

Original Research Article

Induction of hydrolytic enzymes of phytopathogenic fungi in response to *Trichoderma viride* influence biocontrol activity

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ABSTRACT

Trichoderma viride is one of the most extensively studied saprophytic fungi for its potent biocontrol efficiency. The resistance generated by some phytopathogenic fungi against *Trichoderma viride* can be attributed to the inactivation of hydrolytic enzymes of *Trichoderma viride* which mediates mycoparasitism. The studied *Trichoderma viride* exhibited antagonistic against five phytopathogens namely *Aspergillus niger*, *Pythium spp*, *Aspergillus flavus*, *Alternaria spp* and *Fusarium spp*. The presence of coiling suggests effective mycoparasitism in the fungal interactions studied. Hydrolytic enzyme assays of *Trichoderma* co cultured with pathogens shows increased activity of cellulase, chitinase and pectinase over monoculture which confirms positive induction of enzyme secretion by *Pythium spp*, *Alternaria spp* and *Fusarium spp*. The presence of pathogens like *Pythium spp*, *Alternaria spp* and *Fusarium spp* induced the total enzyme secretion of *Trichoderma viride*, which can be correlated to increased antagonistic activity of *Trichoderma viride* against these fungi. Increase in protease enzymes of phytopathogens decreased the total biocontrol activity of *Trichoderma* which can be due to inactivation of hydrolytic enzymes. It can be concluded that the inducible hydrolytic enzymes of phytopathogens namely protease can modulate mycoparasitism of *Trichoderma viride* in a negative manner which can affect the total biocontrol efficiency.

Keywords

biocontrol, mycoparasitism, fungal phytopathogens, hydrolytic enzymes, *Trichoderma viride*.

Introduction

Soil borne fungal phytopathogens like *Pythium*, *Fusarium*, *Alternaria*, *Aspergillus*, *Botrytis*, *Rhizoctonia* and *Phytophthora* cause both post and pre harvest pathologies in agricultural crops (Chet & Inbar, 1994). Traditional pesticides, fungicides and fumigants have drastic effects on the

environment in terms of pollution and toxicity. The generation of resistant strains due to the repeated application of these fungicides also offers serious challenges (Naseby, Pascual, & Lynch, 2001). As an advent to resist ecological impacts of chemical pesticides and fertilizers an array of biocontrol agents and biofertilizers including bacteria such as *Bacillus subtilis*

(Moyné, Shelby, Cleveland, & Tuzun, 2001), *Pseudomonas fluorescens* (Pal & McSpadden Gardener, 2006) and fungi like *Trichoderma viride* (Pal & McSpadden Gardener, 2006) was introduced. The use of *Trichoderma viride* is being considered as the most effective alternative for plant disease control and plant growth promotion (Moreno et al., 2009) over other marketed biocontrol agents (BCA's).

Trichoderma spp. is the most widely studied and used biocontrol agent against plant pathogens because of their ability to reduce the soil borne phytopathogens (Papavizas, 1985). Several species of *Trichoderma* have been commercially marketed as biopesticides, biofertilizers and soil amendments (G. E. Harman, 2000). These are free living, opportunistic, avirulent plant symbiont, highly interactive in root, soil and foliar environment. The antagonism of *Trichoderma spp.* towards the growth of several phytopathogenic fungi emerged them as most effective biopesticide. Moreover their ecological adaptability, wide distribution, tolerance to environmental changes (Elad, 2000), colonization of the rhizosphere, stimulation of plant defense, plant growth promotion especially root and high reproductive capacity are also advantageous (Gary E. Harman, Howell, Viterbo, Chet, & Lorito, 2004).

