Efficacy of *Trichoderma* chitinases against *Rhizoctonia solani*, the rice sheath blight pathogen

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Thirty-five strains of *Trichoderma viride* and *T. harzianum* were screened for their antagonistic ability against the rice sheath blight pathogen, *Rhizoctonia solani*. The strains that inhibited/overgrew the phytopathogenic fungus were considered effective. Light microscopic studies showed the antagonism of the hyphae of effective *Trichoderma* strains towards their host hyphae. Chitinase activity of *Trichoderma* culture filtrates was enhanced, when colloidal chitin was used as the sole carbon source, instead of glucose. Chitinase pattern differed among the four select strains. The chitinase isoforms are induced differentially by carbon sources. The chitin affinity column fraction of *Trichoderma* culture filtrate inhibited, *in vitro*, the growth of *R. solani*.

1. Introduction

Rice sheath blight caused by *Rhizoctonia solani* Kühn is regarded as an important disease world-wide second only to rice blast (Dasgupta 1992). The biocontrol agents, *Trichoderma viride* and *T. harzianum* (Papavizas 1985) have been shown to act as mycoparasites against *R. solani* (Wells *et al* 1972) and also against a range of other phytopathogenic fungi such as *Macrophomina phaseolina* (Elad *et al* 1986) and *Sclerotium rolfsii* (Mukhopadhyay *et al* 1986). *Trichoderma* spp. are known to produce chitinases, β-1,3-glucanases, proteases and volatile and non-volatile antibiotics (Elad *et al* 1982). Relationship between the mycolytic enzymes, viz., chitinases and β-1,3-glucanases of mycoparasitic fungi and their significance in fungal cell wall lysis and degradation has been well established (Elad *et al* 1980, 1983). *Gliocladium virens* that produces an endochitinase, is successfully used as a biocontrol agent against a wide range of phytopathogenic fungi, including *R. solani* (Lumsden and Locke 1989). *T. harzianum*, which produces an endochitinase, chitobiosidase and N-acetyl-β-glucosaminidase, was demonstrated to be a potent biocontrol agent against several phytopathogenic fungi viz., *R. solani*, *Sclerotium rolfsii* (Cook and Baker 1983) and *Pythium ultimum* (Pe'er and Chet 1990). Lorito *et al* (1993a) purified an endochitinase and a chitobiosidase from *T. harzianum* and showed their ability to inhibit spore germination and germ tube elongation in *Botrytis cinerea*, *Fusarium solani* etc. They also demonstrated the higher activity and wider spectrum antifungal nature of the chitinolytic enzymes of *T. harzianum* than those of higher plants or bacteria.

Our frequent encounters with *Trichoderma* strains with reduced or no biocontrol ability in laboratory and field trials, made screening for effective strains the major objective. Dennis and Webster (1971) recommended dual culture technique and Elad *et al* (1982) suggested chitinase assay for identifying effective biocontrol agents. Moreover, our future objective of cloning promising *Trichoderma* chitinases prompted us to carry out the preliminary studies to understand strain specific expression patterns and substrate-induction of chitinases. Purification and characterization of chitinases from an effective strain of *Trichoderma* was also attempted.

2. Materials and methods

2.1 *Screening Trichoderma strains against the pathogen*

R. solani (RS-7) and 34 strains of *T. viride* (G1, G2, G3, G4, G5, G10, M21, MG9, MNT1, MNT2, MNT3, MNT5,

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MNT6, MNT7, MNT8, MNT9, MNT10, MNT20, MUU7, MUU17, MUU28, NG10, NG17, NTG1, NTG2, NTG3, NTG5, NTG10, *Tv*C3, *Tv*C6, *Tv*I, *Tv*LY, *Tv*PDK and UV10) and one strain of *T. harzianum* (*Th*I),were maintained on potato dextrose agar-PDA (Nakkeeran *et al* 1996). Screening for antagonistic ability of *Trichoderma* strains against *R. solani* was done by dual culture technique (Dennis and Webster 1971). One mycelial disc (9 mm) of each fungus, was placed at opposite poles on PDA plates and incubated at 28 ± 2°C. After 2–3 d, the hyphal interactions were studied under Leitz inverted light microscope.

