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**REVIEW ARTICLE**

# *TRICHODERMA* AS A PROFICIENT BIOCONTROL AGENT FOR PHYTO-PATHOGENIC FUNGI – MECHANISM OF ACTION AND GENETIC ADVANCES

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## ABSTRACT

**Plant disease management presently relies heavily on chemical fungicides. However, their harmful impact on human and ecosystem, and development of fungicide-resistant strains has emphasized on biological control as alternative eco-friendly strategy for disease management and sustainable agriculture. *Trichoderma* is a widely used as biocontrol agent against various phyto-pathogenic fungi owing to its effective strategies to suppress plant pathogens. The control phenomenon involves direct mechanisms like mycoparasitism, secretion of hydrolytic enzymes which degrade the cell walls of pathogenic fungi, and indirect mechanisms like competition for resources, induction of plant defense mechanism and antibiosis. *Trichoderma's* multifaceted approach to biological control makes it environment-friendly option for managing plant diseases. Recent biotechnological approaches have simplified the isolation and characterization of efficient biocontrol agents, along with the identification of their genetic by-products. These techniques facilitate the cloning of these microbes in plants, with aim to bolster their resistance to both biotic and abiotic stresses. Biotechnological advances have not only reinforced the symbiotic interaction between microbes and plants but also allowed the modification of processes through microbial biocontrol agents (MCBAs). Genome sequencing of MBCAs has provided valuable insights into their genetic makeup, aiding their characterization. The present comprehensive review provides an insight to the existing and recent molecular advances utilized to enhance the efficiency of MBCAs in managing plant diseases and understanding biocontrol mechanisms through various omics technologies.**

**Keywords:** Biological control, genomics, mutagenesis, phytopathogens, proteomics, transcriptomics, *Trichoderma*

## 1. INTRODUCTION

By 2050, the overall population of world may reach roughly 9.1 billion, thereby increasing the need to directly increase agricultural food production almost by 70% to feed this rising population. As per conservative estimates, plant diseases annually cause 10-15% direct economic losses to major food crops (Peng *et al.*, 2021). Among the diseases of various fungal, bacterial, and viral etiology, more than 10,000 pathogenic fungal species are inflicted to cause 70-80% diseases worldwide. To control

these diseases, inorganic and organic chemical fungicides of different formulations that are capable to inhibit growth and kill the spores/ mycelium of causal organisms are frequently used. However, the use is in an injudicious manner (Sood *et al.,* 2020). These chemical fungicides not only are expensive, but their use in crop protection often build up toxins that are harmful to humans and their ecosystem (Sood *et al.,* 2020). The fungicide use also exhibits numerous detrimental effects on soil, water, environment, and human health and leads to the development of fungicide-resistant strains. For instance, more than 20 plant pathogens like *Colletotrichum graminicola, Pythium aphanidermatum, Pyricularia grisea, Puccinia horiana, Botrytis cinerea, Alternaria alternata, A. solani, Uncinula necator, Plasmopara viticola, Venturia inequalis, Erysiphe (Blumeria) graminis* f. sp. *tritici, E. graminis* f. sp. *hordei, Mycosphaerella graminicola, M. fijiensis, Mycovellosiella nattrassii, Podosphaera, (sphaerotheca) fusca, Pseudoperonospora cubensis, Didymella bryoniae,* and *Corynespora cassiicola* are reported to be strobilurin-resistant worldwide (Singh *et al.,* 2020, Nabi *et al.,* 2023). There are many reports of fungicide/bactericide resistance development in different plant pathogens such as *Magnaporthe grisea* (organophosphorus fungicides, MBI-Ds), *Botrytis cinerea* (Fluazinam), *Pseudoperonospora cubensis, Phytopthora infestans* (phenyl amides), *P. cubensis, Monilinia natrassii, Corynespora cassiicola, Colletotrichum gleosporioides* (QoIs /strobilurins), *B. cineria, Venturia naschicola, M. fructicola, Gibberella fujikuroi, Tapesia yallundae, Fusarium graminearum, Cercospora kikuchii, C. gleosporioides, Elisinoe fawcetti, E. ampelina* (benzimidazoles), *M. grisea, Pseudomonas avenae* (kasugamycin), *Alternaria alternate* (polyoxin), *B. cinerea, A. alternata* (dicarboximides), *Podosphera (sphaerotheca) fuca, Erysiphe (Blumeria) graminis f. sp. tritici, S. aphanis var. aphanis, Mycovellosiella nattrassii* (sterol demethylation inhibitors), *P. glumae, P. avenae* (oxolininic acid), *Xanthomonas campestris* pv. *prunii, P. syringae* pv. *lachrymans* (streptomycin) for which alternative management strategies are required (Nega and Healthcare, 2014). To minimize the use of chemical fungicides, the biological control method is a feasible attractive alternative strategy to combat plant diseases and attain a sustainable agriculture system.

Biological control involves the use microbial antagonists to suppress or manage diseases and pests including weeds. A living organism that controls the pest or pathogen is called a biological control agent. Predominantly, the biological control term is also used for the use of natural products extracted or fermented from various biological sources. In strict sense, biocontrol is the suppression of pest(s) with the help of other organism. As per legal definition, a biological control agent is a plant pest, an invasive alien species, a quarantine plant pest, a plant protection product, a harmful organism, or any combination of these (Ward, 2016) which reduce pests including pathogens, insect pests, and weeds by exploiting living organisms including viruses for the welfare of humans (Stenberg *et al.,* 2021). Biocontrol is used as an abbreviated synonym for biological control agents. There are different types of microbial species used as biocontrol agents, among them *Trichoderma* is readily used as a biocontrol agent against different phytopathogenic fungi (Ghazanfar *et al.,* 2018).

