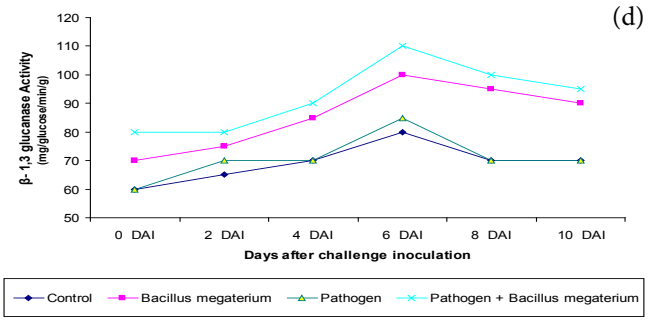
On pathogen challenge in bacterised turmeric tissues, phenylalanine ammonia lyase (PAL) activity started to increase on the second day after the pathogen inoculation and reached maximum on the sixth day after the pathogen challenge. Similarly, turmeric rhizomes inoculated with the pathogen alone-recorded increased activity of PAL but the induction of activity was observed for 2–4 days, there after declined drastically.

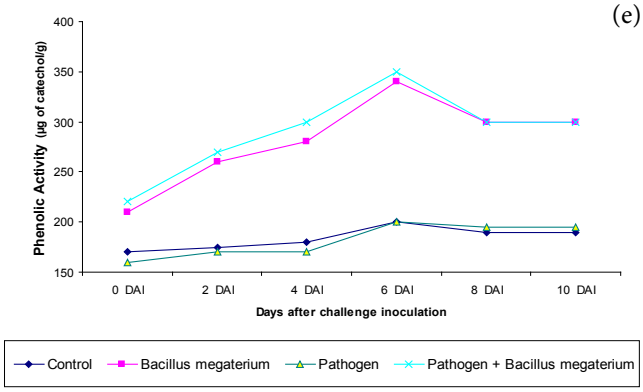
Turmeric plants treated with *B. megaterium*AUM72 alone also had significantly higher PAL activity compared











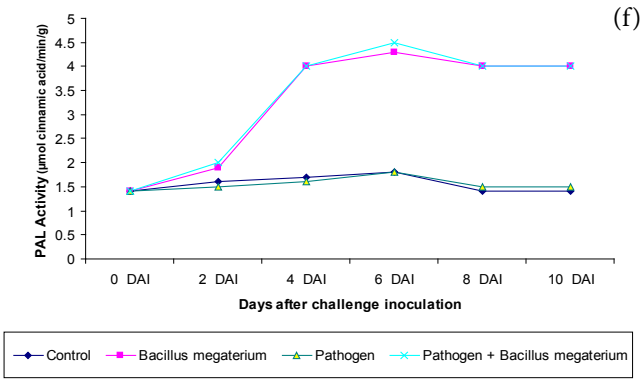


Figure 1: (a) changes in PO activity by rhizome treatment with *B.megaterium* and in turmeric challenged with and without *P. aphanidermatum.*(b) changes in PPO activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum* (c) Induction of Chitinase activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum*. (d) Induction of β-1,3 glucanase activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum*. (e) Influence of rhizome treatment with *B. megaterium* and on PAL activity in turmeric challenged with and without *P. aphanidermatum* (f) Accumulation of Phenolics by of rhizome treatment with *B. megaterium* in turmeric challenge with or without *P. aphanidermatum.*

with untreated control but activity was less compared with challenge-inoculated plants (Figure1(e)).

Defense mechanism

Studies on induction of defense mechanisms revealed that higher accumulation of phenolics was observed in *B. megaterium* AUM72 bacterised turmeric rhizomes challenge inoculated with *P. aphanidermatum*. Accumulation of phenolics started second day after challenge inoculation**.** The maximum accumulation was phenolics activity observed on the sixth day (350) after challenge inoculation. Plants inoculated with the pathogen alone also recorded increased accumulation of phenolics, but accumulation started on the second day after pathogen inoculation and drastically declined days after inoculation. Moreover, the accumulation of phenolics was less compared with bactericide plants challenged with the pathogen (Figure 1(f)).

DISCUSSION

Bacillus species is the major potential unexploited biocontrol agent against soil borne*of P. aphanidermatum* causing rhizome rot of turmeric. The inhibition of pathogen might be due to the production of antifungal metabolites. Production of antibiotics such as Iturin, Bacillomycin, Zwittermycin A and Surfactin is responsible for their antifungal action (Silo-Suh et al. 1994, Asaka and Shoda 1996, Constantinescu 2001, Moyne et al. 2001, Hiradate et al. 2002).

The present study also indicates that application of talc-based formulation of *B. megaterium* AUM72 increases plant growth in the both field experiments and reduced disease incidence. The results are in agreement with the findings of Meena and Mathur (2003) that treating ginger seed rhizomes with bioagents combined with soil application could effectively suppress the rhizome rot of ginger and increase the yield. Previous studies of McKeen et al. (1986) and Abdelzaher (2003) report the successful application of *B. subtilis* to control seedling diseases on a number of crops such as against a number of rhizome and seedling pathogens.