2.2 *Substrate-induction of extracellular chitinases*

Based on their performance against *R. solani* on dual plates, 7 strains were used for studying the substrate-induction of their chitinases. When required, glucose in the basal medium (0·02% $MgSO_4$ $7H_2O$, 0·09% K_2HPO_4, 0·02% KCl, 0·01% NH_4NO_3 0·0002% $FeSO_4$, 0·0002% $ZnSO_4$, 0·0002% $MnCl_2$, 0·5% glucose, pH 6·3–6·8; Elad *et al* 1982) was substituted either with colloidal chitin (Berger and Reynolds 1958) or *R. solani* cell wall preparation (Chet and Huttermann 1980), each to a concentration of 2 mg ml^{-1}. Mycelial discs (9 mm) from 5 day-old cultures of *Trichoderma* strains were inoculated in 50 ml medium and grown at 28 ± 2°C on a rotary shaker for 90 h. The cultures were filtered through Whatman 1 filter paper and the filtrates dialyzed against sterile water, at 4°C, to eliminate residual glucose or N-acetylglucosamine (GlcNAc). The dialysates were tested for chitinase activity (Boller *et al* 1983) and for symptom bioassay, as detailed below. A portion of the dialysate was lyophilized and the proteins were estimated (Bradford 1976) and analysed (20 µg protein/slot) on a 12% SDS-polyacrylamide gel (Laemmli 1970).

2.3 *In vivo efficacy of culture filtrates against the pathogenic fungus*

Efficacy of dialysates of *Trichoderma* culture filtrates on sheath blight symptoms on intact leaf sheaths was studied. Leaf sheaths of the rice cultivar, Co45 were pre-treated by applying onto them a cotton wool holding 500 µl of dialysate. On removal of the cotton wool after 24 h, a 5 day-old mycelial mat of *R. solani* was placed onto the pre-treated leaf sheaths. The mat was protected from dehydration by cotton wool dipped in water. Suitable controls without pre-treatment/inoculation were also maintained. Size (area necrosed in mm^2) of the lesions, developed 7 d after inoculation was measured and the relative efficacy of dialysates in reducing the lesion size, as compared to that in inoculated check not treated with dialysate, was taken as a measure of symptom suppression.

2.4 *Partial purification of chitinases*

For chitinase purification, MNT7 was grown in liquid medium with colloidal chitin (2 mg ml^{-1}) as the sole carbon source. The culture filtrate was centrifuged at

Table 1. Screening *Trichoderma* strains for antagonism towards the rice sheath blight isolate of *R. solani*.

Category	Size of inhibition zone*/Remarks	Effect of culture filtrate-dialysates on sheath blight symptom reduction†
A. Antagonist's inhibition by overgrowth on the mycelium of the pathogenic fungus		
G10	Complete overgrowth on the pathogen 7 d after plating	$1·7 \pm 0·2^e$
MNT2	Complete overgrowth on the pathogen 7 d after plating	$1·9 \pm 0·1^f$
MNT3	Complete overgrowth occurring 10 d after plating	$0·3 \pm 0·1^b$
MNT6	Overgrowth on the pathogen was very slow	$0·5 \pm 0·1^c$
MNT7	Complete overgrowth 7 d after plating	$3·3 \pm 0·3^f$
MNT10	Complete overgrowth occurring 7 d after plating	$2·2 \pm 0·2^g$
NTG3	Overgrowth on the pathogen was very slow	$0·7 \pm 1^d$
*Tv*I	Complete overgrowth occurring 6 d after inoculation with copious sporulation of the antagonist	$2·7 \pm 0·2^h$
UV10	Overgrowth was very slow	$0·7 \pm 0·2^d$
B. Pathogen's uninhibited advancement on the medium coupled with overgrowth on the antagonists' mycelium		
MUU17, *Tv*C3 and *Tv*PDK	No inhibition zone; overgrowth of the pathogen limited to 5 mm	0^a
C. No interaction; Antagonist and the pathogen are mutually exclusive		
G1; G2; G3; G4; G5; M21; MG9; MNT1; MNT5; MNT8; MNT9; MNT20; MNT21; MUU7; MUU28; NG10; NG17; NTG1; NTG2; NTG5; NTG10; *Tv*LY; ThI		0^{a**}

