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Biocontrol of root infecting plant pathogenic fungus, *Sclerotium rolfsii* using mycolytic enzymes and chitin metabolism inhibitors singly and in combination

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ABSTRACT: **A combined effect of mycolytic enzyme mixture and the cell wall chitin metabolism inhibitors to control the growth of a root infecting fungus, *Sclerotium rolfsii* on peanut was studied. Around 10 microbial cultures were screened for their potential to produce extracellularly cell wall synthesis inhibitors, using hyphal tip bursting test with different fungi. Most of them exhibited significant *in vitro* inhibition of enzymes *viz.*, chitin synthase, endo-chitinase or *N*-acetylglucosaminidase activities. In a pot experiment, irrigation with crude culture filtrate of *Bacillus* sp. 102 (1 mg. 50 ml^{-1}) which showed hyphal tip bursting in all the test fungi inhibited 70 percent growth of *S. rolfsii*. Daily irrigation with the mycolytic enzyme preparation of *Myrothecium verrucaria* diluted to give chitinase activity (0.04 U. 50 ml^{-1}) inhibited 40 per cent growth of *S. rolfsii*. The mixture of two *viz. M. verrucaria* and *Bacillus* sp. 102 culture filtrates, controlled 80 per cent *S. rolfsii* infection of peanut seeds. Further fractionation of the inhibitor mixture was also carried out using (60-120 mesh) silica gel column chromatography and preparative TLC. The results with respect to the hyphal tip bursting and enzyme inhibition have been discussed.**

KEY WORDS: *Bacillus* sp., chitin metabolism inhibitor, chitinase, *Myrothecium verrucaria*, *Sclerotium rolfsii*

The biocontrol of root-infecting fungi has been achieved successfully using mycoparasitic fungi like *Trichoderma harzianum* and *Gliocladium virens* (Papavizas, 1985). The relationship between the mycoparasitism and fungal cell wall degrading enzymes, mainly chitinases, β-1,3-glucanases and proteinases has been well established. Lorito *et al.* (1993) demonstrated a wide spectrum of antifungal activity of the chitinolytic enzymes of *T. harzianum*. While using *Serratia marcescens* chitinase it was shown that disease development in beans by *S. rolfsii* was prolonged (Ordentlich *et al.*, 1988). *Myrothecium verrucaria* produced extracellularly a complete complex of mycolytic enzymes *viz.*, chitinase, chitosanase, β-1,3-glucanase, mannanase and proteinase that significantly degraded mycelia of *S. rolfsii* and *Fusarium* sp. (Deshpande, 1999; Vyas and Deshpande, 1989). Another approach

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employed for the pathogen control is the use of antifungal antibiotics, which has been practiced in Japan against rice-plant diseases (Fiedler *et al.*, 1982). The inhibition of cell wall polymer, particularly chitin, synthesis is one of the novel targets suggested for antifungal antibiotics produced by microorganisms (Cohen, 1993; Deshpande, 1998; Groll *et al.*, 1998). In the present investigations, to control the growth of a root-infecting fungus a two pronged attack *viz.*, inhibition of cell wall synthesis and degradation of pre-formed cell walls with special reference to chitin, has been used.

Therefore, the investigations deal mainly with (a) screening of fungal and bacterial cultures for the potential cell wall synthesis inhibitors and (b) the use of *M. verrucaria* mycolytic enzyme mixture alone and with one of the potent microbial preparations of chitin metabolism inhibitors to control root infecting fungus *Sclerotium rolfsii* which causes wilting and stunted growth of peanut.

MATERIALS AND METHODS

Organisms

Myrothecium verrucaria NCIM 903, and *Sclerotium rolfsii*, NCIM 1084 were maintained on potato dextrose agar (PDA2%) slants. The bacterial and fungal cultures screened for anti-fungal activity and three other fungal cultures namely *Benjaminiella poitrasii*, *Aspergillus niger* and *Fusarium* sp. used for hyphal tip bursting test (HTB) were maintained on malt extract-glucose-yeast extract-peptone medium (MGYP) slants that contain: malt extract (0.3%), glucose (1%), yeast extract (0.3%) and peptone (0.5%).

Production of mycolytic enzymes by *M. verrucaria*

Myrothecium verrucaria was grown on a chitin (0.5% w/v) containing medium under shaking conditions (200rpm) at 28°C for 7 days as described earlier (Vyas and Deshpande, 1989). The cell-free broth was used for the estimation of mycolytic enzyme activities *viz.* chitinase, chitosanase, β-1,3 glucanase, protease and mannanase.

