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RESEARCH ARTICLE

STUDY OF CELL WALL PREPARATION OF *PENICILLIUM DIGITATUM* AND LYTIC ENZYME FROM SOME BIOCONTROL AGENTS AND THEIR INTERACTION

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ABSTRACT

Cell wall was prepared from *P. digitatum*, dangerous post harvest disease of *Citrus* sp. The potentiality of lytic enzyme production by three biocontrol agents (*Trichoderma viride* -1, *T harzianum* -1 and *Beuveria bassiana*) was tested *in vitro* in mineral medium. In our experiment all three antagonists or biocontrol agents showed their ability to secrete lytic enzyme. Out of them the enzyme of *T.viride* I lysed 60% of the cell wall after 48 hrs, it was followed by *T. harzianum* I (45%) and *Beauveria bassiana* (37%). Moreover, within 24 hour, at 25^0C, the lysis by enzyme preparation of *T. viride*-I is maximum (30%) followed by 20^0C (26%) 30^0C (22%) and 15^0C (17%) and it indicated that at 25^0C, the efficacy of enzyme secreted by *T. viride*-I is optimum. It revealed that biocontrol agents during interaction with this pathogen may have secreted this enzyme to suppress or kill the pathogen.

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INTRODUCTION

Diverse microorganisms secrete and excrete metabolites that can interfere with pathogen growth and/or activities and one of very important metabolites is lytic enzyme. Lytic enzymes can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly. Control of *Sclerotium rolfsii* by *Serratia marcescens* appeared to be mediated by chitinase expression (Ordentlich *et al*., 1988). And, a β-1, 3-glucanase contributes significantly to biocontrol activities of *Lysobacter enzymogenes* strain C3 (Palumbo *et al*., 2005). While they may stress and/or lyse cell walls of living organisms, these enzymes generally act to decompose plant residues and nonliving organic matter. Currently, it is unclear how much of the lytic enzyme activity that can be detected in the natural environment represents specific responses to microbe-microbe interactions. It seems more likely that such activities are largely indicative of the need to degrade complex polymers in order to obtain carbon nutrition. Furthermore, some products of lytic enzyme activity may contribute to indirect disease suppression. For example, oligosaccharides derived from fungal cell walls are known to be potent inducers of plant host defenses.

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Interestingly, *Lysobacter enzymogenes* strain C3 has been shown to induce plant host resistance to disease (Kilic-Ekici and Yuen, 2003), though the precise activities leading to this induction are not entirely clear. The quantitative contribution of any and all of the above compounds to disease suppression is likely to be dependent on the composition and carbon to nitrogen ratio of the soil organic matter that serves as a food source for microbial populations in the soil and rhizosphere. However, such activities can be manipulated so as to result in greater disease suppression. For example, in post-harvest disease control, addition of chitosan can stimulate microbial degradation of pathogens similar to that of an applied hyperparasite (Benhamou, 2004).

Chitosan is a non-toxic and biodegradable polymer of beta-1, 4-glucosamine produced from chitin by alkaline deacylation. Amendment of plant growth substratum with chitosan suppressed the root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato (Lafontaine and Benhamou, 1996). Although the exact mechanism of action of chitosan is not fully understood, it has been observed that treatment with chitosan increased resistance to pathogens. Therefore, the main objectives of this work are to prepare cell wall from plant pathogen, to produce lytic enzyme from biocontrol agent and finally to evaluate of enzyme action or cell wall and enzyme interaction.

MATERIALS AND METHODS

Preparation of cell wall of the fungal pathogen (*Penicillium digitatum*)

The pathogen growing on PDA slants was harvested after 20 days of growth in 5 ml of distilled sterile water from each slant. An aliquot of 25 ml of pathogen suspension was obtained from 5 slants. They were crushed and homogenized in a homogenizer (A. HT Philadelphia, U.S.A). It was washed by repeated centrifugation (3000 rpm) with 0.1 m NaCl, 0.1M acetate buffer (pH= 5.5) and distilled water until the cells were free from cytoplasmic materials. The wall was inactivated by heating at 100^0C for 30 minutes in a boiling water bath. An aliquot 0.2, µM of Na_2NO_3 was then added to keep the preparation sterile. Preparation of enzyme from antagonistic fungi *Trichoderma viride* 1*, Trichoderma harzianum* 1 & *Beauveria bassiana* were grown separately in 250 ml conical flasks containing 100 ml mineral medium for 8 days at 25 $\pm1^0$C.