*Thickness of the interface; mean of three replicates; †difference in size (mm^2; surface area necrosed) of the lesions on leaf sheaths induced by *R. solani* consequent to pretreatment of the leaf sheaths with the dialysate (see § 2 for details); mean of 5 replicates; entries followed by the same alphabet are not statistically significant. **performed using G1, MNT20 and NG17.

6000 *g* for 10 min at 4°C. The supernatant was precipitated with 80% ammonium sulphate. The pellet obtained after centrifugation at 12000 *g* for 20 min at 4°C was resuspended in a minimal volume of distilled water and dialyzed against 70 mM potassium phosphate buffer, pH 6·0 at 4°C (de la Cruz *et al* 1992). The 80% ammonium sulphate fraction was mixed with sodium phosphate buffer-equilibrated colloidal crab shell chitin. After mixing for 4 h, the mixture was washed thrice with 30 ml sodium phosphate buffer and thrice with 30 ml 25 mM sodium citrate, pH 4·0. Then the mixture was packed into a column at 4°C and the chitin-binding proteins were eluted at the same temperature, with 20 mM acetic acid, pH 3·2 (Swegle *et al* 1992). The proteins were analysed (20 µg protein/slot) on an SDS-polyacrylamide gel. Antifungal activity of this fraction was checked through an inhibition zone technique, with a two day-old *R. solani* culture, using filter paper discs (0·6 mm) impregnated with this fraction with 40 µg of proteins. Filter paper discs with buffer alone served as control. Inhibition zone was visualized after two days.

2.5 *Western blotting*

Western blotting was done according to Winston *et al* (1987) with dialyzed culture filtrates (20 µg protein/slot)