Trichoderma spp. have numerous mechanisms to arrest the proliferation and bioactivity of fungal phytopathogens. These strains are always associated with plant roots and root ecosystems hence induce plant resistance. They colonize root surfaces and causes substantial change in plant metabolism. Common routes of action include competition for space and nutrients (Ozbay Nusret, 2004), production of siderophores for iron chelation (Anke, Kinn, Bergquist, & Sterner, 1991),

(MadhuPrakashSrivastava, RichaTiwari, & Neeta Sharma, 2013), synthesis of inhibitory compounds (pyrone antibiotics) (Benítez, Rincón, Limón, & Codón, 2004) and the release of cell wall lytic enzymes including cellulolytic, chitinolytic, pectinolytic, proteolytic and lipolytic enzymes (Emma et al, 2008). The synergistic effects of these mechanisms elicit potent inhibition against other invading microorganisms.

The major challenge of *Trichoderma* as a potent biocontrol agent is their poor performance under field conditions. The use of *Trichoderma spp.* in commercial agriculture is not yet widespread because of their inefficiency on comparing with synthetic fungicides. Field studies has proven lesser efficacy of *Trichoderma spp.* against many wild pathogenic fungi and bacteria, which limits their application against synthetic fungicides. The decreased efficiency of *Trichoderma spp.* against some pathogens can be correlated to inappropriate substrate for the propagation of the conidia under field conditions. Furthermore the chance of inhibition of *Trichoderma spp.* by the hydrolytic enzymes produced by phytopathogens has not been unveiled yet. Based on this background, the present study attempts to evaluate the enzymatic mechanisms of *Trichoderma viride* and five model phytopathogenic fungi with respect to their synergistic as well as antagonistic effects on its biocontrol efficiency.

Materials and Methods

Isolation and identification of *Trichoderma spp*

Trichoderma spp. used for the current study was revived from a commercial biocontrol agent marketed in Thiruvananthapuram, Kerala. Talc mixed spore suspension was prepared by adding 1g of Biocontrol agent to 100ml of sterile distilled water. The

suspension was serially diluted and spread on a potato dextrose agar (PDA) plate with antibiotics and incubated at 28⁰C for 5 days. *Trichoderma spp.* were identified based on their morphological and cultural characteristics and microscopically by lactophenol cotton blue staining

Isolation of phytopathogens

Infected plant samples were collected from the local markets and fields. The samples were washed with tap water, examined visually and microscopically for infection. Selected samples were subjected to standard tissue isolation on PDA by hyphal tip isolation technique. *Pythium spp.* were observed and identified based on morphology and was further purified on 2% agar plates. *Fusarium spp.*, were isolated from agricultural soil sample by the method of serial dilution. Selected samples were incubated on PDA plates and observed for morphological characters. The isolated colonies were grown on PDA agar plates and identified by their morphological and staining characteristics with lactophenol cotton blue (Chakravarthi, Das, Surendranath, Karande, & Jayabaskaran, 2008).

The phytopathogens *Aspergillus niger*, *Aspergillus flavus* and *Alternaria spp* were also isolated from infected plant parts collected from agricultural field in Thiruvananthapuram as per standard procedures described above.

Inhibitory responses of *Trichoderma viride* against the isolated phytopathogenic fungi

Dual plate technique

Mycelial disc (7mm diameter) was obtained from the peripheral region of 5 days old culture of phytopathogens (*Fusarium spp.*,

Pythium spp., *Aspergillus niger*, *Aspergillus flavus* and *Alternaria spp.*) and placed on a PDA plate (3cm away from the center). The plates were incubated at 28⁰C for 48 hours to initiate growth. Then 7 mm diameter mycelial disc of *Trichoderma spp.* was obtained and placed 3cm away from the inoculated pathogen. The plates were incubated at 28⁰C and the radial growth was measured after 3 days of inoculation. Duplicates were kept for all plates. Radial growth reduction was calculated in relation to growth of the control as follows.

$$S = \frac{C - T}{C} \times 100$$

Where S is the % of inhibition, C is the radial growth of pathogen in the control plate; T is the radial growth of pathogen in the presence of *Trichoderma spp.*

Slide culture for the determination of mycoparasitism

Slides were pre coated with 2% agar and inoculated with *Trichoderma* in one end. The inoculum of *Fusarium spp.*, *Pythium spp.*, *Aspergillus niger*, *Aspergillus flavus* and *Alternaria spp.* Respectively were placed on the other side of the independent slide 1cm apart from each other. The slides were incubated at 28⁰C under moist conditions for 72 hours. After incubation the site of *Trichoderma* pathogen interaction was stained with lactophenol cotton blue and observed under a light microscope. The presence of coiling structure or wall disintegration was taken positive for mycoparasitism.

