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BIOCONTROL MECHANISMS EVOLVED BY *TRICHODERMA* SP. AGAINST PHYTOPATHOGENS: A REVIEW

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ABSTRACT
Plants are the major source of food, fibre, fodder, medicines and many other useful products. Various insects, bacteria, virus, fungi and other pests attack plants at various stages of their development. *Fusarium*, *Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia,* and *Sclerotium* are the major plant pathogens which cause rot and wilt in plants. Approximately 30,000 species of plant pathogens attack Indian crops. For the control of these phytopathogens, different chemical fungicides are used. Chemical based control of phytopathogens is very effective but it has several disadvantages like, environmental pollution, development of resistant strains, short term effect etc. Thus, there is a need for identifying alternative measures, which can be efficiently used for the control of phytopathogens. Biological control is the best answer for this question as it is very effective and environmentally safe. *Trichoderma* is an well known example of biological control. *Trichoderma* exert biocontrol action against soil borne pathogens through different mechanisms such as competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defense mechanisms and antibiosis or directly by mechanisms such as Mycoparasitism.

INTRODUCTION

Plant diseases are the key factor in natural resource destruction. Soil borne phytopathogenic fungi like, *Oxyspporum, Phythium, Phytophthora, Botyris, Rhizoctonia* and *Fusarium* has devasted crops during the last few years (Vipul *et al.,* 2014). Chemical fungicides which are used to control plant diseases have adverse effect on the environment and also lead to the development of resistant strains. On contrary use of biocontrol agents for the removal of phytopathogens have no hazardous effect on the environment and it also promotes degree of disease suppression.

Trichoderma species are the most commonly used biocontrol agents. They can be easily isolated through soil (Harman *et al.,* 2004). *Trichoderma* species are opportunistic avirulant plant symbionts. *Trichoderma* species are among the most studied fungal biocontrol agents. They are commercially available in market as potent biopestides.They are also used as soil amendments (Harman *et al.,* 2004). When *Trichoderma* is applied in fields it colonize the rhizosphere of plants and allow a rapid establishment of microbial community there. It controls phytopathogens by using various mechanisms such as competition, antibiosis, secretion of cell wall degrading enzymes (CWDEs) etc. It also improves the plant health and stimulate the root growth.

Trichoderma species were first described in 1791 in Germany. Gilman and Abbott in 1927 discoverd four species of this genus.These four species were distinguished on the basis colour and shape of the conidia. In 1932, Weinding demonstrated that this genus has parasitic activity against *R. solani*. He was the first person who demonstrate that this genus can be used as biocontrol agent.

The genus *Trichoderma* comprises a great number of fungal strains that act as biological control agents. *Trichoderma* strains exert biocontrol action against fungal pathogens either directly (mycoparasitism) or indirectly (by competing for nutrients and space, modify the environmental conditions, promoting plant growth and eliciting plant defense response) Rifai, M.A. 1969. The indirect and direct mechanisms work synergistically. Activation of each mechanism implies the production of specific compounds and metabolites. These metabolites play an important role in biocontrol activity.

Trichoderma fungi lacks sexual reproduction. Morphologically *Trichoderma* species are similar to anamorph *Hypocrea* and their ITS sequence analysis have reveled their proximity to the *Hypocrea* genus. The most commmon species of *Trichoderma* that are commonly used as biocontrol agents (BCAs) are *T. virens, T. viride* and above all, *T. harzianum*. There are many properties which made *Trichoderma* an excellent BCA. These properties include their high reproductive capability, ability to survive in harsh conditions, efficiency of nutrient utilization, ability to promote plant growth etc. Efficiency of *Trichoderma* is more in acidic soil as compared to alkaline soils.

The main aim of this paper was to study the factors which

affect biocontrol mechanisms.This paper also detail in deatail with the different mechanisms which are employed by *Trichoderma* against plant pathogens.

Factors influencing biocontrol activity

Rhizospheric competence

The ability to colonize and grow in association with plant roots. It is a measure of potential of an isolate to grow and survive in soil.

Temperature

The most important factor, which determines the survivability of bioagent in nature. For a biocontrol agent to be effective and potential it is necessary that it should cover the thermal spectrum of the phytopathogen. *Crinipellis* seems to grow at higher temperature thus limiting the ability of *Trichoderma* to control plant pathogens.