## 2. TAXONOMY OF *TRICHODERMA*

*Trichoderma* was first cited by Persoon in 1794 in his fungal classification but other fungi like *Ascobolus*, *Puccinia* and *Mucor,* and some slime molds like *Stemonitis*, *Trichia* and *Physarum* were also incorporated in his classification. Bisby first proposed *T. viride* as a species of *Trichoderma* from 1939 to 1969 and all the strains were identified as *T. viride* on Bisby's concept. So before 1969, most of the taxa determined are misidentified probably since *T. viride* is relatively a rare species. Rifai in 1969 acclaimed nine "aggregate species" based on an analysis of morphological characteristics namely *T. harzianum* Rifai, *T. koningii* (Oudem.) Duché and R. Heim, *T. viride, T. hamatum* (Bonord.) Bainier, *T. pseudokoningii* Rifai, *T. piluliferum* J. Webster and Rifai, *T. polysporum* (Link) Rifai, *T. longibrachiatum* Rifai and *T. aureoviride* Rifai. In early 1990s, 27 biological species and 5 sections were identified within *Trichoderma* genus. For molecular species identification, the introduction of tools like polymerase chain reaction (PCR) based random amplified polymorphic DNA markers (RAPD), coding sequence variation phylogenetic markers, and restriction fragment length polymorphism (RFLP) markers had a remarkable influence on the

taxonomical development of fungi. From late 1990s to 2002, the number of *Trichoderma* species rose to 47 (Kullnig-Gradinger *et al.,* 2002). *Trichoderma* presently contains at least 469 species names, 382 of which are currently valid. Fourteen species could not be identified even at molecular levels, but 365 species had DNA barcodes for at least one, and usually many, DNA loci. Identification of *Trichoderma* species is a complex and time-consuming operation that necessitates a clear concept in mycology, molecular biology abilities, molecular evolution, and extensive taxonomic literature survey. Modern diversity in *Trichoderma* spp. cannot be recognized using automated sequence similarity searches (NCBI BLAST or MIST BLAST) or oligonucleotide DNA barcodes like TrichOKEY. *In silico,* validation and biological verifications are desired for all molecular identifications. Similarly, *Trichoderma* spp. can't be identified using phylogenetic analysis alone unless the sequence similarity values to the entire collection of closely related species are included. Because of the large number of cryptic and closely related *Trichoderma* species, reliable molecular identification needs the examination of at least three DNA barcodes: ITS, tef1, and rpb2. However, only 273 species have access to the sets of these three loci. Unambiguous identification is only achievable for up to 224 species or 60% of the total number of species because many of these species were identified by insufficient reference sequences or shared tef1 or rpb2 with other species. Currently, 40% of *Trichoderma* species can be molecularly identified with some degree of uncertainty, therefore either high accuracy (lower taxonomic resolution) or precision (lower accuracy) should be chosen (Table 1) (Cai *et al.,* 2022).

**Table 1: Outline of historical developments in *Trichoderma* species**

| S. No. | Major contribution | Reference |
|---|---|---|
| 1. | Coined the term *Trichoderma* | Ch (1794) |
| 2. | *Trichoderma viride* Pers. ex Fries, and notes on Hypocrea | Bisby (1939) |
| 3. | Species aggregates | Rifai (1969) |
| 4. | Cellulase production by *T. reesei* | Montenecourt & Eveleigh (1979) |
| 5. | Mycoparasitism by *Trichoderma* | Chang et al. (1986) |
| 6. | Multigene phylogeny | Kullnig-Gradinger *et al.* (2002) |
| 7. | Release of *H. fecorina/ T. reesei* genome | Martinez *et al.* (2008) |
| 8. | Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma* | Druzhinina *et al.* (2012) |
| 9. | Sequencing the genomes of *T. harzianum, T. asperellum, T. longibrachiatum, T. virens, T. atroviride, T. asperellum,* and *T. citrinoviride* | JGI genome (*https://genome. jgi.doe.gov/portal/2012*) |
| 10. | Sequencing of genomes of *T. helicum, T. koningiopsis, T. pleuroticola* and others | JGI genome (*https://genome.jgi. doe.gov/portal/2018*) |
| 11. | Whole-genome sequence - *T. harzianum* species complex | Baroncelli *et al.* (2015); Fanelli *et al.* (2018) |
| 12. | Complete genome sequence, repeat-induced point mutation, and partitioning of CAZyme gene clusters - *T. reesei* | Li *et al.* (2017) |
| 13. | Evolution and comparative genomics of *Trichoderma* species | Kubicek *et al.* (2019) |

## 3. *TRICHODERMA* AS AN EFFECTIVE BIOCONTROL AGENT

Genus *Trichoderma* belongs to phylum: Ascomycetes, class: Sordariomycetes, order: Hypocreales, and family: Hypocreaceae. *Trichoderma* species are green-spored ascomycetes present in nearly all types of temperate and tropical soils. They are found in decaying plant materials and in rhizosphere. A biologically active volatile compound (6-pentyl-α-pyrone) is responsible in some species for producing a characteristic sweet or 'coconut' odour (Brotman *et al.,* 2012). There are multiple mechanisms whose activation is responsible for the antagonistic properties of *Trichoderma*. Various strains of *Trichoderma* act directly or indirectly as a biological control agent against fungal plant pathogens (Poveda, 2021). Mycoparasitism is a direct mechanism while indirect mechanism involves modification of environmental conditions, induction of plant defensive mechanism, antibiosis and competition for nutrients and space (Gajera *et al.,* 2013).

### *Direct mechanism*

**Mycoparasitism:** Mycoparasitism is a way of living wherein an antagonistic fungus directly parasitizes another fungus. Fossil records reveal it to be a 400 million years old practice (Kubicek *et al.,* 2011). *Trichoderma* species use mycoparsitism approach against pathogens through efficient release of lytic enzymes like β-1,3 glucanase and chitinases, etc. (Gajera *et al.,* 2013). The pathogenic cell wall material speed up this process resulting in mycelial lysis of pathogens due to overgrowth, coiling and penetration of hyphal pegs (Ojha *et al.,* 2011). It is a complex process wherein antagonistic fungus grows chemotropically on the host, recognizes the host and thereafter, the pathogen hyphae attachment and coiling, exudation of extra-cellular enzymes and disintegration and extortion of host cell take place. Different stages are reportedly involved in pathogenic parasitism. The chemotrophic response of antagonistic fungi induced by chemical stimulus from pathogenic fungi, followed by mutual recognition of the two through lectins; and the reciprocation among the hyphae of antagonist and pathogenic fungus. The hyphae of antagonistic *Trichoderma* either coil around the host hyphae or grow along with it and secrete lytic enzymes like glucanase, chitinase and pectinase for proceeding the mycoparacitism. *T. lignorum* supresses the *R. solani* through mycoparacitism. β-1,3-glucanases have the property to degrade cell wall, inhibit spore germination and mycelial growth of pathogenic fungi (Lin *et al.,* 2012). The cell wall and hyphal membrane of pathogen are degraded by proteases produced by *T. harzianum*. *Botrytis cinerea* produce hydrolytic enzymes like exo- and endo-poly-galacturonases which can be deactivated by proteases by the bioagents resulting in decreased grey mould severity. During mycoparasitic activity, the lysis of hyphal cell wall of pathogen occurs by enzymes like β-1,3-glucanases, protease, and chitinases. The growth of *S. rolfsii* was inhibited by *T. viride* and *T. harzianum* that myco-parasitize and compete with pathogens (Kator *et al.,* 2015). *T. longibrachiatum* parasitizes both the aleurioconidia and hyphae of *Thieleviopsis paradoxa* under *in vitro* conditions and produce non-volatile toxic metabolites. Different workers have also recorded the *Trichoderma* spp. having mycoparasitic activity against plant pathogenic fungi (Brotman *et al.,* 2010).

