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Trichoderma for plant disease management: A reality or myth?

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ABSTRACT: *Trichoderma* Spp. are the most widely used biofungicides the world over. These mycoparasitic fungi are effective against several pathogens that cause seed and seedling rot, foliar infections as well as post-harvest decay. We have been working on a particular species of Trichoderma (*Trichoderma virens*) for more than three decades and found it to be one of the most effective biocontrol agents against soil-borne pathogens like *Sclerotium folfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium* spp. In this brief review, I will discuss the progress and potential of biocontrol of *Trichoderma virens* with special emphasis on the strain (IMI 304061) isolated and developed by our group. Two distinct strains of T. virens have been reported – the "Q" strains that produce copious amounts of the antibiotic gliotoxin and the "P" strains that do not produce gliotoxin, but instead produce gliovirin. Our strain belongs to "P" group and is a very rapid colonizer of the sclerotia of S. rolfsii and R. solani, and this mode of antagonism plays an important role in bringing down the inoculum potential. This strain is also effective as an inducer of systemic resistance in plants. Recent advances in molecular genetics have enabled understanding the molecular mechanism of biocontrol and our strain has emerged as a model system in fungal–fungal interactions. Several cDNA, cosmid and BAC libraries have been constructed, ESTs sequences deposited. The role of two mitogen-activated protein kinase (MAPKs) has been studied. The TmkA pathogenicity MAPK was found to be involved in repression of conidiation, parasitism of sclerotia and induced resistance response in plants. Another MAPK, the TmkB cell integrity kinase was also involved in repression of conidiation in addition to antagonistic properties and cell wall integrity. Similarly, a G-protein alpha subunit TgaA was found to be involved in antagonism, though in a host selective manner. Interestingly, even though the two G-proteins studied had no major effects on growth of the fungus, deletion of Tac1, the adenylate cyclase, drastically affected the growth. Deletion of Tac1 also affected the production of antifungal compounds viridin and viridiol. In addition to functional studies, using suppression subtractive hybridization (SSH), genes have been identified that are regulated during MAPK signaling as well as secondary metabolism. These data have opened up enormous potential for genetic improvements of these strains.

Keywords: *Trichoderma virens*; Biological control; Mycoparasitism; Antibiosis; ISR; MAP kinase; G-proteins; cAMP; Signal transduction

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Introduction

Trichoderma spp. (perfect stage, where known, belongs to *Hypocrea*) are widely used as biofungicides. According to a recent estimate, about 60% of all globally registered biofungicides are *Trichoderma* based.[1] It is therefore obvious that *Trichoderma* spp. have become an integrated component of the agriculture world. Despite the fact that *Trichoderma* spp. are, in many cases, not as effective as chemical fungicides due to the living nature of formulations, their performances are often influenced by several biotic and abiotic factors. There is thus a vast scope for genetic enhancement of these biocontrol agents. Here, an understanding of the mechanisms would play an important role in devising strategies for strain selection and improvement based on the knowledge of genetics. Several species of *Trichoderma* are being used for biocontrol and *Trichoderma virens* (earlier *Gliocladium virens*) is one of the successful commercial biocontrol agents (Table 1). *T. virens* strains have been classified essentially based on their ability to produce gliotoxin or gliovirin.[2] The strains producing gliotoxin are classified as "Q" strains, and the ones that produce gliovirin are *T. virens* 29-8 (isolated at Texas A&M University) and *T. virens* strain GVW (IMI 304061) isolated by us at Pantnagar.[3] The purpose of this study is to outline the progress made with biological control and the mechanisms of biocontrol mediated by *Trichoderma virens* IMI 304061 in the past few decades.