Moisture

Moisture is another important factor in determining biocontrol activity as it affects colonization ability of a biocontrol agent. Moisture deficiency limits the spore germination process. *Trichoderma* conidia are very small in size. They must require water and swell before germination.

Nutrients

For spore germination nutrients are required.

Biocontrol mechanisms

Biocontrol agents are living organisms whose activity depends totally upon the environmental conditions. Biocontrol mechanisms of *Trichoderma* involve competition for food and space, secretion of secondary metabolites, antibiosis etc.

Starvation is the most common cause of death. It has been found that some strains of *Trichoderma* have the ability to produce iron chelated compounds called siderophores, thus stop the growth of other fungi as iron uptake is essential for most of the fungi. *T. harzianum* T35 have the ability to control *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients. *Botrytis cinerea* causes a great damage during pre and post harvesting. *Trichoderma* is found to be more promising for the control of *B. cinerea*. It competes with *B. cinerea* for the nutrients and *B. cinerea* is very much sensitive for nutrients concentration. *Trichoderma* has an inherent ability to take and mobilize soil nutrients as compared to other fungi.

Mycoparasitism can be defined as the direct attack of one fungus over other (Fand B. B. *et al.,* 2013). The process of mycoparasitic action involve recognition, attachment and finally killing of the fungi. CWDEs play a major role in the mycoparasitism action. Mycoparasitism action begins with the secretion of chitinase enzyme, which degrade the cell wall of the fungus. As soon as cell wall of the phytopathogen degrades it enters into the lumen of the pathogen and kill that pathogen. In *Trichoderma* there is an evidence for the participation of G alfa unit in coiling.

The heterometric G protein signaling is basically comprise of three parts, a G protein-coupled receptor(GPCR), a heterotrimeric G protein (α, β, γ subunits) and an effector (Neer, 1995). Various fungal genomes are available nowadays and comparative genomics pointed out that receptors can be classified into nine groups (Lafon *et al.,* 2006). Preliminary investigations of the *Trichoderma reesei* (http:// genome.jgi-psf.org/Trire2 /Trire2. home. html) and *T. atroviride* genomes revealed 16 putative proteins with 7-transmembrane domains, well distributed over all nine receptor classes. Highly conserved heterotrimeric G-proteins act as signal transducers that couple cell surface receptors to cytoplasmic effector proteins. G-protein α subunits can be classifi ed into three major subgroups (Bölker, 1998). Further characterisation of the tga1 mutant showed that this G-protein α subunit affects processes like vegetative growth, production of antifungal metabolites, and chitinase formation (Reithner *et al.,* 2005), which are at least partially involved in *Trichoderma* biocontrol mechanism. In liquid culture the tga1 mutant produced strongly decreased chitinase activities and showed a reduced transcription of the nag1 (N-acetyl-glucosaminidaseencoding) and ech42 (endochitinase 42-encoding) genes (Reithner *et al.,* 2005). In antagonistic assays, the tga1 mutant was unable to overgrow and lyse host fungi such as *R. solani, B. cinerea*, and *S. sclerotiorum*, although infection structure formation was unaffected; nevertheless, it displayed an enhanced growth inhibition of the host fungi by over-producing and secreting low molecular weight metabolites. In contrast to the role of Tga1 in influencing growth and conidiation in *T. atroviride*, its homologue TgaA did not affect these properties in *Trichoderma virens*. tgaA mutants grew normally and sporulated like the wild type, but had a reduced ability to colonise *S. rolfsii* sclerotia, whereas they were fully pathogenic against R. solani (Mukherjee *et al.,* 2004). No such host specificity could be observed in the *T. atroviride* tga1 mutant. Mutants of *T. virens* lacking the TgaB protein (belonging to subgroup II Gα subunits) did not show major phenotypic defects: they grew and sporulated like the wild type and biocontrol against *R. solani* and sclerotia of *S. sclerotiorum* was unaffected (Mukherjee *et al.,* 2004).