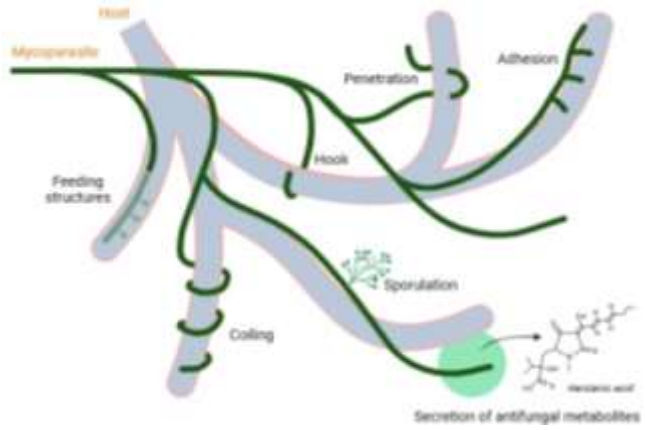


**Fig. 1: Direct mechanism of parasitism (mycoparasitism) by *Trichoderma* for biocontrol of plant pathogens**

### *Indirect mechanisms*

**Competition for food and space, and modification of environmental conditions:** *Trichoderma* species is considered excellent competitors for nutritional and space resources. *T. spirale* reduces disease severity by inhibiting *Corynespora cassiicola* and *Curvularia aeria* pathogens causing leaf spot disease in lettuce by competition, production of cell-wall degrading enzyme and through release of volatile antifungal compounds (Baiyee *et al.,* 2019). Displacement of pathogen occurs due to the competition between pathogen and biocontrol agent. In soil and rhizosphere, *Trichoderma* competes with other fungi for essential elements, food and/or modify rhizosphere by lowering soil pH thus rendering the survival of pathogen difficult. To be rhizosphere competent, *Trichoderma* species should colonize the rhizosphere more than 2 cm deep from seed or its concentration should multiply and exceed the initial population of seed coating. Rhizospheric competence of a *Trichoderma* isolate makes it a triumphant biological control agent. *Trichoderma* species, applied either as seed or soil treatment grow easily together with growing/evolving root system of treated plant ( Khan *et al.,* 2017) and provide defence against the pathogenic infection. *F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis* in rhizosphere of cotton and melon, respectively, are supressed by *T. harzianum* via competition for carbon source. The competition for carbon source is involved in antagonism shown

by several strains of *Trichoderma* spp. against several plant pathogens, especially *F. oxysporum*. Though the competition by rhizosphere competence is not a main mechanism directing the biological control, it is undisputedly a valuable appurtenance to those that cause biological control. One concept related to the rhizosphere competence and competition is the renewal of native fungi on root surfaces.

**Induction of plant defense mechanism:** *Trichoderma* strains when added to the rhizosphere secure plant pathogens such as fungi, bacteria, and viruses through induced resistance mechanisms like induced systemic resistance, hypersensitive response, and systemic acquired resistance (Haggag and Mohamed, 2002). The growth of *T. atroviride* strain TS-treated plants was remarkably stimulated in comparison to the untreated plants (La Spada *et al.*, 2020). *Trichoderma* spp. stimulate host defense response that has been reported to be a vital mechanism for induced disease suppression. Induced resistance has been reported with *T. virens* on cotton, *Trichoderma* spp. on cucumber and *T. harzianum* on bean (Koike *et al.*, 2001). Seedlings of cotton showed higher levels of defence-related compounds like terpenoids and higher peroxidase activity in roots when treated with *T. virens* strains. *T. viride*-treated legume seeds induced systemic resistance by reprogramming their defense mechanism. The levels of defense enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, reactive oxygen species ($H_2O_2$, OH•, $O_2$), antioxidant enzymes superoxide dismutase, catalase, and scavenging activity of antioxidant enzymes were alleviated. This mechanism develops resistance in plants thus imparts protection against pathogenic invasion. *T. virens* induces more peroxidase activity in cotton seedlings than control; and some effective strains stimulate terpenoid, protein or glyco-proteins production as likely elicitors. *T. viride* produces an ethylene-inducing xylanase that elicits phytoalexin resveratrol production in grape vine cells (Hanson and Howell, 2004, Rao *et al.*, 2015).

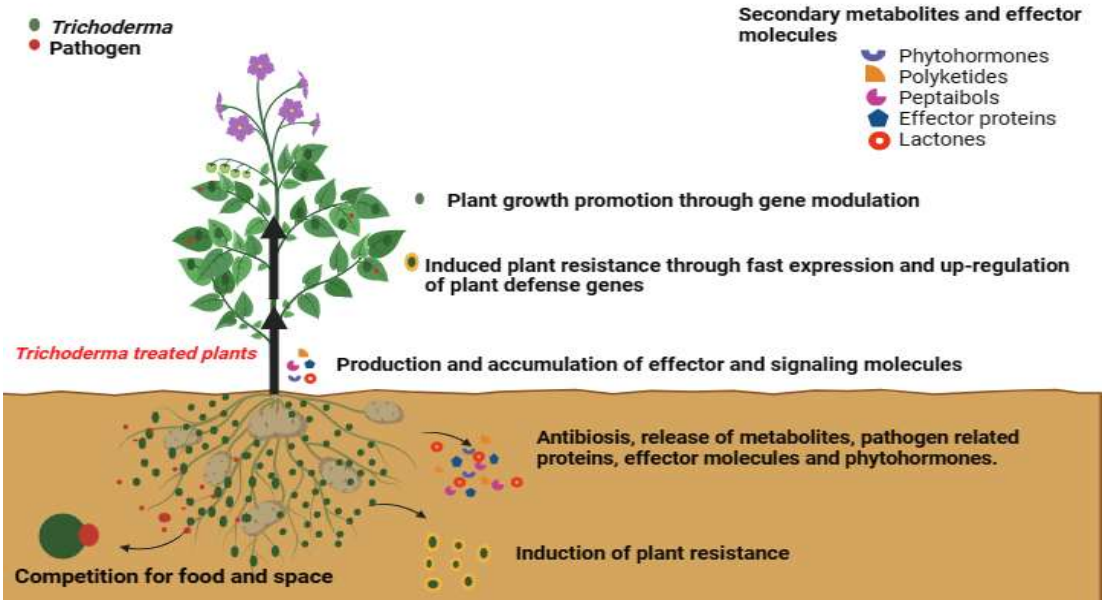


**Fig. 2: Indirect mechanism of parasitism by *Trichoderma* species for biocontrol of plant pathogens**

*T. harzianum* inoculation induces defense response in both the leaves and roots of treated plants. Tomato plants pre-treated with chemical inducers *viz*., methyl jasmonate, salicylic acid and biocontrol agent *T. harzianum* induces defense against *F. oxysporum* f. sp. *lycopersici* by enhancing the expression of several antioxidant enzymes (Zehra *et al.*, 2017). The use of biocontrol agent in conjunction with chemical inducers may be a more promising option for efficient management of plant diseases through different defense mechanisms. The systemic acquired resistance activation is correlated with the expression of genes related to pathogenesis including acidic and basic β-1,3-glucanases and chitinases that act against the pathogenic cell wall. *Trichoderma, Bacillus,* and *Pseudomonas*-treated plants of *Phyllanthus niruri* resulted in higher peroxidase, polyphenol oxidase,