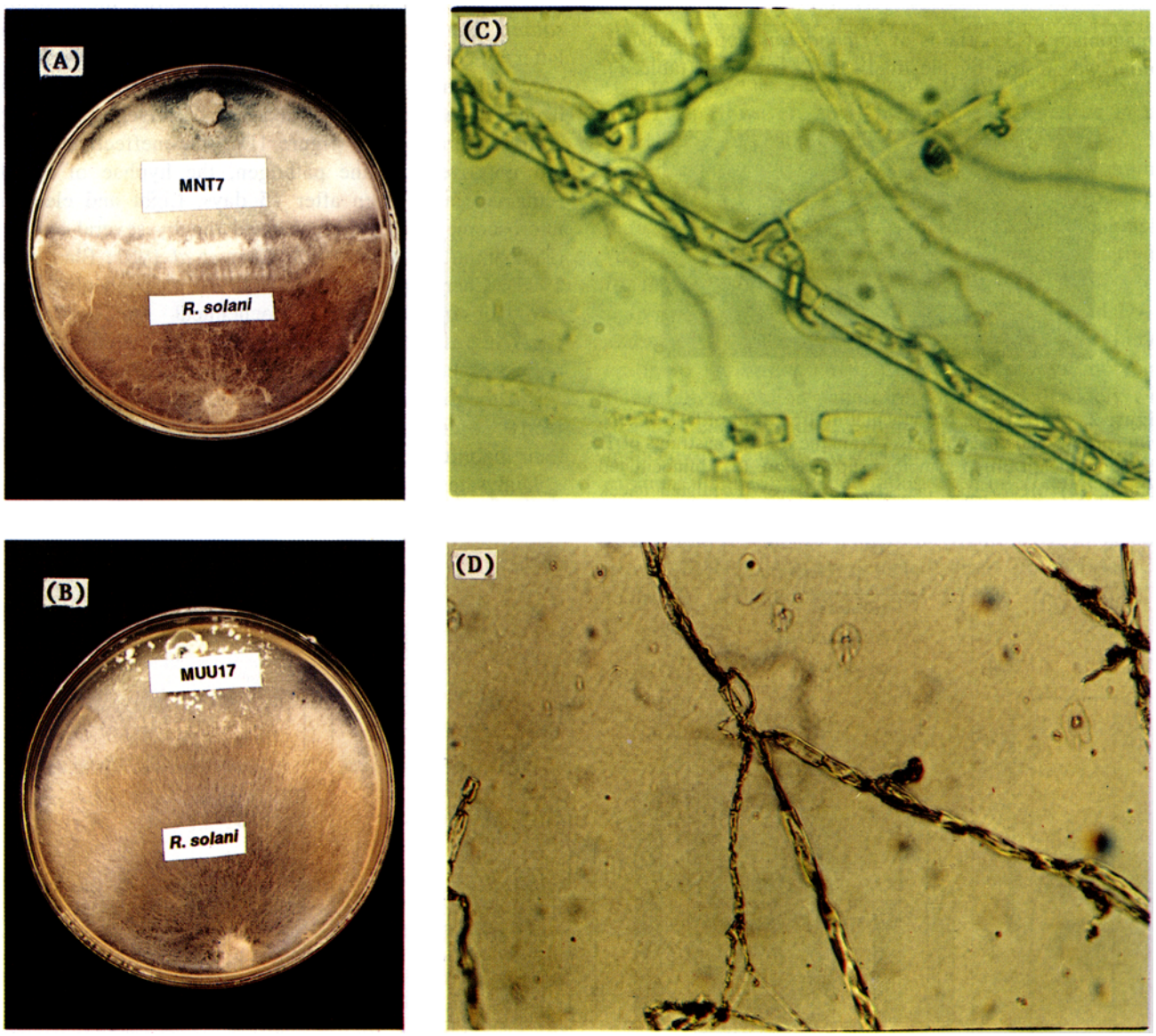

Figure 1. (**A**) Antagonist's inhibition and complete overgrowth exhibited by *Trichoderma* strain MNT7 towards *R. solani*. (**B**) *R. solani*'s uninhibited advancement on the medium coupled with overgrowth on the antagonist MUU17 mycelium. (**C**) Light micrograph showing coiling of intact *R. solani* hyphae by MNT7 hyphae as observed 3 days after plating. (**D**) Light micrograph showing coiling, shrivelling of parasitized host hyphae as observed 7 days after plating.

of *Trichoderma* strains grown on different carbon sources and also with chitin affinity column fraction (10 µg protein/slot) of culture filtrate of MNT7. The primary antibody used in the study was an anti-barley chitinase antiserum, a generous gift from Prof. S Muthukrishnan, Department of Biochemistry, Kansas State University, USA.

2.6 *Statistical analysis*

Duncun's multiple range test (DMRT; Gomez and Gomez 1984) was used to compare treatment means using the software, 'IRRIstat' developed by the International Rice Research Institute, Los Baños, Manila, The Philippines.

3. Results and discussion

Antagonism of 35 strains of *Trichoderma* spp. against *R. solani* was tested by the dual culture method (table 1).

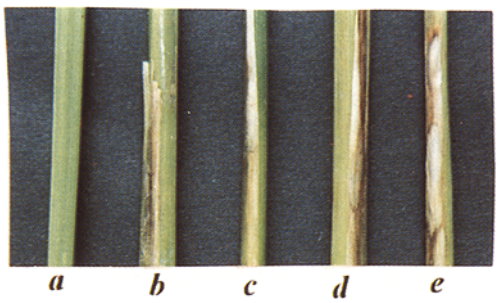

Figure 2. Efficacy of pre-inoculation treatment of leaf sheaths with partially purified chitinases from the following strains of *T. viride* on sheath blight symptom suppression. (**a**) Uninoculated control; (**b**) MNT7; (**c**) *Tv*I; (**d**) *Tv*C3; (**e**) inoculated check without pretreatment.