Stimulation of plant defense mechanism

Trichoderma strains when apply to the fields they colonize the plant roots prior to the protection against plant pathogens. Colonization refers to the ability to adhere and recognize plant roots, penetrate the plant root and tolerate the toxic metabolites produced by plant root in response to invasion (Chet *et al.,* 1997). It has been found that some *Trichoderma* strains produce comopounds that induce localized or systemic plant resistance response (Shahid *et al.,* 2014). The resistance of *Trichoderma* species against the phytoalexins, flavonoids, terpenoids, phenolic derivatives, aglycons and other antimicrobial compounds secreted by plants against invasion is found to be associated with the ABC transport system, Harman *et al.* (2004)

When *Trichioderma* colonizes plant roots it enhances the plant development, root length, productivity, resistance against biotic stress, pathogens etc. It has been find out by many coworkers that when plant roots were treated with *Trichoderma* strains there is an considerable increase in productivity. However there is a very limited data available on the production of plant growth factors (auxins, cytokinens and ethylene). Recently some species have been detected which are found to sercret zeaytn, gibberelilin. It has been found that besides secreting these compounds some *Trichoderma* species have the

tendency to secrete gluconic acid, citric acid, fumaric acid (Osiewacz *et al.,* 2002) and thus have the tendency to acidify the environment (Gómez *et al.,* 1994). The production of the acids results from the metabolism of carbon sources such as glucose and in turn are able to metabolize phosphates, and other micronutrients. Therefore, addition of *Trichoderma* in soils results in the metal solubilization and increase in crop productivity (Harman *et al.,* 2004).

The plant root-fungus association also protects plant against various types of phytopathogens. When *Trichoderma* added to the soil they trigger a defense response in plants against numerous classes of phytopathogens. This defense response results in an increase in the concentration of metabolites and enzymes, which are associated with the defense mechanisms such as phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS), involved in the biosynthesis of phytoalexins (HR response), chitinases and glucanases. Addition of *Trichoderma* metabolites triggers the plant resistance or in the synthesis of cap proteins. Expression of the chitinase Chit42 from *T. harzianum* in tobacco and potato plants resulted in transgenic lines highly tolerant or completely resistant to the foliar pathogens *Alternaria alternata, Alternaria solani* and *Botrytis cinerea* and to the soil-borne pathogen *Rhizoctonia solani*. In some other cases expression of glucanase and pectinases results in reinforcement of enzymatic pool against phytopathogens but not in specific stimulation of plant resistance mechanisms. Addition of laminarin, a â-1,3-glucan, to grapevines induced several defense genes and reduced infection by *B. cinerea* and *Plasmopara viticola*.

Rhizospheric modifications

Soil environment has a great impact on spore germination, secondary metabolite production, antibiotic and enzymes production. It has been already investigated that when BCAs are applied into the soil they modify the rhizospheric environment. p^H is the most important factor that affect the activity of both *Trichoderma* and pathogen. At high p^H antibiotic degradation starts, similarly fungus growth is also inhibited by weak acids, at low pH enzyme degradation starts (Delgado-Jarana., 2000). Therefore the ability to survive over a wide pH range is the important factor that make *Trichoderma* a potent biocontrol agent. Some strains of *Trichoderma* has an ability to control external pH and thus make a suitable environment for their secreted enzymes. At the transcriptional level, several proteases, glucanases, cell-wall proteins and a glucose transporter are pH-controlled, which suggests a pH dependent transcriptionally controlled response of different enzymes.

External p^H is also an important factor in determining the pathogenecity of the pathogen, because their pathogenecity factors are produced only within a narrow pH range.Therefore the pH modification also determines the pathogens ability to colonize the host. *Trichoderma* strains able to modify external pH and to adapt their own metabolism to the surrounding growth conditions would consequently reduce the virulence of phytopathogens because most pathogenicity factors could not be synthesized (McIntyre *et al.,* 2004).

Antibiosis

The antibiosis may be defined as the antagonistic association between an organism and the metabolic substances produced by another. *Trichoderma* species produce numerous volatile and non volatile compounds that decrease the colonization activity of pathogen (Antal Z *et al.,* 2000). The different metabolites that play the key role in phytopathogenic action are harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described. In some cases, antibiotic production correlates with biocontrol ability, and purified antibiotics mimic the effect of the whole agent. However, there are also examples of antibiotic-overproducing strains, such as gliovirin over producing mutants of *T. virens*, which provide control similar to that of the wild-type, and of gliovirin-deficient mutants which failed to protect cotton seedlings from *Pythium ultimum,* whereas the parental strain did. The combined effect of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone. Synergistic effects between an endochitinase from *T. harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols

Table 1: Details of lytic enzymes from *Trichoderma harzianum* that have potential bio-control capability

