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Introductory Chapter: Identification and Isolation of *Trichoderma* spp. - Their Significance in Agriculture, Human Health, Industrial and Environmental Application

Mohammad Manjur Shah and Hamisu Afiya

1. Introduction

The genus *Trichoderma* is a diverse group of free-living fungi in the family *Hypocreaceae*, commonly present in all soils [1–6]. These ascomycetes fungi are opportunistic, avirulent plant symbionts inhabiting root ecosystems [3, 7] and parasites on other groups of fungi [2]. They reproduce by chlamydo-spores and ascospores and proliferate better at mesophilic temperatures (25–35°C) and wide range of pH. Several findings supported this such as [8] who observed no visible growth of conidia at 15°C, but retain growth at 25°C and best results at 30°C [9], evaluated the growth of *Trichoderma* isolates at different temperatures and pH ranges, and reported the highest mycelial growth of *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969, and *T. hamatum* T614 at 25°C and *T. virens* T523 and *Trichoderma* sp. at 30°C. For pH requirement, mycelial growth of *T. hamatum* T612, *T. harzianum* T447, and *T. virens* T523 grew best at pH 5 and *T. harzianum* T969 and *Trichoderma* sp. at pH 7, while *T. hamatum* T614 has best mycelial growth at pH 8. A recent study by [10] reported 25–35°C. Similarly, a pH range of 5.5–8.5 was congenial for *T. harzianum* and *T. hamatum*.

Trichoderma colonizes several ecological niches where they play a vital role; they have been earlier recognized as effective biocontrol agents of plant-pathogenic fungi, producers of secondary metabolites of medical importance [3, 11, 12], and agents of bioremediation. Similarly, their ability to degrade lignocellulosic biomass to produce second-generation biofuels and other value-added products has been widely accepted [3, 12].

2. Identification of *Trichoderma* isolates

Conventional methods for identification of *Trichoderma* spp. using morphological and cultural approach have earlier been used. These include arrangement of conidiophores, phialides, and conidia, while cultural features include linear growth, colony color, growth pattern, and pigmentation of hyphae. The fungus has revealed different morphologies on various cultivation media due to genetic factors and

environmental and nutritional factors. Green colony pigmentation after incubation for 7 days at 28°C on potato dextrose agar (PDA) was observed in *Trichoderma* cultures isolated from soil samples in Kliran [5]. Rhizospheric isolates revealed pale or yellowish color of reverse colonies at 25 and 30°C with rapid growth, loosely arranged conidia, and effused conidiation [13]. An ellipsoidal, obovoid, and bowling pin phialides were observed in *Trichoderma* spp. [14]; 10 isolates from groundnut rhizosphere revealed different morphological and microscopic features as shown in **Table 1**.

S/N	Isolate	Colony color	Colony reverse color	Conidiophore character	Phialide character	Conidia shape	Chlamyospore formation
1	GRT-1	Dark green	Amber	Long infrequently branching and verticillate	Frequently paired, lageniform, and divergent	Globose to ellipsoidal	Infrequent, terminal, and intercalary
2	GRT-2	Dull green to bluish green	Colorless	Broad, verticillate, and frequently branching	Lageniform, divergent, terminal phialid more elongated	Sub cylindrical to narrow ellipsoidal	Frequent, intercalary, and terminal
3	GRT-3	White	White	—	—	No conidia	Abundant, terminal, and intercalary
4	GRT-4	Scattered in minute tufts and pale yellow green	Pale yellowish	Rarely branched and verticillate	Cylindrical or slightly inflated and divergent	Ellipsoidal	Frequently intercalary and terminal
5	GRT-5	Dell green to bluish green	Pale yellowish	Broad frequently branching and verticillate	Ampulliform and divergent	Sub cylindrical	Infrequent, intercalary, and terminal
6	GRT-6	Dark bluish green	Uncolored	Infrequently branching and verticillate	Lageniform and convergent	Globose to ellipsoidal	Frequently intercalary and terminal
7	GRT-7	Dark green producing tufts or pustules fringed by sterile mycelium	Dull yellowish	Frequently branching and verticillate	Ampulliform and convergent	Sub globose to obovoid	Infrequent, internally and terminally
8	GRT-8	Dull green to bluish green	Pale yellowish	Narrow verticillate and frequently branching	Ampulliform and divergent	Sub cylindrical to narrow ellipsoidal	Infrequent, intercalary, and terminal
9	GRT-9	Dark bluish green	Uncolored	Infrequently branching and verticillate	Lageniform and convergent	Globose to ellipsoidal	Infrequent, intercalary, and terminally
10	GRT-10	Compute dull, green tufts or pustules	Discolored	Frequently branching and pyramidal structure	Lageniform and divergent	Obovoid	Frequently, intercalary, and terminal