phenylalanine ammonia lyase, and total phenolic activities (Kamakannan *et al.,* 2004). A small protein 1 (Sm1), an elicitor secreted by *T. virens*, induces resistance in cotton cotyledons against the foliar pathogen *Colletotrichum* spp. by activating host defense mechanism. In plants, the induced systemic resistance by *Trichoderma* is phenotypically similar to the systemic acquired resistance. Certain strains of plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR) can establish systemic resistance to plant immunity. (Contreras-Cornejo *et al.,* 2014). The cellulase and xylanase derived from *Trichoderma* spp. induces ethylene biosynthesis (Martinez *et al.,* 2008).

**Antibiosis:** Saprophytic ability of a fungus is decided by one of the important characteristics known as antibiosis that can be defined as the process in which the growth of a pathogen is inhibited by the production of antibiotics or substances like non-specific or specific metabolites by the antagonist (Haggag and Mohamed, 2002). Only a few species in *Trichoderma* and *Gliocladium* produce various antibiotics resulting in antibiosis (Mukhopadhyay and Kumar, 2020). *Trichoderma* produces secondary metabolites constituting different chemical compounds which help them in competition with other micro- and macro-organisms and also in other interactions like differentiation, symbiosis, and metal transportation (Adnan *et al.,* 2019). *Trichoderma* spp. produce three types of secondary metabolites *viz.,* peptaibols (linear-oligopeptides of 15-22 amino acids long and rich in amino-isobutyric acid, N-acetylated at N-side and an amino-alcohol at C-side); volatile antibiotics 6-pentyl-a-pyrone (an iso-cyanide derivative); and water-soluble compounds koningic acid or heptalic acid (Zeilinger *et al.,* 2016, Adnan *et al.,* 2019). Different types of antibiotics are reportedly produced by numerous biocontrol agents including *Trichoderma* spp. Several soil-borne phytopathogens like *Bipolaris sorokiniana, R. solani, F. oxysporum, Pythium middletonii* and *Phytophthora cinnamomi* were inhibited by koninginin D (Singh *et al.,* 2018). *Trichoderma* spp. are recognized as efficient producers of extracellular enzymes, and a few of these are involved in biocontrol of plant disease. Peptaibol antibiotics (basic serine chitinase and proteinase) are produced by *T. harzianum* strains thus revealing that the factors of virulence involved in biocontrol of fungal pathogens and entomopathogenicity are same. The various antibiotics or volatile compounds produced by *Trichoderma* spp. include koninginin F, pachybasin, trichodermin, succinic acid, harzianolide (3-(2-hydroxy1-propyl))-4(hexa-2″-dienyl2(5H)) furanone, trichorzianines, trichoviridin, propionic acid, 3-(3-isocyano-cyclopent-2-enzylidene), acrylic acid, 3-(3-isocyano-6-oxabicyclo (3,10) hex-2-eh-5-yl), acetic acid, octadecyl ester, acetone, arachidic acid, benzyl butyl phthalate, bicycloheptan-2-ol, butanoic acid, butyl ester, carbolic acid, phenol, lindane, isopropyl ester, 1-methylethyl ester, isopropyl myristate, limonene, isomenthone, isopentyl acetate, menthone, trichodermol, harzianone (harziane diterpene), viridiol, 9-epi-viridiol, harzianum A, chitobiase, trichosetin, gliotoxin, viridian, protease, β-l,3-glucanase, chitin-1-4-*b*-chitobiosidase *n*-acetyl, *b*-d-glucosaminase, endochitinase, dermadin, sesqui-terpene heptalic acid, heptelidic acid, α-glucosidase protein, chitinase, alamethicin, paracelsin, and trichotoxin (Stoppacher *et al.,* 2010, Siddiquee *et al.,* 2012, Miao *et al.,* 2012). *Trichoderma* spp. produce antibiotics like harzianic acid, gliotoxin, alamethicins, viridian (Malmierca *et al.,* 2012).

Antibiotics represent just one of the activities performed by *Trichoderma* against host fungi, in synergism with cell wall degrading enzymes. Gliotoxin, a toxic substance produced by *T. lignorum,* is fatal to *R. solani* and *Sclerotinia americana*. Gliotoxin is produced by *Gliocladium virens* which has now been renamed as *Trichoderma virens*. *T. harzianum* forms one or more antibiotics when incubated with *B. cinerea,* like peptaibols, beta-1,3-glucanase and chitinase. The cultural supernatant of *T. harzianum* inhibited hyphal elongation and spore germination in *B. cinerea* when placed on fresh medium. The non-volatile metabolites in cultural filtrate of *Trichoderma* spp. inhibit the linear pathogenic growth. The inhibition of *F. solani* and *F. longipus* growth by 60-64% was reported by use of cultural filtrates of *T. harzianum*. Gliovirin, an antibiotic isolated from *Trichoderma virens* (GV-P), inhibited *Phytophthora* spp. and *P. ultimum*, however, it was not effective against *Verticillium dahlia, Rhizopus arrhizus, Thielaviopsis basicola, Phymatotrichum omnivorum* and *R. solani*. Two bacteria namely *P. fluorescence* and *B. thuringenesis* were also not inhibited by gliovirin. The damping-off of zinnia caused by *R. solani* and *P. ultimum* complex was reduced by the suppressive