Mineral medium

Glucose (anhydrous) 10 g
Ammonium tartarate2g
$K_2HP0_4$1 g
$MgS0_4$, $7H_20$0.5g
Trace element solution 1 ml
and pH 5.5.
The trace element solution in (mg/L) : $Na_2B_4O_7$, $10H_20$, 100; $ZnS0_4$, 7 H_20, 70; $FeS0_4$, $7H_20$, 50; $CuS0_4$, $5H_20$, 10; $MnS0_4$, $4H_20$, 10; $(NH_4)_6$ Mn_70_{24}, $4H_20$, 10.

The culture filtrate was separated from the mycelium by filtration through Whatman Filter No. 3. The filtrate was centrifuged at 3000 rpm. for 30 minutes. The culture filtrate was then dialysed twice against two changes of double distilled water for 48 hrs. at 4^0C. This resultant preparation was taken as that of enzyme.

Measurement of Lysis

One mg. of cell wall preparation was taken in 1 ml. of 0.05 M borate- citrate-phosphate buffer (pH 5.5). This was inoculated for different times (12hr, 24hr, 36hr and 48hr) and temperature (5^0 C, 10^0 C, 15^0C, 20^0C, 25^0C, 30^0C, and 35^0C) with 1 ml enzyme preparation. Turbidity was measured in Nephelometric turbidity units (NTU) by Nephelometric method by Nephelometer (Systronic, India). The sample was diluted with one or more volumes of turbidity free water until turbidity level fell down within limits of 30 to 40 NTU. Turbidity in original sample was calculated from the turbidity of diluted sample and the dilution factor was known. The sample was, then transferred to the turbidometer tube and direct reading was taken on the scale. The calculation was done by using this formula:

Nephelometric Turbidity Units = A x (B+C)/C where A= NTU found in diluted sample, B= Volume of dilution water in ml; C = sample Volume taken for dilution in ml.

The percentage loss in turbidity indicates the measure of lysis of the cell wall of pathogens by enzymes of different antagonists or biocontrol agents. The boiled enzyme (30 minutes) was taken as control for the experiment.

RESULT AND DISCUSSION

The data in Table 1 indicated the degree of cell wall lysis of *P.digitatum* by enzymes of antagonists. The enzyme of *T.viride* I lysed 60% of the cell wall after 48 hrs, it was followed by *T. harzianum* I (45%) and *Beauveria bassiana* (37%). Earlier author reported the involvement of enzyme in mechanism of mycoparasitism of mycoparasites. Panchenari and Dix (1980) observed that *Gliocladium roseum* parasitized on *B.alli* by glucanase enzyme which degrades glucans of the cell wall. Elad *et al*. (1983) revealed lysed sites and penetration holes at the area of contact of *R. solani* hyphae, parasitized by *Trichoderma* spp.

They detected with scanning electron microscope and fluorescence microscope high amount of secretion of β-1, 3 glucanase and chitinase in dual agar cultures where *T. harzianum* parasitized *S. rolfsii*. The enzyme of *T. harzianum* rapidly digested (86%) the cell wall of *R. solani* within 25 hrs (Srivastava and Singh, 2000). Lytic enzyme of *T. harzianum* digested 76% of the cell wall preparation of *A. rabiei* (Chakraborty *et al*., 2008). In our experiment, enzymes of *T. viride* showed the same trend. The table 2 showed that within 24 hour, at 25^0C, the lysis of enzyme preparation of *T. viride*-I is maximum (30%) followed by 20^0C (26%) 30^0C (22%) and 15^0C (17%) and it indicated that at 25^0C, the efficacy of enzyme secreted by *T. viride*-I is optimum.

Table 1. Degree of lysis (%) of the cell wall preparation of the pathogen by different antagonists at 25±1^{0}C

Antagonists	*Percentage of lysis of cell wall *P digitatum*			
	12 hr	24hr	36 hr	48hr
Trichoderma harzianum I	15	25	35	45
T. viride I	30	37	50	60
Beauveria bassiana	12	25	30	37

*percentage mean data of three replicas

Table 2. Effect of different temperatures on the lyses cell wall by enzyme preparation of *T. viride*-I

Incubation temperature ^{o}C	Lysis (%) of *P. digitatum* cell wall preparation measured after 24 hour of incubation
5	00
10	06
15	17
20	26
25	30
30	22
35	12

Conclusion

In conclusion, all three biocontrol agents secreted lytic enzymes and *in vitro* enzyme–cell wall interaction showed cell wall degradation or digestion happened by this enzyme. It indicated that biocontrol agents during interaction between this pathogen may have secrete this enzyme to suppress or kill the pathogen.

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