Effect of fungal pathogens on Total hydrolytic enzymes of *Trichoderma viride* under co-cultured condition

To study the effect of phytopathogens on the hydrolytic enzyme secretion of *Trichoderma*

viride, a co-culture of *Trichoderma viride* and each of the phytopathogens (*Fusarium spp*, *Pythium spp*, *Aspergillus niger*, *Aspergillus flavus* and *Alternaria spp*) were maintained individually. Enzyme crude extracts were retrieved at regular intervals such as 5th, 7th and 9th day and quantified for the activities of cellulase, chitinase and protease enzymes. To overrule the secretion of hydrolytic enzymes by fungal phytopathogens the enzyme activities of the given enzymes were determined for the monocultures of all the five phytopathogens as per procedures described below. *Trichoderma viride* monoculture was used as control.

Cellulase assay

The culture supernatants were separated from spores by centrifugation and used as crude enzyme source. 1ml of crude enzyme extract was incubated with 0.5ml of 1% carboxy methyl cellulose which was used as substrate for 20 minutes and the reaction was stopped by adding 2ml of 1% TCA. The total amount of reducing sugar formed after cellulose digestion was determined by modified Dinitro salicylic acid(DNS) following previously published protocols using glucose standards (Miller, 1959).

Chitinase assay

Colloidal chitin was prepared from the chitin flakes (Ferrari, Gaber, & Fraaije, 2014) and used as substrate for the assay. For chitinase assay, 2ml of 1% chitin in potassium phosphate buffer (pH 6) and 0.5 ml of enzyme extract was incubated at 25°C for 2 hours in a shaker. After incubation the reaction mixture was kept in boiling water bath for 2 minutes. Filtrate was collected by centrifugation, 1ml DNS was added and boiled for 5minutes. N acetyl glucosamine was used as standard.

Protease assay

The protease activity was measured (Anson, 1938) using casein as substrate. 1ml of 2% casein in phosphate buffer (pH 7-4) and 0.5ml of enzyme was incubated for 10 minutes at 30°C and the reaction was stopped by adding 2.5 ml of 5% TCA. The unreacted casein was precipitated by filtration, 0.5ml of filtrate was mixed with 2ml of 6% Na₂CO₃ and 0.5ml of 2N Folin's reagent was added. After 30 minutes of incubation at room temperature absorbance was measured at 750nm. Protease activity was expressed in enzyme units, where one unit was the amount of enzyme required to release amino acid present equivalent to 1µm of tyrosine under standard condition.

Results and Discussion

Trichoderma viride, were identified based on their macroscopic and microscopic features. Microscopically it showed septate hyaline hyphae. Conidiophores are hyaline, branched and phialides are also hyaline, flask-shaped, and inflated at the base. The color of the conidia is mostly green. The presence of these characteristics confirmed the *Trichoderma viride*.

Pythium isolates were characterized at genus level based on their cultural characteristics such as growth rate, sporangia color and sporulation rate. On PDA it shows fast dense hairy white growth with sporangia consisting of a terminal complex of swollen hyphal branches of varying length, broadly sac shaped antheridia, 24-29 µm sporangia (Plaats-Niterink, 1981).

Fusarium spp. were identified according to the colony morphology and pigmentation on PDA and microscopically by examining the microconidia, macroconidia, phialides and other feature under light microscope .Pink

colored colonies which turns white on further incubation, spindle shaped straight to slightly curved macroconidia produced in the aerial mycelium. Conidiophores are unbranched and monophialides and polyphialides are branched and microconidia are absent (Navi, Bandyopadhyay, Hall, & Bramel-Cox, 1999).