Source: [15].

Table 1.
Morphological characteristics of some Trichoderma isolates.

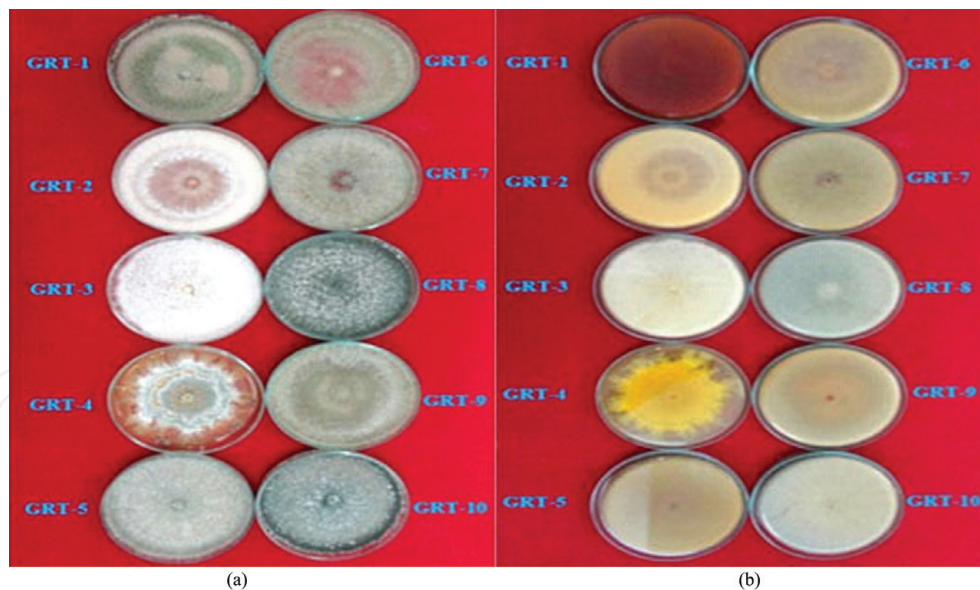


Figure 1.
Trichoderma isolates after 5 days incubation period [15]. (a) Colony color. (b) Colony reverse color.

Isolates GRT-1, GRT-6, and GRT-9 were confirmed as *Trichoderma viride*; GRT-2, GRT-5, and GRT-8 as *Trichoderma koningii*; GRT-4 as *Trichoderma reesei*; GRT-7 as *Trichoderma harizanum*; and GRT-10 as *Trichoderma aureoviride*, while GRT-3 could not be identified up to species level (**Figure 1**) [15]. However, conventional methods for the identification of *Trichoderma* spp. using morphological and cultural methods are prone to error and poor documentation of isolates at the culture collection since isolates are not “adequately differentiated” [16]. The use of molecular phylogenetic features coupled with the conventional methods is a better approach for verification of isolates and identification of novel strains [8] than morphological features alone, as anamorph and teleomorph used for defining species have reached their limits [17]. Several literatures have reported various techniques of molecular characterization to confirm *Trichoderma* isolates, such as “amplifying and analyzing the sequences of internal transcribed spacer gene (ITS) 1 and 2 and translation elongation factor 1-alpha (tef1) encoding gene” and BLAST interface in TrichOKEY and TrichoBLAST [16]; “restriction fragment length polymorphism (RFLP) and DNA sequencing” [18–20]; random amplified polymorphic DNAs (RAPD) and rDNA sequencing [14, 21]; sequence-related amplified polymorphism (SRAP) marker [7]; etc.