Isolation of *T. virens* IMI 304061 (GVW) from Soil and Its Biocontrol Properties

While doing a pot experiment in greenhouse on optimization of inoculum dose for *Sclerotium rolfsii* (inoculated to soil as pre-colonized sorghum grains), we observed some mycoprasite growing on the colonies of this pathogen. The growth was so prolific that witin 7 days, the entire colonies of the pathogen were wiped off (Fig. 1). This green fungus was isolated, purified, identified and

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Table 1: Selected examples of successful biological control by *Trichoderma virens*

Crop(s)	Pathogen(s)	References or further reading
Cotton	*Pythium ultimum, Rhizoctonia solani*	Howell (1982); Howell (1991); Lewis & Papavizas (1991)
Cabbage	*P. ultimum, R. solani*	Lumsden & Locke (1989)
Seasame	*Fusarium oxysporum*	Kang *et al.* (1989)
Apple	*Phytophthora cactorum*	Smith *et al.* (1990)
Tomato	*Sclerotium rolfsii*	Ristaino *et al.* (1991)
Various pulses and oil seed crops	*S. rolfsii, R. solani, F. oxysporum*	Mukhopadhyay *et al.* (1992)
Field pea	*R. solani*	Hwant & Chakravarty (1993)
Tomato	*P. aphanidermatum*	De & Mukhopadhyay (1994)
Poinsettias	*Pythium* sp., *R. solani*	Newman *et al.* (1994)
Tomato, carrot	*S. rolfsii*	Ristaino *et al.* (1994)
Lettuce	*Sclerotinia sclerotiorum*	Budges et al. (1995)
Cotton	*F. oxysporum*	Zhang *et al.* (1996)
Eggplant	*R. solani*	Lewis & Larkin (1997)
Bell pepper	*S. rolfsii*	Ristaino *et al.* (1996)
Sunflower	*Sclerotinia minor*	Burgess & Hepworth (1996)
Cotton	*Verticillium dahliae*	Hanson (2000)
Gladiolus	*Fusarium oxysporum*	Misra *et al.* (2000)
Lentil	*S. rolfsii, R. solani, F. oxysporum*	Singh & Mukhopathyay (2000)
Catharanthus	*P. ultimum*	Burns & Benson (2000)
Soybean	Seed and seedling rot pathogens	Pant & Mukhopadhyay (2001)
Peanut	*Macrophomina phaseolina*	Maheshwari *et al.* (2001)
Chickpea	*S. rolfsii, R. solani, F. oxysporum*	Tewari *et al.* (2002); Tewari & Mukhopadhyay (2003)
Sunnhemp, mungbean	*Pythium aphanidermatum, S. rolfsii*	Piriyaprin *et al.* (2007)
Soybean	*R. solani*	Haikal (2008)
Rice	*R. solani*	Liu *et al.* (2010)
Tomato	*F. oxysporum*	Christopher *et al.* (2010)
Tea	Proia, Black rot	Barthakur *et al.* (in press; submitted in *Int J Tea Sci*)

deposited with IMI as *T. virens* IMI 304061 (then *G. virens*).[3] Subsequently, we developed a selective baiting technique based on this observation for isolation of *T. virens* from soil.[4] A great number of biocontrol assays followed in the Biocontrol Laboratory at Pantnagar and the efficacy demonstrated in farmers' fields. The first report on biocontrol using GVW came in 1992.[5] Subsequently, it was reported to be effective in controlling a variety of pathogens (like *Pythium* spp., *Fusarium* spp., *Rhizoctonia solani* and ββ*S. rolfsii*) in several crops.[6–11] The efficacy of this strain has also been demonstrated in farmers' field. This strain has thus proved to be an ideal model system for studies on the genetic mechanisms as illustrated in the following sections.

Mechanisms of Biocontrol

Trichoderma spp. can help reduce crop damage directly by suppressing the pathogen or indirectly by boosting plant immunity.[11] The direct effects include mycoparasitism and production of antifungal metabolites. Again, the mycoparasitism could be of the resting structures or the active hyphae. The indirect effects include improving plant defenses through the production of elicitor molecules or through improving the general health of plants by increasing nutrients availability.[11] GVW is a very effective mycoparasite on the sclerotia of *S. rolfsii* and *R. solani*, and is a destructive mycoparasite on the hyphae of *R. solani* (Figs. 2 and 3). By comparing a strain of