Alternaria spp. was identified according to the colony morphology. The culture showed abundant mycelium light olive green to brown in color, dark brown hyphae, thick, septate, and branched. Simple, erect clustered conidiophore which produced dark pigmented conidia. Chains of conidia are produced at the beak of a spore (Navi et al., 1999).

Aspergillus flavus was identified on the basis of their colony morphology, pigmentation and microscopic examination which showed yellowish green, compact and velvety with submerged mycelia and Conidiophores is colorless and swollen apically and bear phialides with conidia in long, dry chains. Conidial heads are typically spherical, splitting into several poorly defined columns. *Aspergillus niger* showed white to pale yellow colonies quickly formed jet black conidia., conidiophores long, smooth, and hyaline, Conidia brown to black, very rough and globose. Hyphae is septate and hyaline (Meera Gupta, Kumari Manisha, & Ruby Grover, 2012).

Inhibitory responses of *Trichoderma viride* against the isolated phytopathogenic fungi

Biocontrol efficiency of *Trichoderma viride* was determined using dual culture method against different pathogens studied. *Trichoderma viride* was found to be effective in inhibiting the growth and

progression of all the organisms studied. A maximum rate of inhibition was observed for *Pythium spp.*, in which the organism was completely inhibited at sixth day. *Alternaria spp.* exhibited low resistance towards *Trichoderma* mycelia progression but an 82.4% inhibition was observed at 12th day of incubation (Wani, Taylor, Wall, Coggon, & McPhail, 1971).

The standard slide culture confirmed the mycoparasitism of *Trichoderma viride* against the phytopathogens studied. On staining with Lactophenol cotton blue, a distinct coiling around fungal mycelia was observed. The images were taken microscopically at 40x. All the organisms showed comparatively good mycelial coiling where *Pythium spp.* dominated in this aspect also. The results suggest mycoparasitism as the predominant mechanism of biocontrol activity (Fig 2).

Effect of fungal pathogens on Total hydrolytic enzymes of *Trichoderma viride* under co-cultured condition

Cell wall degrading enzymes especially cellulase, chitinase and protease possess considerable significance in maintaining the biocontrol efficiency of *Trichoderma viride* against many of the phytopathogens.

Cellulase

A maximum activity of 7.9×10^{-1} enzyme units was observed for *Trichoderma viride* which decreased further on incubation. From the results it can be observed that apart from *Trichoderma viride* all monocultures of phytopathogens shown negligible cellulase production. Co culturing of *Trichoderma* with *pythium*spp produced considerable increase in cellulase enzyme secretion where nearly 19% increase was observed on 9th day. *Pythium spp.* alone showed

negligible cellulolytic activity in monocultures (Nemec, 1974). An increase of cellulase activity was observed for *Fusarium spp.* and *Alternaria spp.* at 5th day of incubation suggesting induction of *Trichoderma viride* enzymes by pathogen mycelial mass but *Aspergillus flavus* failed to elicit cellulase enzyme secretion in co cultures.

The role of *Pythium spp.* in *Trichoderma viride* enzyme production can be laid down to findings by Garrett (Garrett, 1951), who reported that *Pythium spp.* rarely produce cellulase enzyme. Many workers had also given evidences that *Pythium spp.* possess little ability to degrade complex structural complexes containing cellulose (Burgess & Fenton, 1953). The increase in enzyme production in *Trichoderma viride* suggests induction of cellulase gene by *Pythium spp.* and other fungi studied, which is concomitant with suggestions of different workers (Chet & Inbar, 1994)(de las Mercedes Dana et al., 2001).