Sl no.	Gene	MW Kda	Activity	Strain
Chitinase				
1	-	102	N-acetylgluco-saminidase	TM-39-1
2	-	73	N-acetylgluco-saminidase	TM-25-1
3	*exc2*	73	-	TM
4	*Excl/nag1*	64-69	-	TM-25-1, Pla
5	-	28	N-acetylgluco-saminidase	T-189
6	-	52	endochitinase	TM-TY
7	*ech42*	*42*	endochitinase	IMI206040a
8	*chit44*	44	-	CECT2413
9	*cht42*	42	-	GV2908
10	*ThEn42*	42	-	Ola
11	-	40	chitobiosidase	Pla
Glucanase				
1	*bgn13.1*	78	β-1,3 endoglucanase	Pla, CECT2413
2	-	74	-	T24
3	-	36	β-1,3 endoglucanase	39.1
4	-	17	β-1,3 endoglucanase	CECT2413
5	*b16.2*	43	β-1,3 endoglucanase	CECT2413

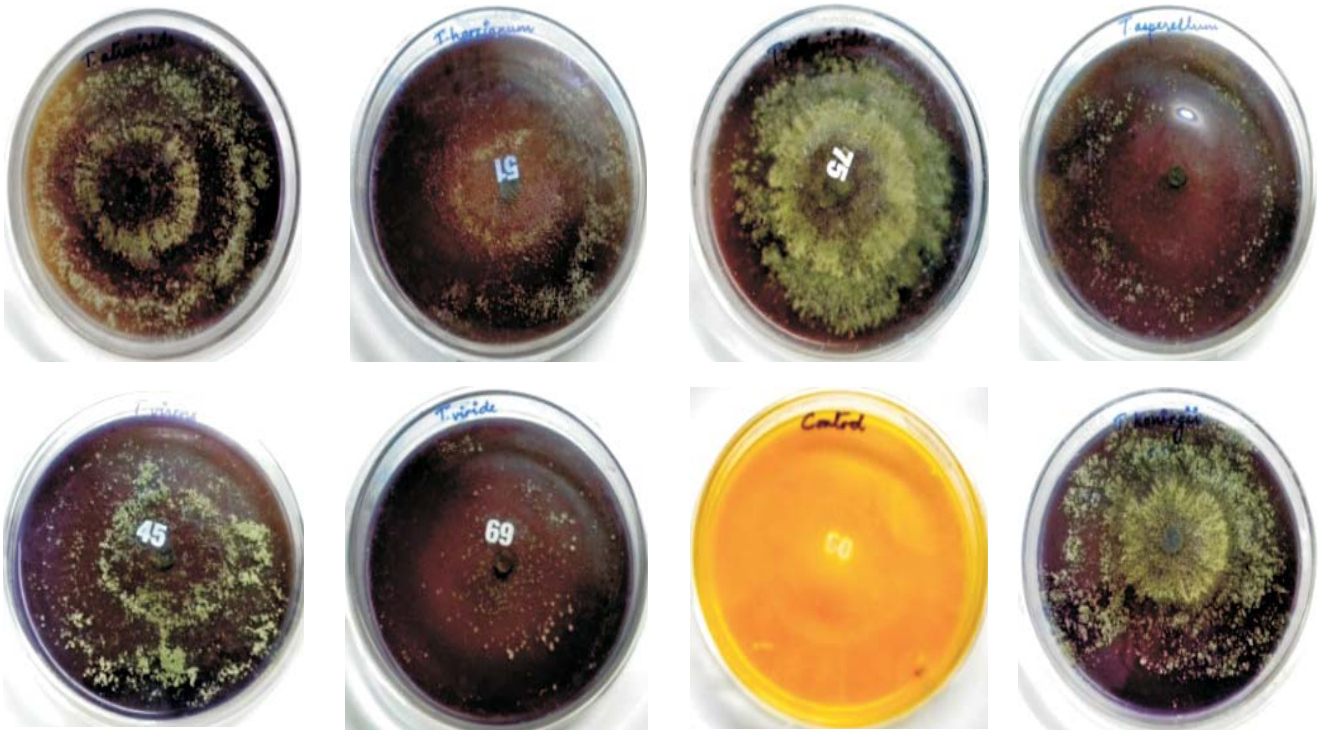

Figure1: Chitinase activity of *Trichoderma* spp.