activity of *T. virens* (GL-21) and correlated for the production of gliotoxin by the bioagent. The production of non-volatile metabolites (NVMs) by *Trichoderma* against various phytopathogenic fungi by filtered liquid extract method on PDA medium has also been reported (Marques *et al.*, 2018). The NVMs produced impeded *S. sclerotiorum*, while two strains of *T. brevicompactum* (CEN1274 and CEN1245) showed broad-spectrum antagonistic activity against *F. oxysporum*, *Cylindrocladium* spp., *Colletotrichum gloesporioides*, *V. dahliae* and *S. rolfsii*. The toxin producing biocontrol agents suppress disease development by either causing the death of pathogenic cells (antibiosis) or by affecting spore germination (fungistasis) (Haggag and Mohamed, 2002). The growth suppression of plant pathogens by *T. asperellum* and inhibition of spore germination (by 30-75%) has also been reported (Win *et al.*, 2021). The antibiotic gliovirin produced by *T. virens* can kill *P. ultimum* by protoplasm coagulation. *Trichoderma* spp. produce antibiotics that include harzianic acid (Vinale *et al.*, 2013), trichoviridin, gliotoxin, viridiol, alamethicins, viridian, etc. *T. longibrachiatum* EF5, an endophytic fungal antagonist, exhibited 58% inhibition against the soil borne fungal pathogen *Macrophominia phaseolina* in dual culture, while crude soluble secondary metabolites showed 61% biological control activity (Sridharan *et al.*, 2021). These antibiotics when combined with cell wall degrading enzymes synergize their pathogen-inhibiting activity (Vinale *et al.*, 2013). *T. harzianum* PC01 produces trichotoxin A50 that inhibited sporangia production and mycelial growth of *P. palmivora*.

**Enzyme production:** *Trichoderma* spp. produce many lytic enzymes that play vital role plant growth promotion and disease suppression (Mohiddin *et al.*, 2010). Chitins, glucans and polysaccharides impart rigidity to the fungal cell wall and these constituents are degraded by various hydrolytic enzymes. Four *Trichoderma* species when grown along with the cell walls of *F. solani*, *R. solani* and *S. sclerotiorum* secreted various cell wall degrading enzymes. *T. harzianum* and *T. asperellum* were most promising biocontrol agents against phytopathogenic fungi (Qualhato *et al.*, 2013). The endo- and exo-chitinases produced by *T. koningii* (Tr5) help in the penetration and destruction of pathogenic cells without causing any harm to the plant tissue. The enzyme preparations of *T. harzianum* inhibited the growth of *S. rolfsii* by 61.8%. Many *Trichoderma* species produce hydrolytic enzymes like, β-1,3-glucanase, chitinase, protease and non-volatile and volatile antibiotics. Chitinases, glucanases and proteases are lytic enzymes secreted by *Trichoderma* to infest various phytopathogenic fungi (Radjacommare *et al.*, 2010), and this step is important for mycoparasitic process. The cell walls and cellular contents of pathogenic fungi are rendered available to *Trichoderma* by lytic enzymes secreted by *Trichoderma*. The hydrolytic enzymes on bean leaves, produced by *B. cinerea*, might be inactivated due to the proteases produced by *T. harzianum* T39. The protease solutions produced by biocontrol fungus when applied to the bean leaves partially deactivated the hydrolytic enzymes and reduced disease severity by 56-100%.

## 4. STRAIN IMPROVEMENT IN *TRICHODERMA* SPECIES

Genomics and transcriptomics have yielded fascinating insights regarding the makeup, structure, and development of *Trichoderma*. It has facilitated deeper probe into the processes like cellulase and glucanase hyper-production, mycoparasitism, etc. through more comprehensive biological approaches. Efficient gene manipulations by molecular tools in any fungal species including bioagents paved the way for strain improvement. In particular, the development of high-throughput methods for gene insertion and/or deletion, utilizing improved deletion fragment construction and strains with a near-perfect integration frequency, highlight the remarkable progress in this field. This breakthrough now enables ambitious projects, such as creating a library of genome-wide knockout strains. Not only do these progresses provide new opportunities for enhancing molecular strain improvement, but they also have serve as a source of inspiration for accelerated research on *Trichoderma* spp. (Bischof and Seiboth, 2014). The enhancement of biocontrol activity in *T. harzianum* strains has been a focal target in research. This involves fortifying the protein and chemical effectors for biocontrol, improving the resilience of strains, and adjusting the gene-regulatory system that governs these processes. The new advanced tools play important role in systematic taxonomy within a fungal community. Numerous

strains once labelled as "*T. harzianum*" have now been re-designated as distinct *Trichoderma* species (Chaverri *et al.,* 2015, Fanelli *et al.,* 2018, Cai and Druzhinina, 2021). The study of diverse structures and functions found in microbial communities and their complex interactions with host is crucial to understand the microbial ecosystems. This diversity is cornerstone for leveraging these ecosystems for biotechnological purposes. Microbes, as key players, have a vital role in shaping the functionality of earth's ecosystems. Focusing on individual proteins or transcripts alone does not provide complete understanding of intricate biological processes. A more comprehensive approach, like conducting omics-based studies, may provide a wealth of information encompassing the genomic, transcriptomic, translational omic, metabolomic, and proteomic data. This encompassing approach is crucial in unravelling the functional pathways of fungi at cellular level. The unique traits of *Trichoderma* strains make them incredibly adaptable, with natural inclination towards increased productivity. Moreover, a plethora of cutting-edge omic techniques have been proposed to further enhance the yields. Omics methodologies provide a comprehensive understanding of *Trichoderma* strain's physiology by using a combination of genomic, transcriptomic, translatomic, and proteomic analyses (Sharma *et al.,* 2017). Extensive molecular studies have delved into the genetic and transcriptional tendencies of various *Trichoderma* strains at atomic level. Targeted molecular methods have flourished the *Trichoderma* strains through the implementation of whole-genome sequencing (Lorito *et al.,* 2010).