Chitinase activity

Trichoderma chitinase is also an important enzyme which plays a major role in cell wall degradation, since most phytopathogens possess chitin as a cell wall constituent. *Trichoderma viride* showed a maximum activity of 7.91×10^{-1} enzyme units. *Pythium spp.* and *Alternaria spp.* exhibited chitinase activity of 6.40×10^{-1} and 5.72×10^{-1} respectively. Other fungi under investigation had shown slight chitinase activity. Chitinase activity of co-cultures had comparatively low activity for *Trichoderma* enzymes. From the results it can be observed that only *Trichoderma viride* produced significant chitinase whereas co cultures showed considerable decrease in total chitinolytic activity. The effect will be due to the inactivation of fungal enzymes of

phytopathogen by *Trichoderma viride* (Viterbo, Ramot, Chemin, & Chet, 2002).

Protease activity

Trichoderma protease was reported to have a role in inactivating enzymes secreted by phytopathogen in co-culturing. Only *Aspergillus flavus* and *Aspergillus niger* showed significant proteolytic activity when grown along with *Trichoderma viride*. *Trichoderma viride* showed minimal or no proteolytic activity in almost all co-cultures (*Pythium spp.*, *Fusarium spp.*, *Aspergillus flavus*, *Aspergillus niger* and *Alternaria spp.*). The least biocontrol efficiency of *Trichoderma viride* against phytopathogens *Aspergillus niger* and *Aspergillus flavus* can be attributed to activation of proteolytic enzymes. The considerable decrease in *Trichoderma* protease can be attributed to that fact that most of the proteolytic enzymes of *Trichoderma viride* is stress or metabolite induced (Kredics et al., 2005).

Trichoderma viride mediates its biocontrol activity via penetrating the mycelia of the phytopathogens and promoting its further coiling. On co-culturing with phytopathogens, there can be a synergistic or antagonistic enhancement in the total hydrolytic enzyme secretion. The hydrolytic enzyme secretion of *Trichoderma spp.* is highly influenced by the presence of other phytopathogens such as *Pythium spp.*, *Fusarium spp.*, *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria spp.* in the rhizosphere which were studied in detail. Here we used the phytopathogens as inducers of enzyme production.

Many species of *Trichoderma* have been used as potent biocontrol agents for a variety of soil-borne phytopathogenic fungi. The response of *Trichoderma viride* to a potential host includes production of

antibiotic compounds, formation of specialized structures and degradation of the host's cell wall followed by the assimilation of its cellular content, namely mycoparasitism. Mycoparasitism has been proposed as the central mechanism accounting for the antagonistic activity of *Trichoderma viride*. (S. Ojha, 2011). Hydrolytic enzymes produced by *Trichoderma viride*, such as chitinases, β -1-3 glucanases, β -1-6 glucanases, and proteases facilitate penetration of the host cell wall but we mainly focused on effect of protease on inhibition of hydrolytic enzymes.

Filamental fungal cell wall also contains lipids and proteins. It is therefore expected that antagonistic fungi synthesize protease which may act on the host cell walls. In the absence of phytopathogens there was no considerable protease secretion. The major polysaccharides of fungal cell walls are chitin and the secretion of chitinase is a prerequisite in this biocontrol activity of *Trichoderma viride*. From the monocultures *Trichoderma viride*, it was observed that *Trichoderma viride* secreted very less amount of hydrolytic enzymes in the absence of pathogens. Most of the pathogens including *Pythium spp.* and *Alternaria spp.*

were hyper producers of chitinase and cellulase (De Marco & Felix, 2002).

Co culturing results suggest significant shift in metabolic activity of phytopathogens and decrease in enzyme secretion. It has already been reported that there exhibits a synergism between lytic enzymes and antibiotics of fungi. It can be concluded that the initial secretion of enzymes triggered this expression of lytic enzymes and antibiotics in *Trichoderma viride* hence activating the mycoparasitism. Similarly chitinase gene induction is reported in *R. solani* (Kullnig, Mach, Lorito, & Kubicek, 2000).

The biocontrol efficiency of *Trichoderma spp.* depends largely on its ability to secrete hydrolytic enzymes and the most the hydrolytic enzymes in *Trichoderma viride* are inducible. The presence of protease producing *Aspergillus niger* and *Aspergillus flavus* significantly altered the hydrolytic enzyme secretion and decreased the biocontrol efficiency of *Trichoderma viride* against these species. The study clearly depicts the significance of pathogenic enzymes in determining the biocontrol efficiency and resistance imparted by *Trichoderma viride* against phytopathogens.