3. Isolation media for *Trichoderma*

The growth of *Trichoderma* has been screened on different culture media for various studies using available, relatively cheaper supporting media such as corn meal agar, oat meal agar, potato dextrose agar, Czapek’s Dox agar, special nutrient media, carrot agar, rose Bengal agar, selective media, etc. However, selective media favor growth of *Trichoderma* strains over other fungi and hence preferred for easy identification of *Trichoderma* isolates over rapidly growing fungi that may overlap it [22].

3.1 *Trichoderma* selective media (TSM)

Trichoderma selective medium (TSM) is recognized for quantitative isolation of *Trichoderma* spp. from soil. It is composed of low glucose level for rapid growth and sporulation of the fungus. Chloramphenicol is used to inhibit the growth of

bacteria, while pentachloronitrobenzene, p-dimethylaminobenzenediazo sodium sulfonate, and rose bengal are used as selective fungal inhibitors [22].

3.1.1 TSM recipe

1. 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
2. 0.9 g of K_2HPO_4 .
3. 0.15 g of KCl.
4. 1.0 g of NH_4NO_3 .
5. 3.0 g of glucose.
6. 0.15 g of rose bengal.
7. 20 g of agar.
8. 0.25 g of chloramphenicol.
9. 0.3 g of p-dimethylaminobenzenediazo sodium sulfonate.
10. 0.2 g of pentachloronitrobenzene.

Recipe is dissolved in 1000 ml distilled water and autoclaved at 121°C , 1.4 kg cm^{-1} for 15 min. Then add 0.25 g chloramphenicol and 0.2 g pentachloronitrobenzene into the solution. Keep/store media at 45°C to prevent solidification.

3.2 *Trichoderma harzianum* selective medium (THSM)

Selection of THSM enables comparison between aggressive and non-aggressive *Trichoderma* groups. The antimicrobials chloramphenicol, streptomycin, quintozone, and propamocarb are added to the medium to highly select *T. harzianum* in compact colonies without visible contamination [23].

3.2.1 Recipe for THSM

1. 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
2. 0.9 g of K_2HPO_4 .
3. 1.0 g of NH_4NO_3 .
4. 0.15 g of KCl.
5. 0.15 g of rose bengal.
6. 3 g of glucose.
7. 20 g of agar.
8. 950 mL of distilled water.

Media is autoclaved at 121°C, 1.4 kg cm⁻¹, for 15 min, and 0.25 g of chloramphenicol, 9.0 mL of streptomycin, 1.2 mL of propamocarb, and 0.2 g of quitozene are added.

3.3 Rose Bengal agar (RBA)

RBA is a nonselective medium for isolation of *Trichoderma* which is developed by Jarvis in 1973, for enumeration of molds and yeasts from food. The medium is suitable with protein foods and tolerates high temperatures. Chloramphenicol or chlortetracycline is added to suppress the growth of bacteria [24].

3.3.1 Recipe for rose Bengal agar (RBA)

1. Mycological peptone 5.0 g
2. Rose bengal 0.05 g
3. Glucose 10.0 g
4. Chloramphenicol 0.1 g
5. Dipotassium phosphate 1.0 g
6. Agar 15.0 g
7. MgSO₄·7 H₂O 0.5 g
8. Distilled 1000 mL

Add the ingredients to the distilled water and boil to dissolve completely. Add 10 mL of chloramphenicol or chlortetracycline; shake and autoclave at 121°C for 15 min. Store in the dark at 4°C for further use.

4. Method for isolation of *Trichoderma*

Several methods are available for the isolation of *Trichoderma*; however, one of the commonest methods reported in literature is the serial dilution of samples [22, 25–28]. This technique is simple, cost-effective, and appropriate to handle large samples.