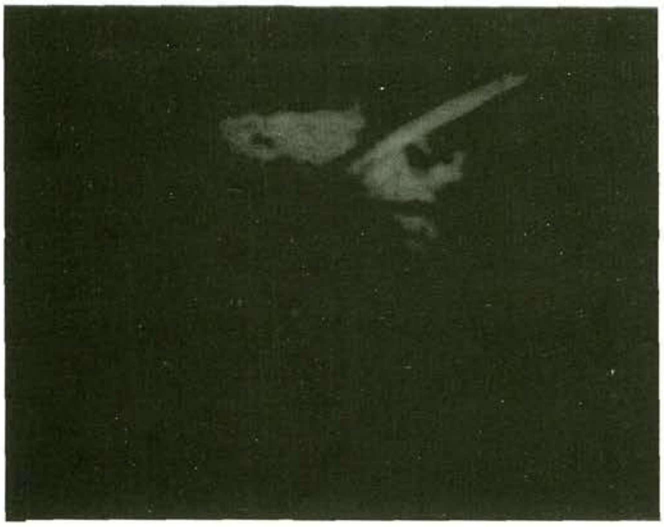

Figure 1. *Trichoderma virens* IMI 304061culture on soil (*Source*: Mukherjee *et al*., 1993).

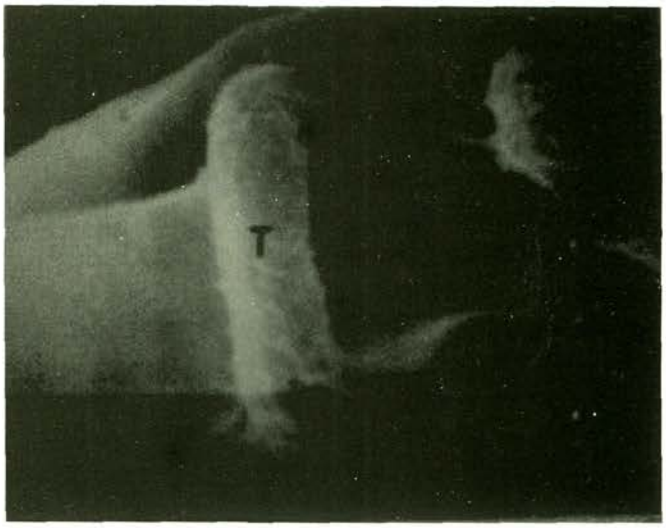

Figure 2. Mycoparacitism of *Trichoderma virens* on *Rhizoctonia solani*.

Trichoderma harzianum with GVW, Mukherjee *et al.*[12] concluded that parasitism of sclerotia is the principal mechanism of biocontrol of GVW on *S. rolfsii* and *R. solani*. Howell *et al.*[2] classified isolates of *T. virens* into two defined strains: the "P" strains that produce gliovirin and the "Q" strains that produce gliotoxin. By HPLC, we have confirmed that GVW is a "P" strain which does not produce gliotoxin, but produces plenty of the antimicrobial viridin and viridiol.[13] The direct role of antibiosis in biocontrol has not been established, but recent work on fungal secondary metabolites suggest that they might play other roles like induction of defense in plants[14,15] and also in survival potential in soil. In addition to direct effects on the pathogens, it has also been established that *T. virens* in an inducer of systemic defense responses in plants.[14,16]

Molecular Mechanisms of Biocontrol

Hydrolytic Enzymes

Hydrolytic enzymes play important roles in biocontrol, and among the hydrolytic enzymes, are chitinases and glucanases. In order to understand the role of the 42-kDa endochitinase, the coding gene *ech42* was deleted or over-expressed in *T. virens*. As a consequence, the biocontrol potential against *R. solani* in cotton were significantly decreased and enhanced, respectively.[17] The overexpression of a serine-protease encoding gene *tvsp1* significantly enhanced the ability of *T. virens* to protect cotton seedling against *Rhizoctonia solani*.[18] Constitutive overexpression of two β-glunasase genes *tvbgn2* and *tvbgn3* improved the biocontrol potential of *T. virens* against *P. ultimum*, *Rhizopus oryzae* and *Rhizoctonia solani*.[19] Recently, the role of a laccase gene *lcc1* in sclerotial parasitism has been suggested.[20]

