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ENZYMATIC AND SAPROPHYTIC ABILITY OF *Trichoderma* SPECIES FOR BIOLOGICAL CONTROL OF FUNGAL PLANT PATHOGENS

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ABSTRACT

***Trichoderma* species have been reported as most potential biocontrol agents against several fungal plant pathogens. Fungal cell walls are complex structures constituted mainly of polysaccharides such as chitin, β-glucan and proteins. These polysaccharides act as inducers of hydrolytic enzymes and thus cell wall-degrading enzymes such as chitinases and β- 1, 3-gulcanases play an important role in the mycoparasitism. The enzymatic activities of selected strains of *Trichoderma* species such as chitinase, β- 1, 3- glucanase and protease. Maximum chitinolytic activity was observed in case of *T. harzianum* BHU after 7 days from their inoculation. Maximum activity of β 1, 3- gulcanase was observed in the case of *T. harzianum* BHU at 4-days after inoculation of spore inoculum. Maximum protease activity was observed in case of *T. harzianum* BHU at 6-days after inoculation of spore inoculum. *Trichoderma harzianum* have high competitive saprophytic ability (CSA) than other strains of *Trichoderma* species. The maximum colonization was found due to *T. harzianum* BHU (90.0%) at the concentration 4 log cfu/g soil which was followed by *T. harzianum* IVRI (78.3%).**

KEYWORDS: *Trichoderma*, Biological Control, Enzymatic and Saprophytic Ability

Trichoderma species is free-living fungi that are highly interactive in root, soil and foliar environments. The species of the genus *Trichoderma* have been reported as most potential biocontrol agents (Lewis and Papavizas, 1991; Haran *et al*. 1996a; Haran *et al*. 1996b; Elad, 2000; Hermosa *et al*. 2000; Kredics, *et al*., 2003; Joshi, *et al*., 2010; Hermosa, *et al.*, 2012; Keswani *et al*., 2015; Bastakoti *et al*., 2017; Hyder *et al.*, 2017; Sridharan *et al*., 2020; Chandra, 2021) due to their ability to successfully antagonize other fungi.

In order to solve the national and global problems of environmental hazards due to application of chemicals for disease control, antagonistic microbes have been considered as prospective agents for the purpose (Cook, 1985). Chemicals are necessary for control of different diseases but its adverse effect on human and animal health, environmental contamination, phytotoxicity, development of resistance against pathogens and their high cost (Mulder, 1979; Mukherjee and Garg, 1983) make their application difficult to be continued in future.

Several modes of action have been proposed to explain the suppression of plant pathogens by *Trichoderma* these modes of action include production of antibiotics, competition for key nutrients and space, production of cell wall degrading enzymes, stimulation of plant defence mechanisms, and a combination of these possibilities (Neethling and Nevalainen, 1995; Martinez-Medina *et al*., 2016). Modern approaches to the use of this fungus to control pathogenic fungi have largely been based on the direct use of inoculants. They grow toward hyphae of other fungi, coil around them in a lectin mediated reaction, and degrade cell walls of the target fungi. This process (mycoparastism) limits growth and activity of plant pathogenic fungi. In addition to or sometimes in conjunction with mycoparasitism, individual strains may produce a wide range of antibiotic substances (Sivasithamparam and Ghisalberti, 1998; Martinez-Medina *et al*., 2016) and that they parasitize other fungi. They can also compete with other microorganisms for example, they compete for key exudates from seeds that stimulate the germination of propagules of plant-pathogenic fungi in soil (Howell, 2002) and more generally compete with soil microorganisms for nutrients and or space (Elad, 1996). Furthermore, they inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi, such as *Botrytis cinerea* for penetrate leaf surfaces (Zimand, 1996).

These direct effects on other fungi are complex and remarkable and until recently were considered to be the bases for how *Trichoderma* species exert beneficial effects on plant growth and development. Research on these topics has generated a large body of knowledge including the isolation and cloning of a range of genes that encode proteins which have antimicrobial activity genes that encode fungi toxic cell-wall-degrading enzymes that can be used to produce transgenic plants

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resistant to disease (Bolar *et al.,* 2000; Bolar *et al.,* 2001; Lorito, 1998) and the discovery of enzymes that are useful in the bioprocessing of chitin (Donzelli *et al*., 2003; Gajera *et al.,* 2012).