Screening of microorganisms for cell wall synthesis inhibitors using hyphal tip bursting test

All the microbial cultures were grown in a medium containing soyabean meal (1%), starch (0.5%), yeast extract (1%) and mannitol (1.5%) under orbital shaker conditions (200 rpm) at 28°C for 96h. The supernatant was separated by centrifugation at 10000 rpm for 10 minutes and was used for further studies.

For the hyphal tip bursting test the four fungi namely, *B. poitrasii*, *S. rolfsii*, *A. niger*, and *Fusarium* sp. were selected for their distinct features. *S. rolfsii* is a pathogen under investigation and has chitin-rich cell wall; *A. niger* and *Fusarium* sp. reported to have high glucan contents. To obtain actively growing hyphal tips the fungal cultures were inoculated on MGYP agar plates and these plates were incubated at 28°C for 16-18h. The hyphal tip elongation of *B. poitrasii* was approximately 1.5 divisions/2 min, while time period required to advance one division was 3-5 min for *S. rolfsii*, *A. niger* and *Fusarium* sp. The culture filtrate (10ml) of the potential antifungal organism in presence of sorbitol (0.6M) was added to the plates. The bursting of the hyphal tips was monitored microscopically up to 3h. The hyphal tips (15-20) per field were counted and the number of tips bursted in 10 fields was counted to find HTB (%) in a stipulated time as indicated.

Enzyme assays

The extracellular chitinase, β-glucanase, *N*-acetylglucosaminidase and protease activities were estimated using acid swollen chitin, laminarin, *p*NP-*N*-acetylglucosaminide and casein as substrates (Vyas and Deshpande, 1989; Vyas and Deshpande, 1993; Mendonsa *et al.*, 1996) and chitosanase using acid swollen chitosan (Patil *et al.*, 2000). The mannanase activity was estimated using yeast mannan (1%) as a substrate at 50° C for 1h. One unit of enzyme activity was defined as the amount of enzyme that liberated 1μmole of the product per min from the respective substrates

under assay conditions. The intracellular chitin synthase of *B. poitrasii* mycelial cells was estimated using UDP-C^{14}-*N*-acetylglucosamine (Deshpande *et al.*, 1997), and chitinase and *N*-acetylglucosaminidase activities were measured with 4-methyl-umbelliferyl-β-D-*N*-acetyl-glucosaminide (4-MU-GlcNAc)$_3$,4-MU-β-D-*N, N', N"*-triacetyl chitotrioside (4-MU-(GlcNAc), respectively as described earlier (Ghormade *et al.*, 2000). The crude culture filtrates (10-20 ml) of the organisms showing high percentage of hyphal tip bursting were added to check their effect on enzyme activities.

Pot experiment

The plant pathogen, *S. rolfsii* was grown in yeast extract- peptone- glucose (YPG) medium for 48h at 28°C. The garden soil was sterilized by autoclaving and then infested with mycelial mass of *S. rolfsii*. In each pot four peanut seeds were sown. The plants were irrigated with, (a) culture filtrate of *M. verrucaria* diluted to give chitinase activity, 0.04U50ml^{-1}; (b) culture filtrate of organism containing potential cell wall synthesis inhibitor (1mg 50 ml^{-1}) and (c) combination of a and b. Two control pots *viz.*, seeds sown in sterile soil and infested soil were irrigated with tap water. The observations were noted everyday up to 21 days.

Isolation of cell wall synthesis inhibitor(s)

The 10 times concentrated culture filtrate *Bacillus* sp. 102 by freeze-drying was used for liquid-liquid extraction for 14h using different organic solvents (8 times) such as ethyl acetate, chloroform, hexane and benzene at room temperature. The two layers were separated, solvent was evaporated and the residue was dissolved in the distilled water containing 0.6 M sorbitol for HTB test.