Earlier, Bell *et al* (1982) classified *Trichoderma* isolates based on their ability to overgrow *R. solani.* An isolate that overgrew the pathogen, covering completely or at least two-thirds of the medium surface, was considered antagonistic. In the present study, the strains, G10, MNT2, MNT7, MNT10, and *Tv*I) induced well-defined inhibition zones between the advancing frontiers of their mycelial growth and that of the pathogen, inhibiting the latter's growth, covering more than three-fourths of the surface (table 1; figure 1A). These effective strains were selected for further studies. Such antagonism was not noticed with MUU17, *Tv*C3 and *Tv*PDK (table1; figure 1B).

Observation of interacting mycelia under Leitz inverted light microscope revealed the antagonism of the hyphae of effective strains of *Trichoderma* towards those of *R. solani.* The former established close contact with the latter by coiling around them tightly, even at early stages of cocultivation (figure 1C). In later stages, *R. solani* hyphae showed extreme shrinkage and shriveling, probably an irrecoverable state (figure 1D). The ineffective strains did not overgrow the pathogen, the hyphae of which remained intact even after 15 days. Light and electron microscopic studies were carried out by several authors (Benhamou and Chet 1993; Chet *et al* 1981; Elad *et al* 1982). Cherif and Benhamou (1990), through ultrastructural observation and cytochemical localization of GlcNAc residues, concluded that a chitinolytic enzyme played a major role in the parasitism of *Trichoderma* on *Fusarium oxysporum.* Bell *et al* (1982) distinguished ineffective strains of *Trichoderma* from the effective ones, by their inability to invade the pathogen.

Dialysates of the culture filtrates of the antagonistic strains were tested for their efficacy to reduce the symp-

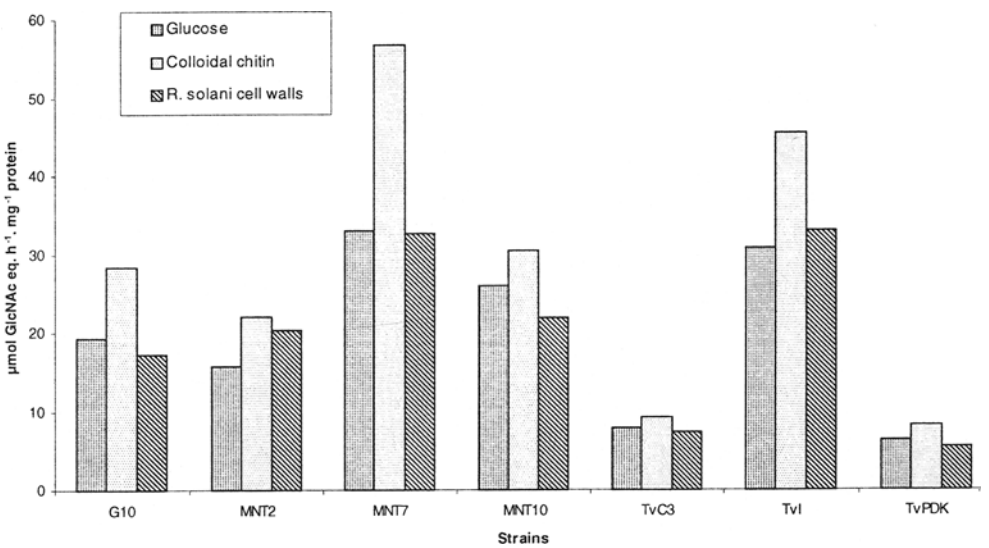

Figure 3. Specific chitnase activity of strains of *Trichoderma* grown on synthetic medium amended with different carbon sources.

tom induction by the pathogenic fungus on rice leaf sheaths (table1; figure 2). Dialysates of the culture filtrates of category A strains could limit the development of necrosis to varying degrees, while those of category B or C strains did not show any discernible effect. Among the strains tested, MNT7 recorded the greatest reduction in lesion size.