However, it is becoming increasingly clear that our understanding of the mechanisms of biocontrol has been incomplete. In addition to the ability of *Trichoderma* species to attack or inhibit the growth of plant pathogens directly recent discoveries indicate that they can also induce systemic and localized resistance to a variety of plant pathogens. Moreover, certain strains also have substantial influence on plant growth and development. Their role in plant growth promotion has been known for many years and can occur in both axenic systems (Lindsey and Baker, 1967; Yedidia *et al*., 2001) and natural field soils (Chang *et al*., 1986; Harman, 2000). These new findings are dramatically changing our knowledge of the mechanisms of action and uses of these fungi.

An effective antagonist should be able to persist at high population density for adequate biocontrol activity after introduction into soil, rhizosphere, phyllosphere or carposphere. Numerous biotic factors (nature of the target organism and of the host plant, presence of predators, parasites or antagonistic microorganisms among the resident micro flora) and a biotic factors (nature of the soil or substrate, humidity, availability of nutrients, temperature, radiations, salinity, pH) may reduce growth and establishment of biocontrol fungi in soil ecosystems (Dandurand and Knudsen, 1993; Eastburn and Butler, 1988a, b; Hubbard *et al*., 1983; Knudsen and Bin, 1990; Papavizas, 1985). These may have negative influence in the biocontrol efficacy of *Trichoderma* strains therefore it is important to collect information about the effects of environmental factors on the different activities of *Trichoderma* strains that possess biocontrol potential.

MATERIALS AND METHODS

Source of the *Trichoderma* species

The pure culture of different strains of *Trichoderma* species were obtained from Laboratory of Applied Mycology and Plant Pathology, Department of Botany, Banaras Hindu University, Varanasi where the cultures were maintained from the collection centers of Institute of Microbial Technology (Chandigarh), National Botanical Research Institute (Lucknow), Indian Agricultural Research Institute (New Delhi), Indian Institute of Vegetable Research (Varanasi). Local species/strains of *Trichoderma* were isolated from soils of various locations from and around Banaras Hindu University Campus, Varanasi on the *Trichoderma* Selective Medium (TSM). The cultures were maintained on PDA by periodically sub culturing and were stored in a refrigerator at 4 ^{o}C.

Assay of enzyme activity of the selected *Trichoderma* species

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) contained $MgSO_4.7H_2O$ (0.5 g), KH_2PO_4 (1.0 g), NH_4NO_3 (2.0 g), KCl (0.5 g), $FeSO_4$ (10 mg), $ZnSO_4$ (10 mg), $CuSO_4.5H_2O$ (5.0 mg). The medium was supplemented with appropriate carbon source. The pH of the medium was adjusted to 6.0 and sterilized by autoclaving at 15 psi for 20 min. The medium was inoculated with conidial suspension of ~5×10^6 per ml and centrifuged at 150 rpm at 25^{o}C. The cultures were harvested at 1, 2, 4, 7, 9 and 10 days respectively and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4 ^{o}C to get cell free culture filtrate.

Chitinase Assay

Chitinolytic activity was assayed by measuring the release of free N-acetyl glucosamine (NAG) from colloidal chitin by following the method of Miller (1959). The assay mixture contained 0.5 ml of 0.5% colloidal chitin (MERK), suspended in 1M sodium acetate buffer (pH 4.7) and 1 ml of enzyme solution. The reaction mixture was incubated for 6 h at 40^{o}C with continuous shaking and then centrifuged at 11200 ×g for 5 min at 4^{o}C. The reaction was stopped by adding 3 ml of 1% DNS (Dinitrosalicylate) reagent in shaking and was stopped with 3 ml of 10% trichloroacetic acid (TCA). The reaction mixture was allowed to stand for 1 h at 4^{o}C and then centrifuged at 8000×g for 15 min to precipitate the undigested protein. The absorbance of the 1 M NaOH and followed by heating for 10 min. at 100 ^{o}C. While it was hot 1ml of 40% Rochelle salt was added. The solution was again centrifuged at 10000 rpm for 5 min at 4^{o}C and absorbance of the reaction mixture at 582 nm (A_{582}) was measured. Chitinolytic activity was expressed in NAG units with one NAG unit (NAG unit = 1 µ mol of NAG) under the assay conditions. Specific chitinolytic activity (A_{582}, NAG- U.h^{-1}. ml^{-1}) was defined as quantity of NAG units released by 1 ml of enzyme solution per hour under the assay condition.