## 5. BIOTECH APPROACHES TO IMPROVE *TRICHODERMA* EFFICACY

***Directed mutagenesis technique***

The exposure to ultraviolet (UV) radiations and physical/chemical mutagens induce mutations in microbial biological control agents (MBCAs). These mutations may cause changes in specific bases within the double-stranded DNA molecule. Such mutations can be achieved by using compounds like ethyl methane sulfonate (EMS) or N-methyl N′-nitro-N-nitrosoguanidine (NTG). UV radiation can cause the formation of dimers between adjacent pyrimidines, resulting in incorrect base insertion at dimer site. Further, either type of mutagen can trigger frame shifts, duplications, or deletions in DNA sequence. To enhance the capabilities of MBCAs and their antifungal production, different mutagenesis techniques from physical mutagens like γ-irradiation and UV radiation, to chemical mutagens are used. Among these techniques, the isolation of tvk1 gene from *T. virens*, which encodes a mitogen-activated protein kinase, has been found effective. The mycoparasitic abilities, conidiation, and biocontrol efficiency of tvk1 null mutants showed a significant increase in the expression of genes involved in mycoparasitism when exposed to *R. solani*. Moreover, unlike wild-type strain, the null mutants displayed a remarkable increase in protein production with high lytic enzyme concentrations in culture supernatant (Mukherjee *et al.,* 2003). The γ-rays were able to generate *T. harzianum* mutants with strong inhibition against *Macrophomina phaseolina* (Abbasi *et al.,* 2014). The melon plants treated with *Trichoderma* mutants had significantly 28% lower disease incidence of charcoal rot as compared to the control. So efforts were made to improve the antagonistic properties of *T. harzianum*-1432 and *T. atroviride* against *S. rolfsii* causing chickpea collar rot, by using the mutagen NTG (Singh *et al.,* 2016). The γ-radiation of a Co-60 γ-radiator caused mutations in *T. harzianum*, ultimately enhancing its ability to combat pathogen *F. oxysporum* f. sp. *radicis-cucumerinum* (Sahampoor *et al.,* 2020). To generate more robust strains of *T. viride*, varying concentrations of EMS was used to induce mutations. The resulting mutants showed impressive abilities to combat *F. oxysporum* f. sp. *ciceris* (Dandale *et al.*, 2017). Improved effectiveness and resilience of *T. asperellum* strains was successfully achieved through NTG-induced mutations, resulting in enhanced carbendazim tolerance. Notably, N2-1, N2-2, N2-3, and N1 mutants demonstrated exceptional antagonistic properties against *F. oxysporum* f. sp. *ciceri*, *R. bataticola*, and *B. cinerea*. These mutants displayed their ability to sporulate when exposed to high dose of carbendazim (2000 µg $mL^{-1}$), highlighting their robustness against fungicide pressure (Naik *et al.,* 2023). It is worthwhile to note that the genetic approaches employed in these studies did not involve recombinant DNA methods, but rather imitated natural processes. As a result, these methods are not subject to the regulations governing the use of genetically modified organisms, a quality making them more readily acceptable for purposes of disease management.

Several new mutant strains of bacterial bioagents have been created through random integration of transposable elements like Tn5 or Tn3 into an organism's genetic code so as enhance biocontrol abilities. For instance, Luo *et al.* (2023) explored the potential of *B. velezensis* Bs916 in enhancing surfactin production, a valuable bioactive compound known for its powerful antimicrobial properties, and identified 20 variants through transposon mutagenesis, with derivative H5 [carrying a mutation in GltB gene (δGltB)], yielding remarkably 7-fold surge in surfactin production over original strain. Transposon mutagenesis has promising potential in *Trichoderma* in developing efficient mutants.

***Protoplast fusion technique***

Protoplast fusion methods have been employed to combine beneficial characters of diverse strains, with the aim to improve the biocontrol abilities of MBCAs. The processes of protoplast isolation, fusion, and regeneration have effectively been carried out in multiple *Trichoderma* strains. The production of lytic enzymes involved in the breakdown fungal cell walls, including chitinases and endo-glucanases, is a vital mean of biocontrol by *Trichoderma* species. By merging protoplasts from *T. harzianum* and *T. viride*, a stronger strain namely *Trichoderma* HF9 fusion was developed. This unique combination displayed remarkably enhanced activity, surpassing both parent strains, in total and specific chitinase activity (Balasubramanian *et al.,* 2012). The protoplast fusants of *Trichoderma* species with improved ability to withstand multiple stressors have successfully been developed Out of 36 fusants, 20 fusants displayed remarkable qualities that were inherited from only one of the parent strains. Interestingly, the remaining fusants were heterozygous, demonstrating a combination of traits inherited from both parental sources (Hirpara *et al.,* 2019). The protoplast fusion from endophytic *T. harzianum* strains yielded a powerful fusant, FU3, which showed superior antagonistic properties against *A. flavus, S. sclerotiorum,* and *R. solani* (Dolatabad *et al.,* 2019). *T. harzianum* mutants produced by γ-radiation showed a remarkable increase in chitinase and endoglucanase as compared to the original strains, and these modified strains effectively suppressed the growth of *R. solani* (Ghasemi *et al.,* 2019). The protoplast fusion of *T. harzianum* NBAII T 1 with *T. viride* NBAII Tv 23 boosted the antagonistic potential against soil-borne pathogens like *R. solani, S. rolfsii, F. oxysporum,* and *M. phaseolina* causing root rot and wilt diseases (Lakhani *et al.,* 2016).

***Genetic recombination and transformation***

One way to boost the effectiveness of a MBCA is by introducing a foreign DNA, carrying the genes responsible for producing biocontrol metabolites, into its genome. Further, disabling or deleting certain genes within such biocontrol strain may improve its long-term efficacy. This approach has successfully been implemented to combat *Agrobacterium tumefaciens*-mediated crown gall disease. Specifically, a modified version of *A. radiobacter* K84 was used to prevent the emergence of agrocin 84-resistant *A. tumefaciens* strains by introducing plasmid genes (de Boer *et al.,* 2003). The genetic makeup of *T. viride* contains a powerful endo-chitinase gene. This 1672 bp long DNA fragment contains a promoter and mRNA coding section. After being combined with the promoter and terminator sequences of *Aspergillus nidulans* Trp synthetase, the gene has successfully been inserted into *Chaetomium globosum* CG10. An impressive 33% modified strains displayed a noteworthy surge in endo-chitinase activity, showcasing its efficacy. Recently, the genes responsible for producing xylanase in *T. harzianum* kj81197 have successfully been isolated (Ellatif *et al.,* 2022). Of these genes, xyn2 showed highest xylanase activity, surpassing that of the wild type. Remarkably, this gene also displayed impressive antifungal properties, effectively combating pathogenic fungi such as *Alternaria* spp., *F. oxysporum, Corynespora cassiicola,* and *Botrytis cinerea*.

Mycoparasitic antagonists combat pathogens through cell wall degradation of pathogen by producing enzymes like chitinases, proteases, and glucanases. These chitinases have successfully been cloned from various microbes such as *Serratia marcescens* and *T. harzianum*. In fact, in *T. virens* strains from cotton, the presence of two copies of ech42 gene has shown greatly enhanced antagonistic activity against *R. solani* (Baek *et al*., 1999). Further, a defective ech42 gene fragment led to decrease in antagonistic activity. The constitutive expression of chit33 in *T. harzianum* on agar plates proved to be a successful strategy in enhancing biocontrol activity against *R. solani* (Limón *et al.*, 1999).