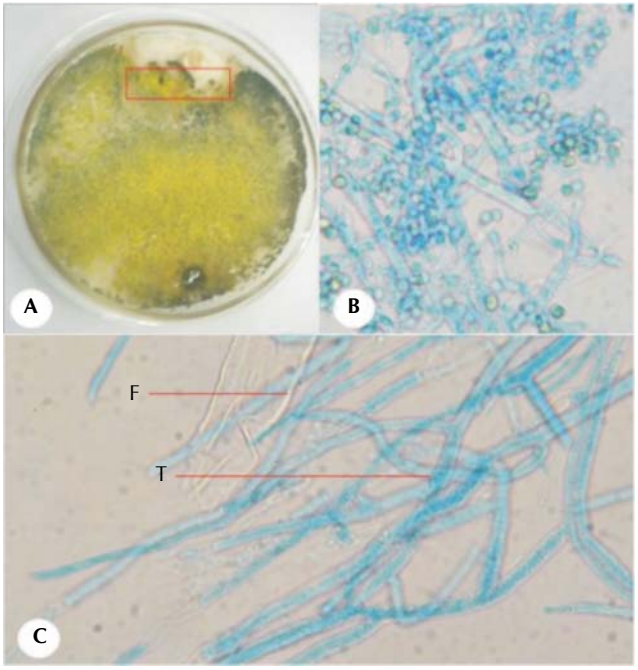

Figure 2: *Tichoderma* on petri plate, B.Slide view of *Trichoderma* C. Mycoparasitic action of *Trichoderma* against *Rhizoctona*

on conidial germination of *B. cinerea* is well known (Howell, 2003). When combinations of antibiotics and several kinds of hydrolytic enzymes were applied to propagules of *B. cinerea* and *F. oxysporum*, synergism occurred, but it was lower when the enzymes were added after the antibiotics, indicating that cell-wall degradation was needed to establish the interaction (Howell, 2003). Peptaibols are a class of linear peptides that generally have strong antimicrobial activity against gram positive bacteria and fungi act synergistically with cell wall degrading enzymes to inhibit the growth of phytopathogens and elicit plant defense response against pathogens. In tobacco plants, exogenous applications of peptaibols trigger a defense response and reduce susceptibility to tobacco mosaic virus (Wiest *et al.,* 2002). A peptaibol synthetase from *T. virens* has recently been purified, and the corresponding gene, which has been cloned, will facilitate studies of this compound and its contribution to biocontrol. An extensive review on antibiosis and production of *Trichoderma* secondary metabolites is provided by Howell (Howell 1998; Howell, 2003).

Mycoparasitism

Mycoparasitism can be defined as the attack of one fungus on another. This process is a very complex which includes various steps such as recognition, attack, penetration and killing of the host. The pattern of induction differs from one species to another. Mycoparasitism involve morphological changes such as coiling and formation of appressoirum like structure which aid in the penetration of the host cell wall. *Trichoderma* attaches to the pathogen cell wall. Once *Trichoderma* is attached with the host it coils around the pathogen and forms the appresoria. The following step consist of production of Cell wall degrading (CWDEs) and peptaibols, Which facilitate both the entry of *Trichoderma* hyphae into the leumen of pathogen and asssimilation of cell wall contents. In *Trichoderma*, there is biochemical evidence for the participation of G-α unit in coiling, since thereis an increase in coiling around nylon fibers was detected after the addition of activators of G-protein (mastoparan and fluoroaluminate)

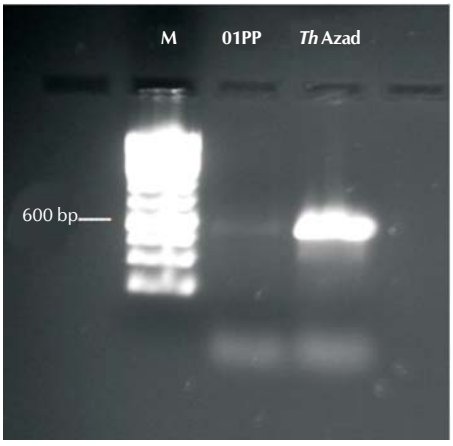

Figure 3: Amplified gene fragment of chit-HAR3 gene present in *T. harzianum Th* Azad only