β-1, 3 Glucanase Assay

β-1, 3 glucanase was assayed by measuring the release of reducing sugar with DNS (Nelson, 1944). One ml of enzyme sample was incubated with 1 ml 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH 4.8) at 50 ^{o}C for 1 h. After incubation 2 ml of copper reagent were added and boiled for 10 min. in a water bath. The

tubes then cooled completely and added 2 ml of Arsenomolybdate reagent, vertexed and adjusted the final volume to 25 ml with distilled water. The solutions were centrifuged at 8000×*g* for 5 min and the aliquots of supernatant was measured the absorbance at 500 nm. The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1, 3 glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of glucose equivalents per min.

Protease Assay

Protease activity of culture filtrate was determined by a modified Anson's Method (Yang and Huang, 1994). The substrate used (1% casein in 50mM phosphate buffer, pH 7.0) was denatured at 100^{o}C for 15 min in a water bath and cooled at room temperature. The reaction mixture containing 1 ml of substrate and 1 ml of enzyme solution were incubated at 37^{o}C for 20 min with liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280 nm equivalent to 1 μmol of tyrosine in one minute under the assay condition.

Competitive saprophytic ability (CSA) of the selected *Trichoderma* species

Competitive saprophytic ability (CSA) of the selected *Trichoderma* species was assayed by following Cambridge method (Garrett, 1965), modified by Ahmad and Baker (1987). Strains of the *Trichoderma* species were grown on PDA medium. The plates were incubated at 25± 2^{o}C for 4 days, which was then flooded with sterile distilled water and conidia were gently freed from the culture with a brush. The suspension was sieved through four layers of cheesecloth, centrifuged at 2,500 x g for 15 min and resuspended in sterile distilled water thrice. The number of conidia in the suspension was counted with a haemocytometer and then adjusted to the desired concentration. The freshly harvested conidia of the *Trichoderma* species were mixed in the natural soil at the rate of 10^1, 10^2, 10^3 and 10^4 conidia per gram of soil. No conidia were added in controls. Twenty pieces of sterilized wheat straw (2 cm in length) were buried in 500 ml conical flasks containing inoculum mixture of antagonist for each dilution.

The pots were covered with a plastic cover to conserve moisture and incubated at 25± 2^{o}C. The wheat straw pieces were removed after 4 weeks from the treatment and washed with sterilized distilled water followed by surface sterilized with 0.1% sodium hypochlorite solution and 5% ethanol for 5 min. Five segments of each treatment and control were transferred separately in Petri dishes containing 20 ml of *Trichoderma* selective medium (TSM) and incubated at 25± 2^{o}C. Per cent colonization of the wheat straw pieces by the test antagonists was recorded at 2, 4, 6 and 8 days of incubation.

RESULTS AND DISCUSSION

Fungal cell walls are complex structures constituted mainly of polysaccharides such as chitin, β-glucan (Debono and Gordee, 1994) and proteins (Peberdy, 1990). These polysaccharides act as inducers of hydrolytic enzymes and thus cell wall-degrading enzymes such as chitinases (Lima *et al*., 1999; De Marco *et al*., 2000; De Marco *et al*., 2002) and β 1, 3- gulcanases (Lorito *et al*., 1994; Schirmbock *et al*., 1994) play an important role in the mycoparasitism. Filamentous fungal cell wall also contains lipids and proteins (Hunsley and Burnett, 1970) and therefore it is expected that antagonistic fungi produce proteases which may also act on the host cell-wall during mycoparasitism (Sivan and Chet, 1989; Flores *et al*., 1997).

Present study was performed to identify the enzymatic activities of selected strains of *Trichoderma* species such as chitinase, β 1, 3- glucanase and protease. In this study considerable variation has been observed among various species/strains of *Trichoderma* (Figure 1). The differences observed depend on the ability of each isolate to produce extracellular fungal cell wall hydrolyzing enzymes such as chitinases, ß -1,3-glucanases and proteases, or the specific isoenzyme pattern expressed by each isolate (Grondona *et al*, 1997). The production of the hydrolytic enzymes has been shown to be affected by culture conditions and the type of the host (de la Cruz *et al*., 1992; Lorito *et al*., 1994).

Maximum chitinolytic activity was observed in case of *T. harzianum* BHU. The enzymatic activity in case of all the strains of *Trichoderma* species was maximum after 7 days from their inoculation. This suggests that chitin is able to induce chitinase genes from very beginning of the growth of *Trichoderma* and after 7 days due to their limitation in culture filtrates the enzymatic activity got reduced.