Fig 1. The isolated colony of *Trichoderma viride* (A) showing hyphal coiling suggesting mycoparasitism (B).

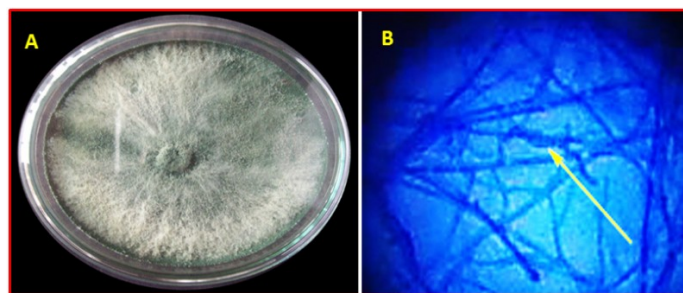


Fig.2 Dual plate technique showing antagonistic activity of *Trichoderma viride* against phytopathogens viz. *Alternaria* spp., *Fusarium* spp., *Pythium* spp., *Aspergillus* spp. and the respective control plates of the phytopathogens.

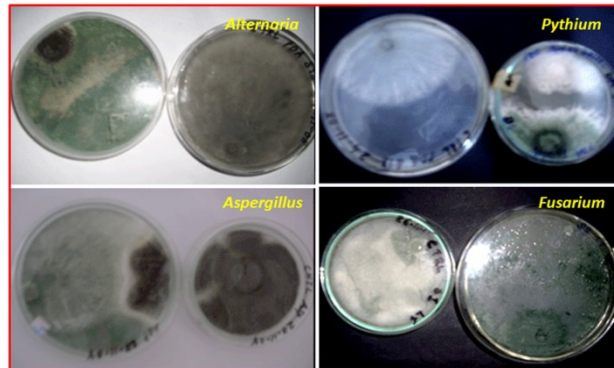


Fig 3.Co-culture of *Trichoderma* with phytopathogens

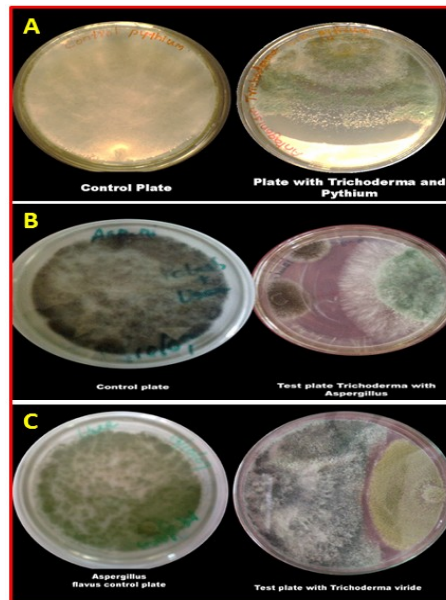


Fig.4 Percentage inhibition of the growth of phytopathogens upon co-culture with *Trichoderma*

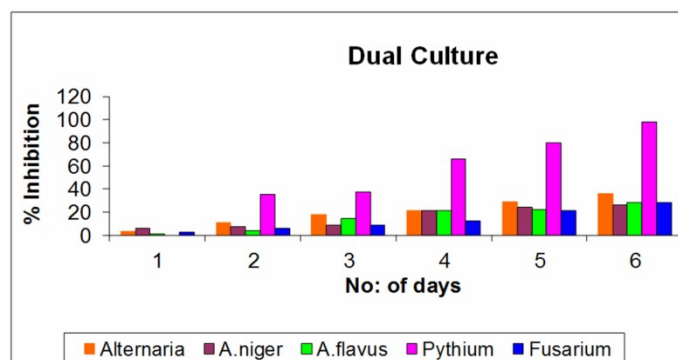


Fig 5.Determination of cellulase activity.Co culturing of *Trichoderma* with *Pythium* spp showing nearly 19% increase on 9th day of culturing