***Cloning and insertion of genes for antibiotic/antifungal enzymes production***

An effective method for expanding the range of a biocontrol agent involves the transfer of genes associated with antibiotic production from a hostile strain to another. A prime example of this approach is successful cloning and expression of trichodermin gene (*tri5-trichodiene synthase*) from *Trichoderma brevicompactum* strain, which resulted in a significant increase in trichodermin production. This compound has shown powerful antifungal properties against *Aspergillus fumigatus* and *Fusarium* spp. which highlights its potential in combatting the infectious fungi (Majumdar, 2023). Exo-enzymes, especially chitinases, cellulases and β-1,3-glucanases, are involved in the breakdown of fungal cell wall components that contribute to the pathogenic ability to harm plants. Both bacteria and fungi used as MBCAs produce these enzymes. Due to their single-gene regulation, the genes coding for these enzymes can easily be isolated and transferred to other MBCAs. The genes responsible for xylanase production were effectively cloned from *T. harzianum* kj81197 (Ellatif *et al.,* 2022). The cloned gene *xyn2* notably exhibited augmented xylanase activity in comparison to the original strain. Additionally, *xyn2* showed exceptional effectiveness in inhibiting the growth of *Alternaria* spp., *F. oxysporum, Corynespora cassiicola* and *B. cinerea*. The ability of *T. harzianum* to control *R. solani* was improved by inserting *chit33* gene into the fungal strain and testing its effectiveness on agar plates.

***Improved root colonizing abilities***

Some soil bacteria called 'plant growth-promoting rhizobacteria' (PGPR) positively impact the plant growth without forming a symbiotic bond. These PGPRs occur in soil near and inside the plant roots and help the plants to thrive by inhibiting the growth of harmful bacteria. The amplification of genes responsible for PGPR's ability to fight plant pathogens is possible. One way is to improve their competing ability for nutrient (C, N, and Fe) acquisition, which may boost their ability to colonize the roots. Other way is to manipulate the genes associated with signalling pathways involved in colonization. These pathways govern the processes like motility, chemotaxis and biofilm formation, so their manipulation can enhance the colonization abilities of antagonists. Chi *et al.* (2023) delved into the functional analysis of enzyme glycosyl transferase *Taugt17b1*. This enzyme is crucial in enhancing the root-colonizing ability of *Trichoderma* species. The over-expression of *Taugt17b1* significantly boosted growth rate in *T. atroviridae*, bolstered its root colonization ability and effectively triggered systemic defense responses in plants, thereby providing effective protection against plant diseases.

***Improving biocontrol potential of Trichoderma through advanced omics approaches***

**Genomics:** The term "omics" refers to a diverse spectrum of scientific fields including, peptidomics, transcriptomics, proteomics, metabolomics, phenomics, secretomics, genomics, and interactomics (Sharma *et al.,* 2017). By employing the cutting-edge omics techniques, the metabolomic and transcriptomic profiles of various MBCAs including *Trichoderma* have successfully been identified and analysed. Different omics techniques have significantly contributed to our understanding of the complex and diverse interactions involving MBCAs (Lorito *et al.,* 2010). The studies on genetic spectra namely genomics is recognized as a subfield within the realm of omics. Through microbiome study of MBCAs, we can effectively suppress the pathogenic activity and develop new consortia. The implementation of genome mining techniques has aided in identifying the multiple genes in *Trichoderma* strains, providing better insight into their genomic data. Further, this approach has unravelled the previously unknown orphan biosynthetic cluster genes, making it easier to recognize related secondary metabolites in bioagent strains (Oni *et al.,* 2015).

**Transcriptomics:** Transcriptome studies offer a valuable approach to identify the potential transcripts involved in a variety of biological processes. Techniques like ESTs, subtractive libraries, and cDNA microarrays can be used to study comprehensive transcriptomic reactions to various stimuli, connecting them with specific biological functions (Herrera-Estrella, 2014). Numerous transcriptome studies have explored the intricate relationships between *Trichoderma*, plant and pathogen. A comprehensive transcriptomic analysis has been conducted in *T. harzianum* T4 focusing specifically on mycoparasitism related genes (Wang *et al.,* 2023). The study showed that *T. harzianum* exhibited impressive control

over tomato grey mould pathogen *B. cinerea*. A total of 2871 differentially expressed genes (DEGs) were identified in *T. harzianum* at various time of growth (12, 24, 48, and 72 h) in presence of the pathogen's cell wall. Notably, the top ten significantly upregulated genes encompassed a wide range of functions, including glycoside hydrolases, cutinases, proteases, secondary metabolite synthesis, and signal transduction proteins. The transcriptome of *T. atroviride* IMI206040 was studied during its interactions with *R. solani, Phytophthora capsici*, and *B. cinerea*. Quantitative reverse transcription PCR (qRT-PCR) was used to assess the expression levels of 13 genes, revealing significant variations in presence of *R. solani* (Reithner *et al.,* 2011). These genes were mainly responsible for expansin-like proteins, acetyl xylan esterases, aspartyl proteases, and trypsin-like proteases. Further, the relative gene expression analysis at different stages of parasitism against *B. cinerea* and *P. capsici* showed a collaborative increase in the transcription of several genes involved in degradation of fungal cell walls.

**Secretomics:** Secretomics is a specialized field of proteomics focused on studying secreted proteins. This approach is incredibly useful in understanding the full range of proteins produced by MBCAs, such as *Trichoderma*. Different species of *Trichoderma* play a vital role in the colonization of host plants aiding in their defense against harmful infections. *T. harzianum* produces a significant amount of proteins while combatting the plant pathogen *Guignardia citricarpa*. These excreted proteins played a vital role in inducing plant's defense mechanisms and halting the progress of pathogenic invasion (de Lima *et al.*, 2017). da Silva *et al.* (2022) showed that *T. harzianum* TR 274 secretomes mined from culture filtrates in presence and absence of *Phaseolus vulgaris* host revealed the secretion of 124 and 190 fungal proteins, respectively. The most prominent functional proteins identified were glycoside hydrolases, proteases, and oxidoreductases, which play vital role in modulating the host's defense responses. Secretome analysis has also unravelled the biocontrol mechanisms of MBCAs. The mycoparasitic activity of *T. harzianum* ALL42 was found linked to several key proteins such as chitinases, glucoamylases, β-1,3-glucanases and proteases. These proteins were activated after 24 incubation upon the introduction of *F. solani* cell wall in the culture medium (Ramada *et al.,* 2016).