Various studies on chitinase and their genes have been described which are responsible for antagonism of *Trichoderma* species (Haran *et al*., 1996; Kim *et al*., 2002; Harman *et al.,* 2004). Resent study confirmed that a 73 kDa *nag1* is responsible for triggering chitinase gene expression and the chitin present in cell wall of the pathogen induces this gene (Carsolio *et al*., 1999; Mach *et al*., 1999). The role of extracellular

chitinase in biocontrol activity of *Trichoderma virens* was examined using genetically manipulated strains (Baek *et al*., 1999; Woo *et al*., 1999). *T. virens* strains in which the chitinase gene (cht42) was disrupted or constitutively over-expressed significantly decreased and enhanced, respectively in their biocontrol activity against *Botrytis cinerea* and *Rhizoctonia solani* when compared with the wild type strain. Viterbo *et al*. (2001) reported that *T. harzianum* Rifai T M transformants overexpressing chit36 chitinase inhibited *Fusarium oxysporum* and *Sclerotium rolfsii* more strongly than the wild type strains. Moreover, culture filtrates inhibited the germination of *Botrytis cinerea* almost completely.

Maximum activity of β 1, 3- gulcanase was observed in the case of *T. harzianum* BHU. All the strains of *Trichoderma* species showed maximum enzymatic activity at 4-day after inoculation of spore inoculum. This suggests that the genes responsible for the production of this enzyme induced very rapidly on the substrate laminarin and after 4 days due to substrate limitation their activity got reduced.

β 1, 3- gulcanases play a nutritional role in saprophytes and mycoparasites (Chet, 1987; Sivan and Chet, 1989), and therefore, this enzyme is responsible for antagonism of *Trichoderma* species which is used as biocontrol of plant pathogens. Cell wall preparations in case of different pathogens have been shown to induce different levels of β-glucanase activity (Viterbo *et al*., 2002). Direct evidence for the involvement of glucanases in mycoparsitism has been demonstrated by Lorito *et al*. (1994). It has been shown that β 1, 3- gulcanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases (Benitez *et al*., 1998; El-Katatny *et al*., 2001) and antibiotics (Carsolio *et al*., 1999; Howell, 2003).

Maximum protease activity was observed in case of *T. harzianum* BHU. All the strains of *Trichoderma* species showed maximum production of protease at 6-days after inoculation of spore inoculum. Different strains of *Trichoderma* species growing in casein-containing liquid medium produced substantial proteolytic activity. The supernatant of the cultures were then used to purify the protein showing proteolytic activity against casein.

An extensive analysis of *T. harzianum* proteases under different metabolic conditions has been conducted by Delgado-Jarana *et al*. (2000). The role of proteases in mycoparasitism was found to be reinforced with the isolation of new protease overproducing strains of *T. harzianum* (Szekeres *et al*., 2004). Proteases involved in the degradation of heterologously produced proteins have been characterized (Delgado-Jarana *et al*., 2000). Role of protease in antagonism has been reported by Elad and Kapat (1999), in which the susceptibility of *Fusarium oxysporum* hyphae to chitinase and β-glucosidase was increased after pretreating hyphae with protease (Sivan and Chet, 1989).

The effectiveness of biocontrol agents depends on their ability to survive, multiply and colonize the rhizosphere (Papavizas, 1992). A *Trichoderma* strain for its successful efficacy must be rhizosphere competent i.e. the agent must be colonizing the rhizosphere to a depth greater than 2 cm from the seed or proliferate to a concentration that exceeds from the initial population of *Trichoderma* applied. The strains without CSA could not survive in the soil ecosystem (Nakkeeran *et al*., 2005), therefore, CSA was included in this study. Wheat straw pieces rich in cellulose are commonly used to determine CSA of fungi. Isolation of fungi from baits of dead plant materials buried in natural soil provides evidence that recovered fungi can colonize these substrates as competitive saprophytes (Garrett, 1965). The result obtained in the present study revealed that strains of *T. harzianum* have high CSA than other strains of *Trichoderma* species. The maximum colonization was found due to *T. harzianum* BHU (90.0%) at the concentration 4 log cfu/g soil which was followed by *T. harzianum* IVRI (78.3%). It was found that colonization in the substrate could be directly correlated with their capacity for utilization of the substrate components based on their capacity to produce hydrolytic enzymes (mainly cellulase). Ahmad and Baker (1988b) reported the increased production of cellulase by NTG derived mutants of *Trichoderma harzianum* than the wild type strain. These strains were rhizosphere competent, because of increased enzyme activity that resulted in higher CSA for utilization of cellulose substrate on or near the root surface. Davet (1987) correlated the effective antagonism of *Trichoderma* species with high CSA against sclerotial fungi. Therefore, high CSA of *T. harzianum* might be one reason for its effectiveness as biocontrol agent.