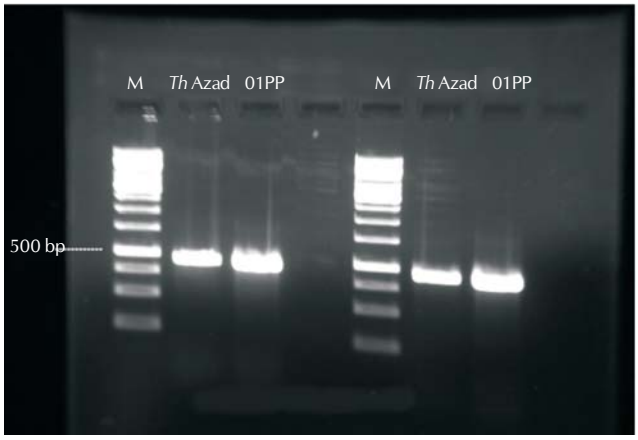

Figure 4: Amplified gene fragment of ech-42 gene present in *T. harzianum Th* Azad and *T. viride* 01PP

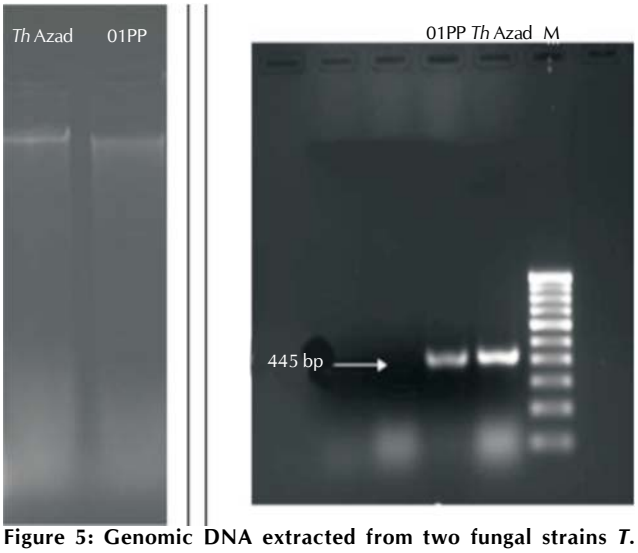

Figure 5: Genomic DNA extracted from two fungal strains *T. harzianum* (*Th.* Azad), *T. viride* (01PP) and their amplified fragments with xyn2 primer

(Omero *et al.,* 1999). The chitinolytic system of *Trichoderma* comprises many enzymes and the list of its components is rapidly being updated as new enzymes and genes are reported. Chitinases are divided into 1,4-β-acetyl glucosaminidases (GlcNAases), endochitinases and exochitinases. Chitinase enzyme occur in a awide variety of microorganisms including bacteria, fungi, insects etc. In fungi, chitinase are believed to have autolytic, nutritional and morphogenic roles. In mycoparasitic fungi chitinase are associated with the degradation of cell walls (Mukesh *et al*., 2014 and Pandey *et al.,* 2014 d).

Trichoderma spp are the most commonly used biocontrol agents against several soil borne fungal plant pathogens such as *Fusarium oxysporum*, *Sclerotium rolfsi, Rhizoctonia solani* and *Pythium spp* (Singh S. P et al.2013 and Vipul et al.,2015). Members of the fungal genus *Trichoderma* spp. produce cell wall degrading enzymes such as glucanase, chitinae, xylanse and protease, cellulose, lipase. Chitinase enzymes are of great importance as compared to other mycolytic enzymes, as fungal cell wall is made up of chitin thats why chitinase degrade phytopathogenic fungi easily. Biocontrol of *B. cinerea* by *T. harzianum* has been attributed in part to the action of proteases produced by the BCA that inactivate hydrolytic enzymes, produced by this pathogen on bean leaves (Howell, *et al.,* 2003). It has been found that Prb1 from *T. harzianum* IMI 206040 play an important role in phytopathogenic action. The gene for an extracellular serine protease (tvsp1) has been cloned from *T. virens* (Pozo, et al., 2004) and its over expression significantly increased protection of cotton seedlings against *R. solani*. This gene shows great potential in improving biocontrol ability, as serine proteases are effective against Oomycetes (Dunne *et al.,* 2000) and nematodes (Howell *et al.,* 2003,,Bonants *et al.,* 1995). Serine protease of 28-kDa with trypsin activity isolated from strain 2413 also reduced the number of hatched eggs of root-knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain (Suárez *et al.,* 2004). Sonika et al a,b,c, 2014 found xylanase enzyme activity bands 43 kDa while in case of glucanase enzyme activity bands were observed at 55 kDa in *Trichoderma* sp.