The role of glucanases and chitinolytic enzymes in fungal cell wall degradation during mycoparasitism by *Trichoderma* spp. is well documented (Lorito *et al* 1993b; Ridout *et al* 1986; Sivan and Chet 1989). In the present study, the extracellular chitinase activity was taken as a measure of antagonistic efficacy of *Trichoderma* spp. Chitinase assays (Elad *et al* 1982) were carried out with the filtrates of 4 day-old cultures, grown on different carbon sources (figure 3). In all the strains, colloidal chitin encouraged highest chitinase induction. The effective strains recorded higher specific activity, with MNT7 reaching the maximum, 56·8 µmol GlcNAc equivalents h^{-1} mg^{-1} of protein, while the ineffective *Tv*PDK recorded only 8·28 µmol GlcNAc equivalents h^{-1} mg^{-1} of protein. Earlier, chitinases were shown to be inducible by chitin or fungal cell walls (Monreal and Reese 1969; Leake and Read 1990; Ulhoa and Peberdy 1993). The reduced levels of chitinase activity associated with glucose, in the present study, could be attributed to probable repression of chitinase by glucose, as was observed in a chitinase over-producing mutant of *Aphanocladium album* (Vasseur *et al* 1990).

Analysis of extracellular proteins of *Trichoderma* strains, grown in a medium with colloidal chitin, revealed over-expression or specific expression of certain proteins (figure 4) by the effective strains (G10, MNT7, MNT10, and *Tv*I). However, apart from these differences in the level and distribution of proteins, the profiles are similar in general. Among the polypeptides found in the culture filtrates of MNT7, those of 30, 42 and 64 kDa were enriched upon chitin affinity column chromatography (figure 5A). This fraction also showed antifungal activity, *in vitro* (data not shown).

In Western blotting analyses (figure 4B, C and D) of extracellular proteins of *Trichoderma,* the anti-barley chitinase antiserum (ABCA) detected several polypeptides, the expression pattern of which varied widely with different carbon sources, suggesting that chitinases were induced differentially by carbon sources. The detection of minimal number of polypeptides, even in effective strains, when grown on glucose, suggested that chitinases may be susceptible to glucose repression (figure 4B). In glucose grown MNT7, ABCA detected three polypeptides of molecular weights, 30, 42 and 50 kDa. In MNT10, a