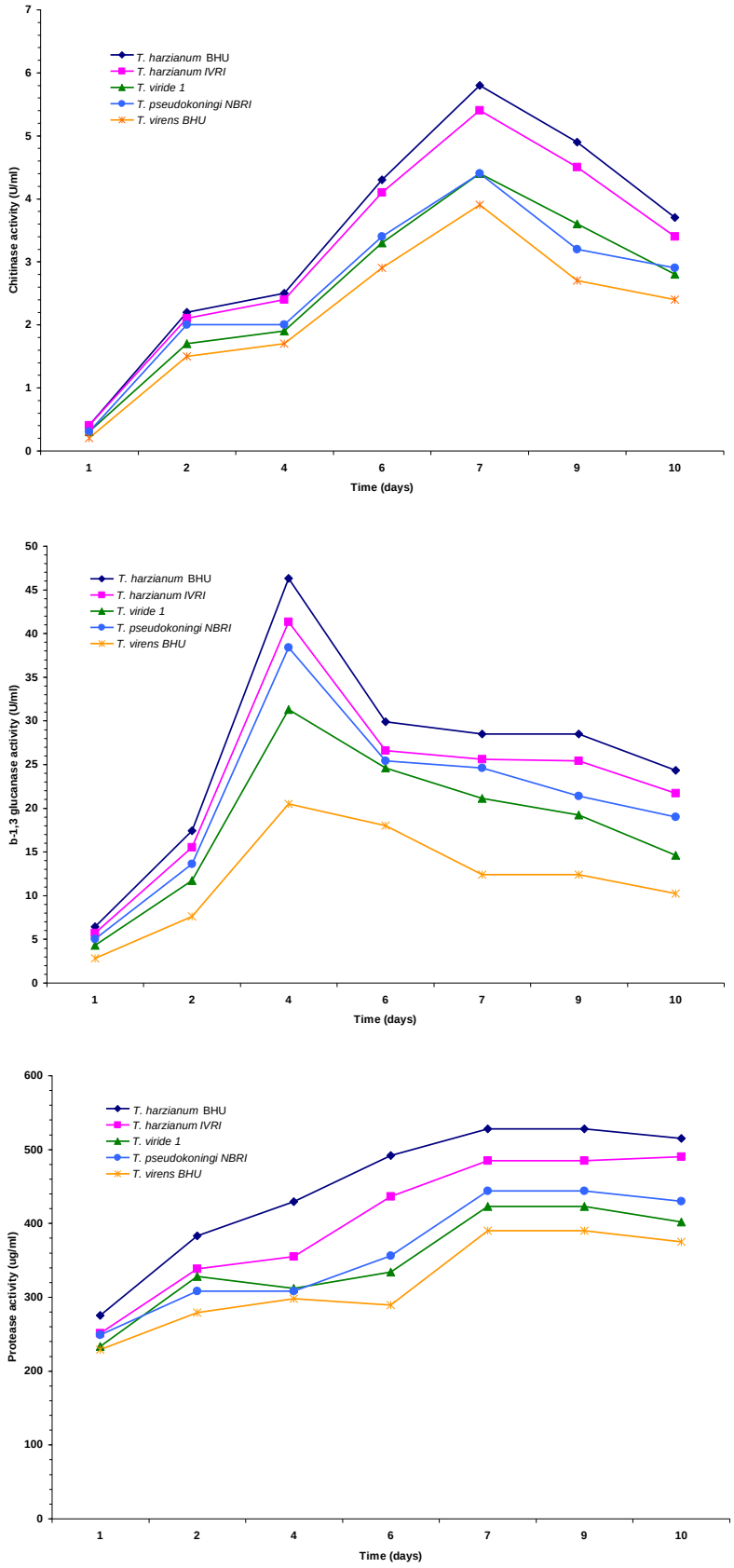

Figure 1: Assay of enzymatic activities of selected *Trichoderma* species at different days. A – Chitinase; B – β-1, 3 glucanase; C - Protease