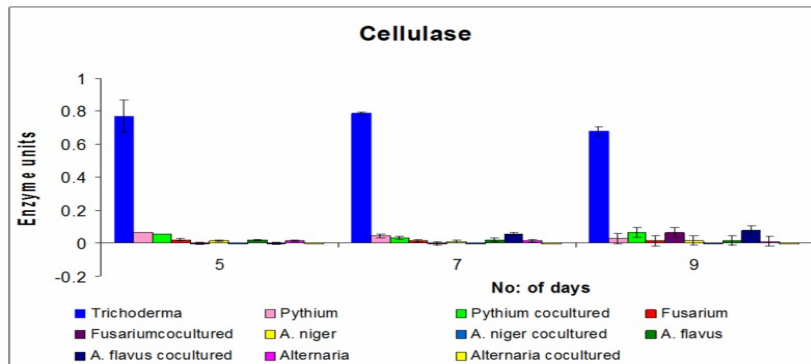


Fig 6.Determination of chitinase activity.*Trichoderma viride* showed higher chitinase activity when compared with co cultures

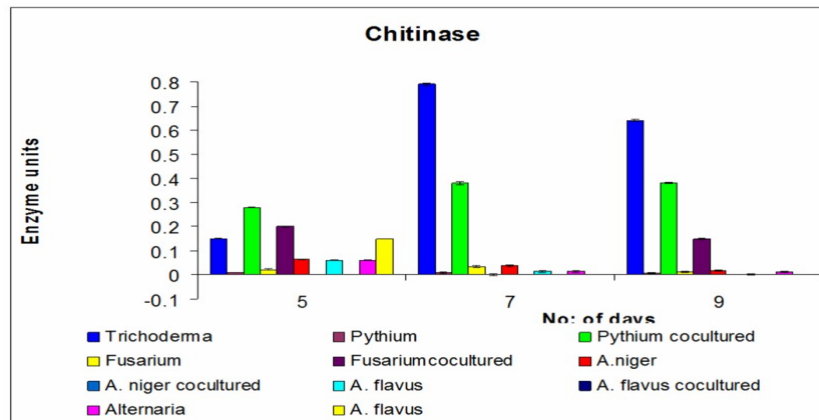
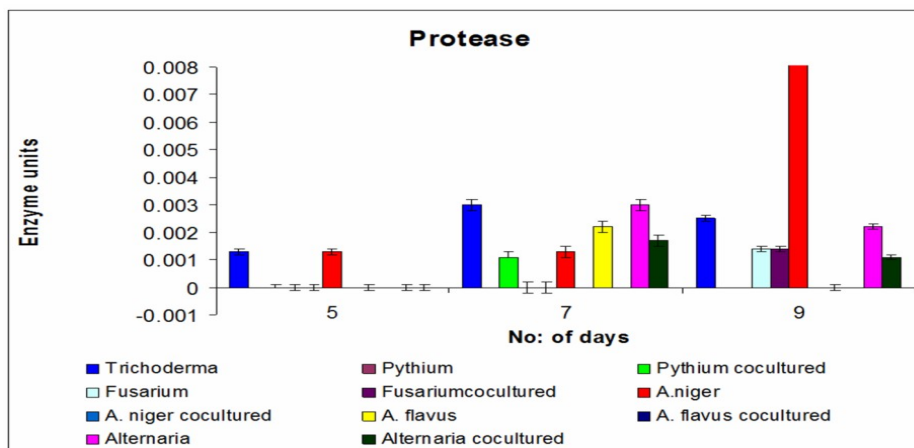


Fig.7 Determination of cellulase activity. *Aspergillus flavus* and *Aspergillus niger* showing increased proteolytic activity on co-culture with *Trichoderma viride* in comparison with co-cultured *Pythium* spp., *Fusarium* spp., *Aspergillus flavus*, *Aspergillus niger* and *Alternaria* spp.



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