**Proteomics:** Proteomics has proved a vital tool in exploring the complex interactions between hosts, pathogens, and microbial biocontrol agents. The isolation and identification of novel proteins that can be used in combating plant diseases is possible through proteomic analysis. While the earlier studies on biocontrol activities relied on gel-based techniques, the use of modern gel-free mass spectrometry-based methods has revolutionized the proteomic research on MBCAs. These advanced methodologies have greatly enhanced protein analysis as compared to the traditional gel-based approaches (Otto *et al.,* 2012). The use of liquid chromatography and tandem mass spectrometry has wide applications during the studies on properties of MBCAs. These techniques not only provide valuable information about MBCA proteomic fingerprints but also offer insights into the underlying biological processes. By using advanced proteomic techniques, it is easy to accurately identify the specific proteins involved in biocontrol mechanisms, demonstrating their potential for biotechnology and showcasing their differential expression (Esposito *et al.,* 2016). Interactions between *B. cinerea* and *T. harzianum* triggered the production of several cell wall degrading enzymes (CWDEs) in nutrient-rich environment (Yang *et al.,* 2009) including β-1,6-glucanases, proteases, chitinases and xylanases. *Trichoderma* species produce 3 unique CWDEs in response to *R. solani*. These novel proteins including N-acetyl-β-d-glucosaminidase and endochitinase, were identified through advanced proteomic analysis techniques (Grinyer *et al.,* 2005). Moreover, proteomics plays crucial role in understanding the impact of MBCAs specifically *Trichoderma*, on plant immunity. Lamdan *et al.* (2015) revealed the presence of both positive and negative protein effectors in an interaction between maize roots and *T. virens*. These effectors effectively suppress defense pathways and trigger systemic resistance highlighting the important role of proteomics in uncovering the mechanisms of plant-MBCA interactions.

**Genome sequencing of MBCAs:** The promising potential of MBCAs was revealed through whole-genome sequencing. The paired-end whole-genome shotgun sequencing was used to examine the distinct traits of several microbes, especially rhizospheric bacteria *Pseudomonas* spp. Cab57 (Table

2) (Takeuchi *et al.*, 2014). Through the strategic implementation of gap-spanning PCR products, the researchers were able to bridge the gaps between contigs successfully. This advanced analysis revealed 4 crucial gene clusters (phl, prn, plt, and hcn) responsible for producing antibiotics, facilitating nitrite/nitrate absorption, and regulating the Gac/Rsm signal transduction pathways. Moreover, the study unveiled that these specific genes greatly enhance bacterial ability to suppress the proliferation of harmful microbes. The genome sequencing assembly and characterization of two yeast cells of the *Metschnikowia fructicola* have shown exceptional biocontrol abilities, efficiently combatting post-harvest infections and successfully preserving the freshness of fruits and vegetables (Piombo *et al.*, 2018). The biocontrol abilities of yeast strain were uncovered through secretion of CWDEs, activation of defence signalling genes, possession of Fe-binding compounds, and production of superoxide anions.

**Table 2: List of various *Trichoderma* species with whole genome sequencing**

| S. No. | Microbial biocontrol agents | Accession No. | Reference |
|---|---|---|---|
| 1. | *Trichoderma harzianum* T6776 | JOKZ00000000 | Baroncelli *et al.* (2015) |
| 2. | *Trichoderma virens* FT-333 | JTGJ00000000 | Kuo *et al.* (2015) |
| 3. | *Trichoderma gamsii* T6085 | JPDN00000000 | Baroncelli *et al.* (2016) |
| 4. | *Trichoderma* sp. ITEM908 | PNRQ00000000 | Fanelli *et al.* (2018) |
| 5. | *T. harzianum* B97 | MRYK00000000 | Compant *et al.* (2017) |
| 6. | *Trichoderma afroharzianum* T11-W, and *T. cyanodichotomus* TW21990-1 | WUWT00000000, and WXUD00000000 | Zhou *et al.* (2020) |
| 7. | *Trichoderma reesei* QM6a | PRJNA225530 | Martinez *et al.* (2008) |
| 8. | *Trichoderma parareesei* CBS125925 | LFMI00000000 | Yang *et al.* (2015) |
| 9. | *Trichoderma hamatum* GD12 | ANCB00000000 | Studholme *et al.* (2013) |
| 10. | *T. virens* Gv29-8 | PRJNA264113 | Kubicek *et al.* (2011) |

## 6. FUTURE CHALLENGES

Improvement of the strains of *Trichoderma* spp. for biocontrol, industrial, and environmental purposes has great potential. Past initiatives have primarily focused on developing fungicide-resistant biocontrol mutants, with only a few instances highlighting the progress in studies related to the role of hydrolytic enzymes. Due to the gradual elimination of various fungicides and the limited discovery of new chemical solutions, the future of farming appears likely to rely on bio-pesticides. The changing climate has also emphasised the needs to develop such microbial pesticides that can withstand abiotic pressure and excel in controlling various pests. Despite the potential for genetic engineering to create new strains, there is no guarantee that these modified organisms will overcome regulatory barriers and be approved for use in field. Hence, mutation becomes a critical element in efforts to create improved *Trichoderma* strains and other microbial pesticides, establishing them as dependable substitutes for chemical pesticides (Mukherjee *et al.*, 2012). *Trichoderma* spp. boast impressive efficacy in a wide range of pathosystems under various cultural conditions. However, their antagonistic progress may not be at par with the chemical fungicides. These organisms require some time adopt a new niche and establish their abilities to manage other microbial populations. In addition, environmental factors such as moisture level, temperature, and fungicide compatibility may potentially hinder their effectiveness. Though chemical fungicides are more effective and cost-efficient, but pose serious threat not only to the environment but also to non-target microbes and animals including humans (Bhandari *et al.*, 2021). There is need to develop strategies for the identification and/or development of fungicide-resistant strains of bio-control agents which may be compatibly integrate in IPM and IDM modules. Molecular techniques have successfully been employed to enhance the effectiveness of MBCAs and comprehend their biocontrol mechanisms against plant pathogens. Despite advancements, there is still much to uncover about the mechanisms governing beneficial microbe-plant interactions. Despite market potential, several challenges such as limitations in fermentation, formulation, registration, and commercialization, need resolution. The evolution of next-generation technologies has provided valuable insights into the interactions between plants and beneficial microbes. The characterization of these MBCAs necessitates a comprehensive

understanding of their genomes. Therefore, to achieve a profound understanding of biocontrol strains, it is advisable to first identify their properties using genomic, transcriptomic, and proteomic approaches. This basic information aids in identifying crucial pathways in biocontrol mechanisms. Recently, various omic methods have unveiled mechanisms employed by MBCAs, and genetic manipulation through molecular techniques hold the potential to create new biocontrol strains with enhanced tolerance to biotic stresses and increased production of toxic compounds against plant pathogens. However, it is crucial to note that genetic manipulation of MBCAs is in its early stages, and regulatory considerations significantly impact its acceptance. There is need for global attention on the necessity to monitor genetically modified organisms under diverse environmental conditions. The expanding biotechnology knowledge can contribute to the development of methods for monitoring the genetic and environmental effects of genetically modified MBCAs, facilitating their long-term use in managing plant diseases.

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