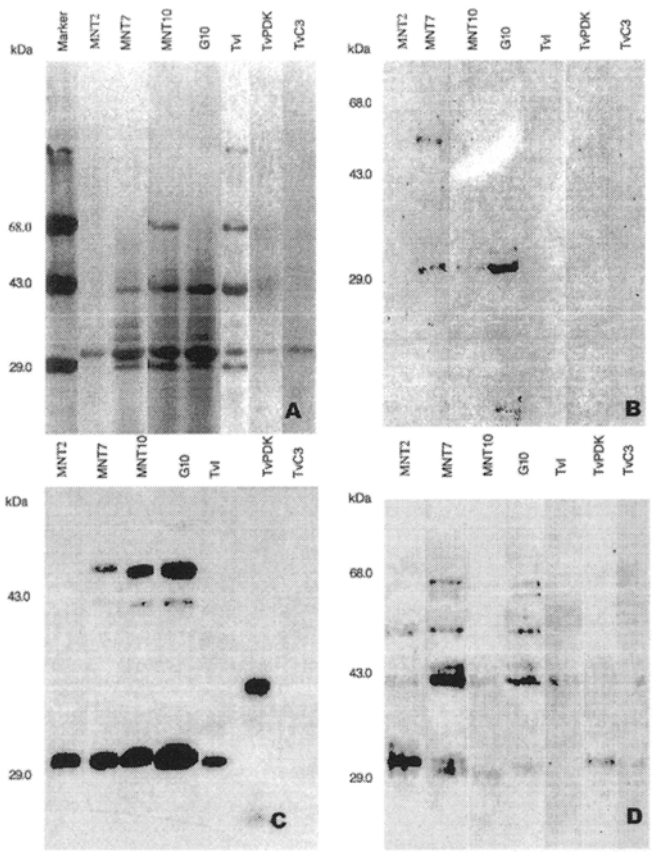

Figure 4. (**A**) Extracellular protein profile of strains of *Trichoderma* grown on synthetic medium amended with colloidal chitin as carbon source. (**B**) Western blot showing extracellular chitinase pattern of strains of *Trichoderma* probed with barley chitinase antisera grown on synthetic medium amended with glucose as carbon source. (**C**) Western blot showing extracellular chitinase pattern of strains of *Trichoderma* probed with barley chitinase antisera grown on synthetic medium amended with colloidal chitin as carbon source. (**D**) Western blot showing extracellular chitinase pattern of strains of *Trichoderma* probed with barley chitinase antisera grown on synthetic medium amended with *R. solani* cell walls as carbon source.

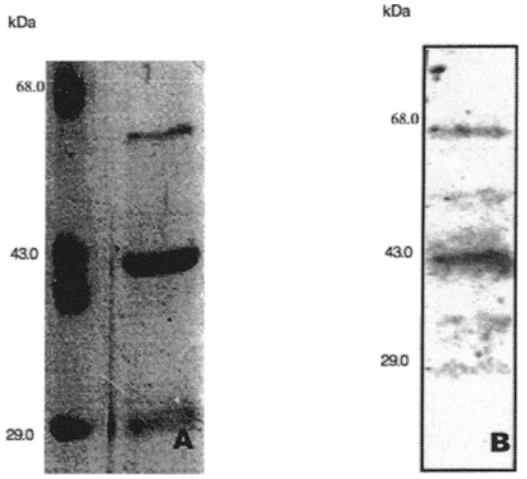

Figure 5. SDS-PAGE (**A**) and Western blot (**B**) of chitin affinity column fraction of MNT7 culture.

30 kDa polypeptide was detected. Along with the 30 kDa polypeptide, a 15 and 32 kDa polypeptides were also detected in G10. In other strains, ABCA did not detect apparently any polypeptide.

ABCA detected a number of extracellular polypeptides in *Trichoderma* grown on colloidal chitin or *R. solani* cell walls (figure 4C and D). Being the substrates for chitinases, these carbon sources induced many polypeptides, detectable by ABCA. In all the effective strains, colloidal chitin induced a 30 kDa polypeptide. Likewise, *R. solani* cell walls induced a 30 kDa polypeptide in all the strains except *Tv*I, an effective strain. *R. solani* cell walls also induced a 42 kDa polypeptide in all the strains, except in ineffective *Tv*PDK. Along with the 30 and 42 kDa polypeptides, a number of polypeptides were detected in effective strains grown on these substrates. Generally, the effective strains expressed more number of ABCA-detectable polypeptides, probably chitinases, when grown on these substrates. For instance, G10 and MNT7 expressed six polypeptides of molecular weights, 30, 42, 44, 50, 60 and 64 kDa. These results showed the substrate inducibility and glucose repressibility of *Trichoderma* extracellular chitinases. The 30, 42 and 64 kDa polypeptides, enriched by the chitin affinity column (figure 5A), were detected clearly by ABCA (figure 5B).

SDS-PAGE (figure 4A) and western blotting analyses (figure 4C and D) pointed at the 30 and 42 kDa polypeptides (based on their association with almost all the effective strains) as major chitinases. However, the contribution by other chitinases can not be ruled out. The chitinase nature of 30, 42 kDa polypeptides, along with a 64 kDa one, were suggested also by their enrichment by chitin affinity column (figure 5A) and by their ABCA detectability (figure 5B). This apart, the enriched fraction also showed ability to inhibit the pathogen *in vitro* (data not shown).

The present study identified a set of highly effective strains of *T. viride*. Field trials are being performed with these strains, with a view to introduce them in agricultural practice. Efforts are on the way to purify and further characterize the major chitinases (30, 42, 64 kDa), identified in this study. Our ultimate aim is to identify the genes encoding these chitinases and eventually introducing into indica rice genome so as to evolve sheath blight resistant cultivar.

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