The increase in the hydrolytic enzymes in turmeric plants via challenge inoculation of AUM72 might help in defending the ingress of *Pythium* into host system. Kloepper at al. (1991) reported that ISR induced by fluorescent *Pseudomonas* was associated with induction of chitinase, as a promising technology for the management of red rot of sugarcane. In the present study, the activity of chitinase and β-1,3-glucanase was higher in biocontrol agents pre-treated plants challenge inoculated with *P. aphanidermatum*, which might have resulted in the lysis of invading pathogen. PO and PPO catalyse the last step in the biosynthesis of lignin and other oxidative phenols. Increased activity of cell wall bound PO has been elicited in different plants such as cucumber (Chen et al. 2000), rice (Reimers et al. 1992). In bean rhizosphere, colonisation of various bacteria induced the PO activity (Zdor and Anderson 1992). In the present study, PO activity was significantly increased from second day after challenge inoculation. The PO activity was elevated on fourth day after challenge inoculation in turmeric rhizome. Radjacommare (2000) reported that *P. fluorescens* isolate Pf1 induced the activities of PPO in rice against *R. solani*. Similarly, Meena et al. (2000) reported that the *P. fluorescens* induced the activities of PPO in response to infection by *C. personata* in groundnut. Similarly, significant increase in the activity of PPO was observed in green gram plants treated with P. fluorescens along with chitin (Saravanakumar 2002). Increases in PO activities were detected in foliar extracts of tomato whose seeds had been microbiolised, with Rhizobacterium B101R, against *Alternaria solani* (early blight), *Corynespora cassiicola* (foliar blight), *Oidium lycopersici* (powdery mildew), *Stemphilium solani* (leaf spot) and *Xanthomonas campestris* pv. *vesicatoria* (bacterial spot) of tomato (Silva et al. 2004). In the present study, higher PO activity were detected in *B. megaterium* AUM72 treated plants challenge inoculated with *P. aphanidermatum*.

In the present study, treatment with B. megaterium AUM72 induced the plants to synthesise PAL when the plants were challenge inoculated with *P. aphanidermatum*. The activity reached the maximum level on sixth day after challenge inoculation in turmeric rhizome pre-treated with the AUM72 which was challenged with *P. aphanidermatum*. Invasion of rhizome tissues by the pathogen might have resulted in decreased activity of

Table 3: Efficacy of talc based formulation of *Bacillus megaterium* (AUBM 29) on the management of rhizome rot of turmeric under field conditions

Treatments	Trial-1			Trail-2		
	Rhizome rot incidence (%)	Plant height (cm)	Yield (g/plant)	Rhizome rot incidence (%)	Plant height (cm)	Yield (g/plant)
Bacillus megaterium	29.00	85.50	436.00	30.00	86.00	490.40
Control	52.00	63.10	296.00	51.00	62.20	310.10

PAL whereas earlier and increased activity of PAL due to treatment with *B. megaterium* AUM72 might have prevented fungal invasion and thus the activity was maintained at the higher levels. When groundnut plants were sprayed with P. fluorescens, increased activity of PAL was observed (Meena et al. 1999, 2000).

Increased activity of PAL was observed in *P. fluorescens*-treated tomato, pepper, mint, rice and ragi plants in response to infection by *F. oxysporum* f. sp. *lycopersici*, *C. capsici* and *R. solani* (Meena et al. 1999, Ramamoorthy et al. 2002a, Radjacommare et al. 2005).

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, rhizome treatment with *B. megaterium* (AUM72) resulted in increased accumulation of phenolic substances in response to infection by the pathogen. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al. 1996). Benhamou et al. (1996) reported that an endophytic bacterium, *Serratiaplymuthica* induced the accumulation of phenolics in cucumber rhizomes following infection by *P. ultimum*, *P. fluorescens* Pf1 isolate also induced the accumulation of phenolic substances and PR-proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato (Ramamoorthy et al. 2002a), *Pyricularia grisea* in ragi (Radjacommare et al. 2005).

CONCLUSIONS

In conclusions, the present study implies that earlier and higher accumulation of enzymes involved in phenylpropanoid metabolism and PR-proteins has been found in turmeric rhizome tissue treated with *B. megaterium* AUM72 in response to invasion by *P. aphanidermatum*. The plant-pathogen interactions have also triggered the activities of defense enzymes initially but later the activities drastically declined when the pathogen colonised the root tissues. Accumulation of phenolics, PAL, β-1,3-glucanase and induction of *B. megaterium* (AUBM29) in turmeric rhizome tissues might have collectively contributed to induced resistance in turmeric plants against *P. aphanidermatum.*

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