To isolate the active principle, the culture filtrate of *Bacillus* sp.102 was used for further liquid-liquid extraction. The HTB with *B. poitrasii* was used as a test to monitor the performance of the extraction procedure. Among the organic solvents used, 70-80 per cent activity as compared to the original culture filtrate was observed in ethyl acetate and chloroform extracts, while HTB was not detected in the benzene and hexane extracts (Table 2). Chloroform was selected for further large-scale extraction. On a large scale, the 1000 ml cell-free culture filtrate was concentrated to 100 ml and extracted for 15h with 500ml chloroform at room temperature. The chloroform layer was separated, dried over anhydrous sodium sulfate and evaporated to dryness under *vacuo*. The crude residue (1.2 g) revealed 6 different spots by Thin Layer Chromatography (TLC). Further separation was achieved on Silica Gel (60-120 mesh) column eluted with an increasing gradient elution of ethyl acetate: petroleum ether (1:9 → 10:0) and then with methanol: ethyl acetate (1:9 → 5:5). Total 30 fractions of 50ml each were collected, using TLC analysis, which were pooled in 6 fractions and tested for antifungal activity using HTB test. One fraction (Fraction 1, Chart 1), which exhibited high HTB activity was further purified by preparative TLC [Silica Gel PF 254, solvent system ethyl acetate: petroleum ether (20:80)]. This fraction furnished two components. The one major spot was characterized using Infrared (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectrometry. All experiments were carried out three times in duplicate, unless otherwise mentioned.

RESULTS AND DISCUSSION

Mycolytic enzyme activities of *M. verrucaria* NCIM 903

The extracellular culture filtrate of *M. verrucaria* had high chitinase (1.8±0.2 U ml^{-1}) and appreciable amount of chitosanase, β-1, 3-glucanase, mannanase (0.012±0.003, 0.214 ± 0.03, and 0.019 ± 0.002 U ml^{-1}, respectively) and alkaline protease (0.007±0.002 U ml^{-1}) activities.

Screening of micro-organisms for cell wall synthesis inhibitors

The supernatants of 4 days old cultures were used for the hyphal tip bursting test using 4 different fungi (Table 1). Among the tested

bacterial cultures, two *Bacillus* strains, 101 and 102 showed positive hyphal tip bursting test for all the four fungal species. While *Streptomyces* isolate NCL1 exhibited HTB for *B. poitrasii* and *S. rolfsii*. The *Actinomyces* isolate NCL2 and fungal isolates, *Aspergillus* MY2 and *Chaetomium* MY3 showed positive test against *B. poitrasii* only. *M. Verrucaria* culture filtrate did not indicate any positive HTB in *B. poitrasii* even after longer incubation (1h). It can therefore be attributed to the absence of cell wall synthesis inhibitor in the culture filtrate.

The *Bacillus* sp. 102 though produced chitinase activity; it was 50 fold less (0.028± 0.002 U ml^{-1}) as compared to *M. verrucaria* when grown under the same experimental conditions. The chitosanase, protease and mannanase activities were not detected. However, it produced marginally higher β-glucanase (0.323±0.08 IU ml^{-1}) activity as compared to *M. verrucaria Bacillus* sp.102 culture filtrate exhibited maximum HTB when it was heated at 90° C for 10 minute. It showed 70 ± per cent HTB with *B. poitrasii*. (Table 1).

Pot experiments

In the sterilized-non-infected soil the germination of peanut seeds was 90 per cent. The maximum development of disease was in the infested soil irrigated with water. As shown in the Fig. 1, the irrigation with a mixture of two culture filtrates was useful to protect peanut plants from the *Sclerotium* attack (85 %). The irrigation of infested soil separately with mycolytic enzyme preparation of *M. verrucaria* and crude inhibitor preparation of *Bacillus* sp.102 resulted in the germination of peanut seeds 40 and 70 per cent, respectively. Earlier, Ordentlich *et al.* (1988) reported that *Serratia marcescens* chitinase prolonged the disease development in beans by *S. rolfsii*. The effectiveness of chitinase, therefore, was attributed to the chitin contents of the pathogen. While it was reported that *T. harzianum*, a mycoparasitic fungus produced high chitinase and other mycolytic enzymes (Schirmbock *et al.*, 1994). Furthermore, it also produced antibiotics like trichorzianines, which affect lipid membranes. Schirmbock *et al.* (1994) therefore, suggested that both mycolytic enzymes and antibiotic acted synergistically against phytopathogenic fungi.