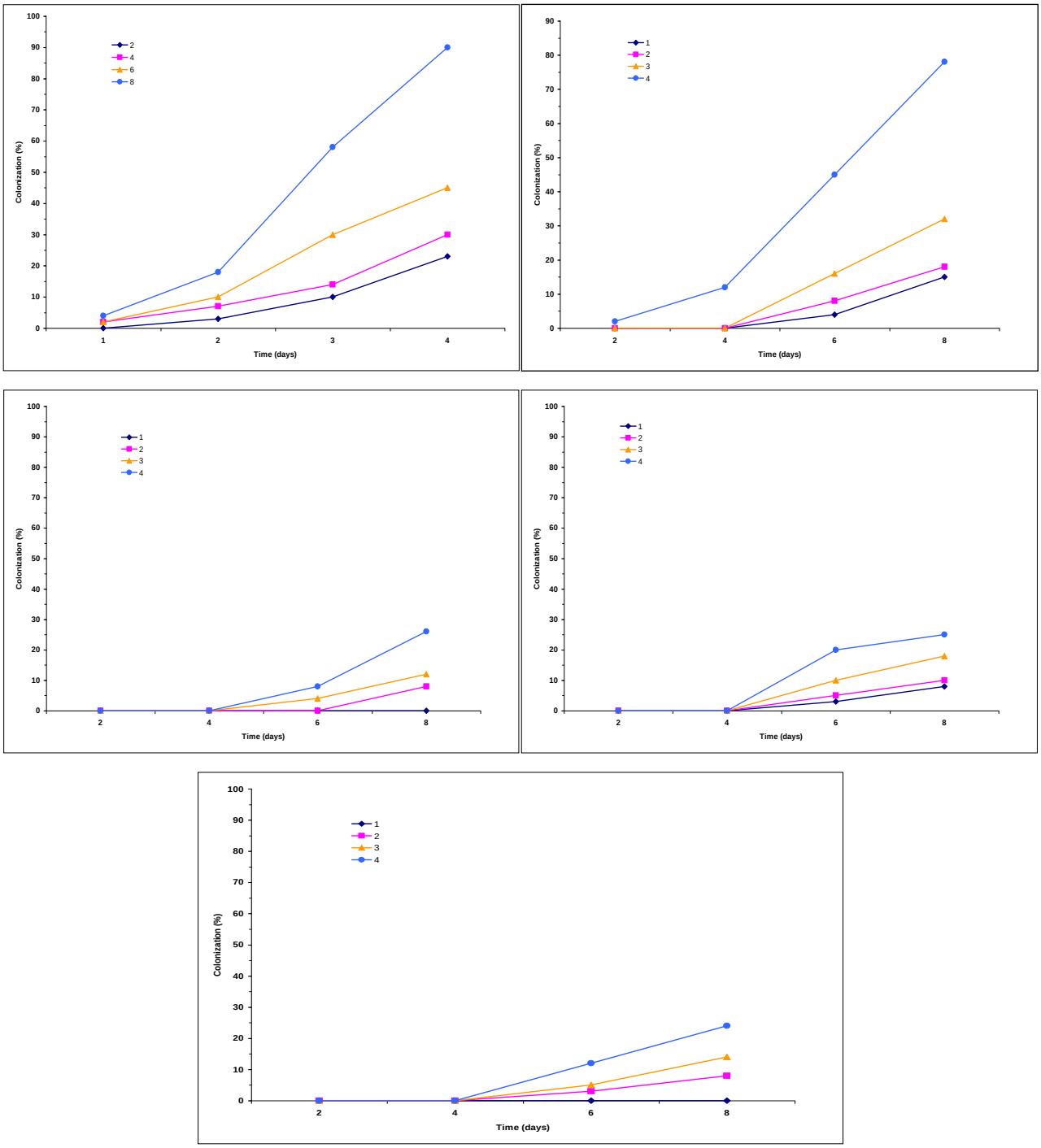

Figure 2: Competitive saprophytic ability of selected *Trichoderma* species at different time intervals (days) with different concentrations (log cfu/g soil). A- *T. harzianum* BHU; B- *T. harzianum* IVRI; C- *T. viride* 1; D- *T. pseudokoningii* NBRI; E- *T. virens* BHU

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