Signal Transduction

Cellular signalling plays an important role in sensing the environment as well as hosts. Taking cues from plant pathogenic fungi on how they sense the host plants during pathogenicity, we investigated the roles of the signalling proteins (G-proteins, adenylate cyclase, MAP kinases) in GVW by gene knockout approach. For this purpose, a cosmid and a cDNA library were constructed and an ESTs database (available in the GeneBank) developed. Using gene knockout, we have studied the role of two MAP kinases, two G-proteins and the adenylate cyclase in growth, morphogenesis and biocontrol properties. The deletion of the *T. virens* MAPK TmkA and TmkB both resulted in de-regulated conidiation.[21,22] The loss of TmkA was associated with loss in mycoparasitism against the sclerotia of *S. rolfsii* and partial loss in mycoparasitism of *R. solani* sclerotia. However, the hyphal parasitism against this pathogen was un-altered. Interestingly, loss of this gene resulted in a reduction in ability of GVW to induce systemic resistance against the foliar pathogenic baterium *Pseudomonas solanacearum* py. *lachrymans* in cucumber, and a loss of biocontrol against *S. rolfsii*.[16] Interestingly, deletion of the same MAP kinase (Tvk1) improved the biocontrol potential of a "Q" strain of *T. virens* against *R. solani* and *Pythium ultimum*.[22] These studies thus indicated multiple roles of the MAPK pathways in regulation of conidiation and biocontrol properties. We have also identified an expansin-like protein Mrsp1 to be repressed by TmkA MAP kinase.[23] In contrast to MAP kinases, the deletion of two

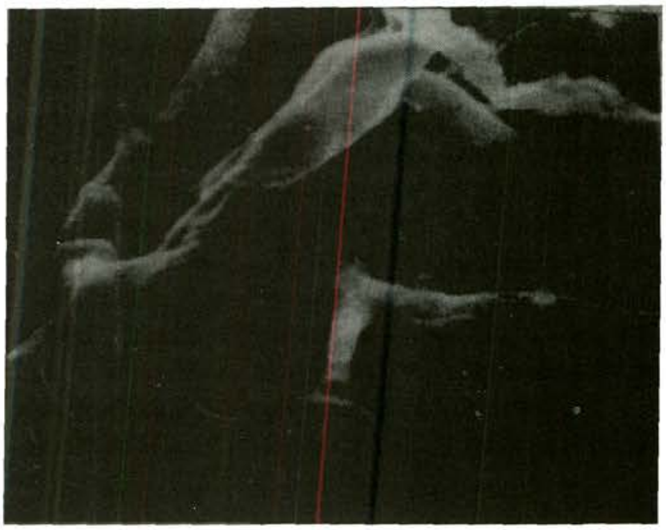

Figure 3. Paracitism of sclerotia of *Sclerotium rolfsii* and *Rhizoctonia solani* by *Trichoderma virens* (*Source*: Mukherjee *et al.*, 1995).

G-proteins did not have any major effects on the morphogenesis and biocontrol of GVW; although there was a reduction in the ability to parasitize the sclerotia of *S. rolfsii* and *R. solani*. However, deletion of the adenylate cyclase gene (*tac1*) resulted in a drastic effect on growth of *T. virens*.[24] The mutants were extremely slow growing, had colonial growth habit and also had reduced antibiotic potential. Using a suppression subtractive hybridization (SSH), some genes for secondary metabolism that are down-regulated in the mutants were identified. These observations indicate that it would be possible to manipulate the signalling pathways to fine-tune a balance between conidia production and biocontrol.

Secondary Metabolism

Trichoderma spp. produce more than 100 different secondary metabolites, some of them having antimicrobial properties.[25] *T. virens* is one of the most extensively studied species for the role of secondary metabolism in biocontrol. *T. virens* isolates are known to produce seven major secondary metabolites: the 18-, 14- and 11-residue peptaibols, gliotoxin, gliovirin, viridin and viridiol.[2,26,27] Gliotoxin is produced by the "Q" strains of *T. virens*[28] and the compound was detected in the rhizosphere.[29] However, the relevance of gliotoxin in biocontrol has not yet been clearly established.[2,30–32] Gliovirin, produced by the "P" strains has been suggested to be involved in biocontrol of *Pythium* spp.[33] *T. virens* also produces the fungistatic and anti-cancer steroidal compound viridin,[2] which is reduced to viridiol in the same culture and has berbicidal properties.[34] Using a non-producing mutant and suppression subtractive hybridization (SSH), Mukherjee *et al.*[35] identified a *T. virens* gene cluster that includes the terpene cyclase VIR4 and cytochrome P450s. The adenylate cyclase gene *tac1* has recently been demonstrated to regulate the biosynthesis of bioviridian/viridiol and the expression of the genes in this cluster.[24]