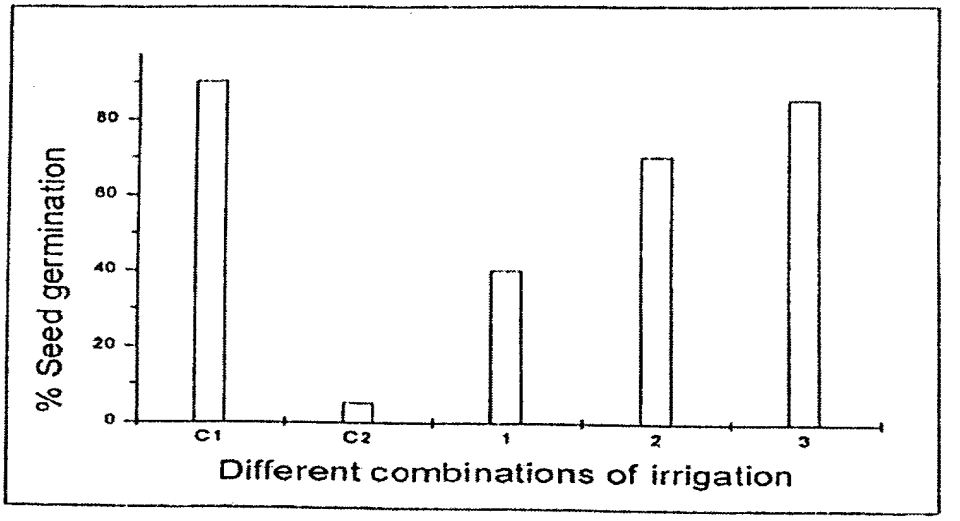

Figure 1. Effect of irrigation with mycolytic and cell wall synthesis inhibitor preparations, on the peanut seed germination in the infested soil with *S. rolfsii*

C1= seeds sown in sterile soil irrigated with tap water
C2= seeds sown in infected soil irrigated with tap water
1-3= irrigation of infested soil with *M. verrucaria* and *Bacillus* sp.102 culture filtrates, and their mixture

Isolation of chitin metabolism inhibitor(s)

The *Bacillus* sp. 102 preparation exhibiting maximum HTB (Table 1) was tested for its inhibitory effect on intracellular chitin synthase, endo-chitinase and *N*-acetylglucosamindase activities of a test fungus *B. poitrasii* along with other crude preparations. The culture filtrates of other isolates, such as *Bacillus* sp. 101, *Streptomyces* sp. NCL1 and *Chaetomium* sp. MY3 inhibited the chitin synthase activity to 20-50 per cent. However, *Bacillus* sp. 102 preparation did not inhibit chitin synthase activity. Instead it inhibited endo-chitinase and *N*-acetylglucosamindase activities to 30-40 per cent as compared to the control. The effect of *Bacillus* sp. 102 culture filtrate on *M. verrucaria* chitinase activity was checked and found to be unaffected when the total chitinase activitiy on acid swollen chitin and *N*-acetylglucosamindase on *p*NP-*N* acetylglucosaminide were measured. Koga *et al.* (1987) had also reported such type of selectivity in the inhibition of insect chitinase by allosamidin (a specific endo-chitinase inhibitor).

Using silica gel column chromatography, 6 fractions were tested for antifungal activity using HTB test with *B. poitrasii*. The fractions

Table 1. Hyphal tip bursting test of extra cellular broth of microbial cultures

Culture	Hyphal tip bursting test (%)			
	B. poitrasii (3-5 min)	*S. rolfsii* (50-60 min)	*A. niger* (8-10 min)	*Fusarium* sp. (5-7 min)
Bacillus sp.101	50 ± 10	40 ± 10	25 ±5	25 ±5
Serratia sp.1	-	-	-	-
Bacillus sp.102	80±5*	70±5	45±5	55±5
Streptomyces sp. NCL1	45± 5	20 ± 5	-	-
Actinomyces sp. NCL2	20 ±5	-	-	-
Actinomyces sp. NCL 3	-	-	-	-
Rhodococcus sp. No. 2	-		-	-
Trichoderma sp. MY 1	-	-	-	-
Aspergillus sp. MY 2	25 ± 5	-	-	-
Chaetomium sp. MY3	60 ±10	-	-	-

\- Not detected.

* The culture filtrate heated at 90° C for 10 min also showed 70 ± 10 % HTB with *B. poitrasii*

(1,2 and 5) showed good HTB activity table 2. The first fraction exhibiting maximum HTB showed two spots in preparative TLC. Further characterization of the major fraction using IR, NMR and Mass Spectrometry revealed that it had characteristics similar to oleic acid. It was confirmed by comparing the spectral data of the standard oleic acid as well as overlaying HPLC. The HTB activity using *Benjaminiella* of the fraction 1 was maximum without separation (Chart 1).