The ech42 primer was used to detect the endochitinase gene in two of the potential strains of *Trichoderma* species viz. *T. harzianum Th* Azad and *T. viride 01PP*. From the figure given above, it is quite clear that the endochitinase gene is present in both species thus this can be used for the gene identification purpose

The chit-HAR3 primer was used to detect the chitinase gene in two of the potential strains of *Trichoderma* species viz. *T. harzianum Th* Azad and *T. viride 01PP*. From the figure given above, it is quite clear that the chitinase gene chit-HAR3 is present only in *T. harzianum (Th. azad)* thus this can be used for the gene identification purpose.

The primer xyn2 was used to detect the xylanase gene in two of the potential strains of *Trichoderma* species viz. *T. harzianum Th* Azad and *T. viride 01PP*. From the figure given above, it is quite clear that the xylanase gene is present in both species thus this can be used for the gene identification purpose.

It has been found that α-1,3-glucanases inhibit spore germination or the pathogen growth in synrgistic cooperation

with chitinase and antibiotics (Cohen-Kupiec., 1999). bgn13.1 (Benítez *et al.,* 1998) and lam1.3 (Cohen-Kupiec *et al.,* 1999) from *T. harzianum*, glu78 (Donzelli *et al.,* 2001) from *T. atroviride* and Tv-bgn1 and Tv-bgn2, these glucanases have been isolated and cloned. In addition, three α-1,6-glucanases have been purified from strain 2413 (Benítez, *et al.,* 1998, De la Cruz *et al.,* 1999, Elad *et al.,* 2000). BGN16.2 exhibited antifungal properties alone or in combination with chitinases (Benítez et al., 1998) and reduced the growth of *B. cinerea* and Gibberella fujikuroi (De la Cruz., *et al.,* 1999). Transformants producing BGN16.2 controlled *R. solani* and *B. cinerea* growth (Benítez *et al.,*1998). Cellulases (α-1,4-glucanases), comprising cellobiohydrolases, endoglucanases (egl1, egl2) and α-glucosidases, have not been widely studied for biocontrol purposes, although cellulose is abundant in oomycetes (Bartnicki-García *et al.,*1968). Howell *et al.,* in 2003 obtained transformants with greater biocontrol activity than the wild-type against *P. ultimun* on cucumber seedling. *T. harzianum* T3 produces a variety of cellulases, which make this isolate very effective in the control of *P. ultimun*. Other hydrolases, such as β-1,3-glucanases, have been purified from strain 2413, and their genes isolated and overexpressed, which resulted in increased biocontrol activity of the transformant strains (Ait-Lahsen *et al.,* 2001).

Trichoderma species are well known for their metabolite production. These secondary metabolites have various applications in medical and Agricultural sectors. Some volatile compounds have phyto and mycotoxic effects as well as can be used as antibiotics and immunosuppressive drugs. These volatile compounds are belong to various structure classes such as ketones, lactones, esters, mono or sesquiterpines. These volatile compound of *Trichoderma* help in phytopathogenic action against pathogens, have plant growth effects etc.

More then 300 volatile compounds have been investigated in *Trichoderma*,out of these different compounds only Propeonic acid, Acetic acid, Tetradecane, Pentadecane, 6PP, Butanoic Acid, and 2-H-Pyran-2-one are the major compounds present in most of the species. Out of these compounds only 6PP and 2-H-Pyran-2-one has been found to be associated with phytopathogenic action.

Cyclosporines are a member of the group of cyclic peptides and are composed of 11 amino acids Cyclosporine A is widely produced by submerged fermentation of aerobic fungi identified as *Trichoderma polysporum* but currently identified as *T. inflatum*. Asma Azam., 2012 reported that *T. harzianum* can also be used for cyclosporine production (44.06 µg/ml).

The biocontrol mechanisms employed by *Trichoderma* against plant pathogens is very complex and varies with the kind of pathogen and host plant involved in interaction. Biocontrol mechanisms are also influenced by temperature, moisture, pH and other members of microflora. Our Knowledge of the complexity of the biocontrol systems is currently very limited. A great deal of research will have to be undertaken in order to understand the complexity of the biocontrol mechanism.

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to have autolytic, nutritional and morphogenic roles. In mycoparasitic fungi chitinase are associated with the degradation of cell walls (Mukesh *et al.,* 2014 and Pandey *et al.,* 2014 d).

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