Interaction with Plants

Many *Trichoderma* spp. can internally colonize roots and develop "intimate" association with plants.[36] The "symbiosis-like" association is driven by nutrient (sucrose) flow from plant to the fungus and induction of resistance against invading pathogens by *Trichoderma*. The role of a *Trichoderma* invertase and a sucrose transporter has been studied recently.[37,38] Interestingly, the hydrolysis of sucrose was also important for regulation of the elicitor protein Sm1. The ability of *Trichoderma* to enhance root growth is well-known, perhaps this also benefits the fungus during symbiotic interactions. For long, it was speculated that *Trichoderma* might produce some phytohormones. In a recent publication, Contreras-Cornejo *et al.*[39] demonstrated that *T. virens* produces auxins and they also identified genes responsible for auxins biosynthesis in this beneficial fungus. *Trichoderma* spp. induce resistance in plants through the production of elicitor molecules. The most well-characterized elicitor protein Sm1 is produced by *T. virens*.[40–43] This is a hydrophobin-like small secreted cysteine-rich protein that, in pure form can elicit resistance response (production of reactive oxygen species, induction of defense-related genes) in both monocots and dicots.[40] Using gene deletion, it was shown that Sm1 is required for ISR response in maize,[44] and the elicitation potential is dependent on the ability of this protein to remian in monomer form.[42] In a significant development, Sm1 has been expressed in yeast and purified in active form.[43] The role of an 18-residue peptaibol in ISR has also been demonstrated.[14]

T. virens as a Source of Transgenes

The first gene to be transferred from *T. virens* to plants happen to be *ech42*, the gene encoding a 42-kDa endochitinase.[45] Transgenic cotton plants expressing this gene showed significant resistance to both *Rhizoctonia solani* and *Alternaria alternata*. Biochemical and molecular analyses showed rapid/greater induction of ROS, expression of several defense-related genes and activation of some PR enzymes and the terpinoid pathway.[46] Interestingly, even in the absence of a challenge from the pathogen, the basal activities of some of the defense-related genes and enzymes were higher in the endochitinase-expressing cotton plants. This elevated defensive state of the transformants may act synergistically with the potent, transgene-encoded endochitinase activity to confer a strong resistance to *R. solani* infection. The same gene was also introduced in rice, and high level of resistance against *R. solani* could be obtained.[47] We have cloned an *ech42* gene from our isolate of *T. virens* and expressed in transgenic tobacco and tomato.[48] The transgenic plants exhibited resistance against the fungal pathogens *Alternaria alternata*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. In another significant finding, a glutathione transferase gene from *T. virens* (TvGST) was cloned and transferred to transgenic tobacco. The transgenic plants, thus generated, were tolerant to several abiotic stresses including cadmium and the pollutant chemical anthracene.[49,50] The transgenic plants, under stress, showed lower levels of lipid peroxidation, compared to non-transgenic plants. The transgenic plants not only tolerated high levels of anthracene, but also degraded it to non-toxic naphthalene derivatives.[50]

Future Outlook

Trichoderma spp. are undoubtedly the most successful biocontrol agents utilized all over the world. In India alone, there are nearly 300 commercial *Trichoderma*-based biogungicides. The tremendous success of *Trichoderma* in the past three decades has turned the critics into admirers of biocontrol. Biological control with *Trichoderma* is no more a myth, but a reality, and this "gift of God to mankind" is here to stay as a main component of not only plant disease contro, but also overall crop management. *Trichoderma* spp. being active not only as antagonists, but its all-out impact on soil and plant health, including growth promotion, improved nutrients update, imparting resistance to biotic and abiotic stresses, bioremediation, and many more desired attributes. The era of genomics has set in and the sequence of *T. virens* has recently been published (http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html). It is now up to the scientists to make use o these huge data to understand in further details the mechanisms of action of *Trichoderma* on plants and pathogens and to design novel strains with improved potential.

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