Though fractions 1, 2 and 5 only showed positive HTB test, all the fractions inhibited endo-chitinase and *N*-acetylglucosaminidase activities to a large extent (30-70%). It has been suggested that chitin hydrolysis may be one of the regulating processes for chitin synthesis in fungal cell wall (Adams *et al.*, 1993; Gooday *et al.*, 1992; Ghormade *et al.*, 2000). Therefore, hyphal tip bursting can also be correlated to the inhibition of *N*-acetylglucosaminidase and endo-chitinase activities which by supplying *N*-acetylglucosamine contribute in hyphal tip growth.

The mycolytic enzymes and chitinase inhibitor exhibited a better control of the spread of actively growing mycelium of *S. rolfsii* in combination than in isolation. The identification and structure-function relationship studies of the

Chart 1. Isolation of chitinase inhibitor(s) of *Bacillus* sp. 102

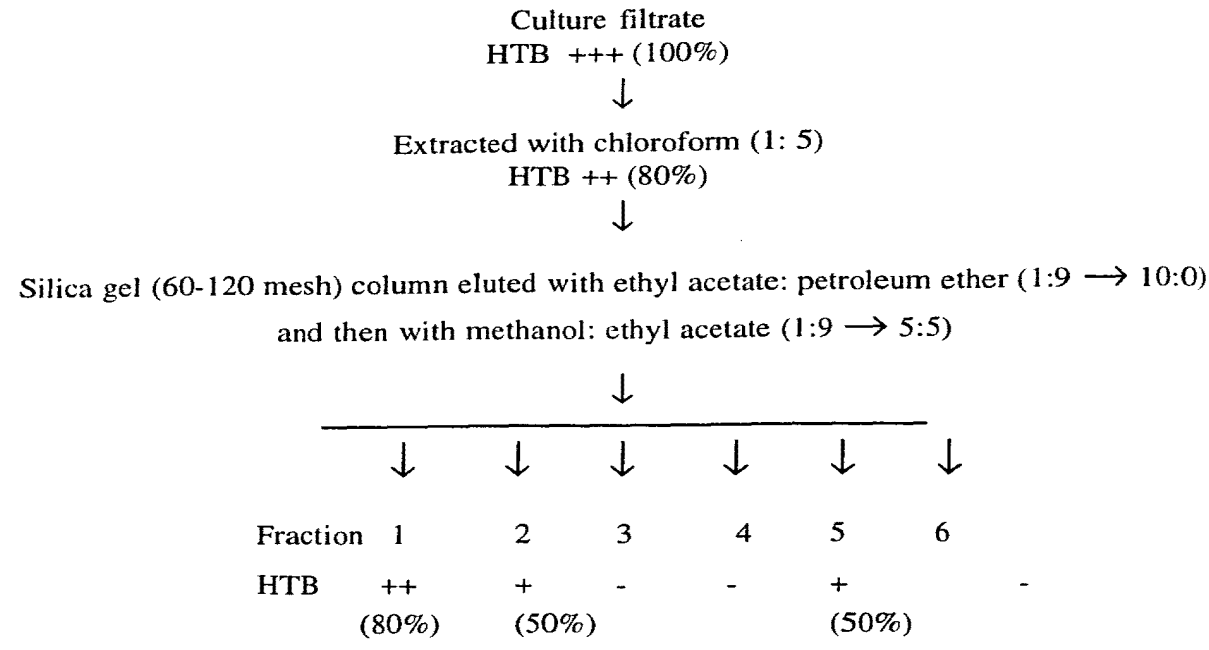

Table 2. Extraction of chitinase inhibitor(s) of *Bacillus* sp. 102

Organic solvent	Hyphal tip bursting test (%)
Culture filtrate	80 ± 5
Ethyl acetate	55 ± 10
Benzene	ND
Hexane	ND
Chloroform	70 ± 5

ND, not detected

pure inhibitor from *Bacillus* sp. 102 will be useful to identify its role explicitly. Koga *et al.* (1987) have reported the inhibitory effect of allosamidin on insect chitinase. Therefore, the further investigations on the contribution of chitins inhibitor from *Bacillus* sp.102 for the control of insect pest will be useful to design an integrated approach.

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