Soil samples are collected, air dried, and ground into powder. Stock solution of sample is prepared by dissolving 10 g of powdered soil sample into 90 mL of distilled water. Next, serial dilution of samples were prepared as 10⁻¹, 10⁻²...10⁻⁵. One milliliter of each of the prepared dilution is spread evenly on a suitable medium on a petri dish at 28 ± 1°C for 7 days.

5. Agricultural significance of *Trichoderma* spp.

Continuous use of chemical pesticides to manage fungal pathogens (which are known to cause major diseases in agriculture) has led to destruction of soil structure, soil infertility, and accumulation of toxic compounds on crops. Moreover, “chemical fungicides have less influence on pathogens due to their diversity,

adaptability and increasing resistance” [4]. Various microbial biocontrol agents serve a solution for management of the aforementioned to attain a sustainable agriculture for future generations [29].

Knowledge about biocontrol potential of the fungus *Trichoderma* spp. has been recognized as early as 1920 [30, 31], although it received researcher’s interest with advances in genetic engineering [12]. This technology has made it easy to isolate, characterize, clone, sequence, and express the roles of specific genes in the biocontrol mechanism. The genes encoding the enzymes play vital roles in biotic and abiotic stress tolerance, growth of hyphae, degradation of cell wall, and antagonistic activity against plant pathogens [29]. *Trichoderma harzianum* (Th. Azad) and *Trichoderma viride* (01PP) are used as biopesticides and biofertilizers [32, 33], growth promoters, and inducers of disease resistance in plants [12, 33]. The former is the main antagonist utilized in management of plant diseases in agriculture [34, 35] due to its cost-effectiveness and minimal effects on the ecological balance [34].

Trichoderma is efficient in improving vegetative growth of plants and nutrient content of soil through decomposition and biodegradation [33]. Active substance such as fungal spores is applied as foliar sprays and pre- and post-planting treatments, during watering and transplanting. *Trichoderma*-based products are marketed worldwide and applied in fields, nurseries, and horticulture for management of fungal soil-borne pathogens such as *Pythium* and *Rhizoctonia* [33, 35]. It is a safe and environmentally friendly method to reduce the detrimental effects of chemical pesticides [36].

Various articles reported on the role of *Trichoderma* spp. as antagonist to plant pathogens such as *T. harzianum*, *T. asperellum*, and *T. virens* against *Phytophthora capsici* in red pepper [16]; *Trichoderma* isolates against *Sclerotium rolfsii*, *Colletotrichum gloeosporioides*, *C. capsici* [37], *S. minor* and *S. sclerotiorum* in the in vitro experiments [38]; *T. atroviride* SY3A and *T. harzianum* SYN were effective biological control agents of *R. solani* damping-off of cucumber [39]; *Trichoderma* isolates were antagonist to soil-borne phytopathogenic fungi (*Fusarium graminearum*, *Rhizoctonia solani*, *Macrophomina phaseoli*, and *Phytophthora cactorum*) [9]; *Trichoderma* species was antagonist to anthracnose of strawberry [5]; *Trichoderma* isolates inhibit and control the growth of *Fusarium oxysporum* with *Trichoderma harzianum* being the most effective [40]; *T. viride*, *T. polysporum*, and *T. harzianum* inhibit more than 60% growth of *C. paradoxa* [19]; *T. hamatum* LU593 and *T. virens* LU556 delayed aphids manifestation on cabbage [41]; *Trichoderma* isolates against *Sclerotium rolfsii* [6]; *Trichoderma* isolates against *Fusarium sambucinum* [42]; and *Trichoderma* spp. and *Bacillus* spp. in seed treatment against root knot nematode *Meloidogyne javanica* [43].

6. Mechanism of biological control

Several researches have revealed the mechanism of biocontrol in *Trichoderma* via mycoparasitism (by coiling around the host, formation of appressoria and breakdown of the host cell wall), antibiosis, and competition for resources (space and nutrients). A peptaibol from *Trichoderma* may induce apoptosis in plant-pathogenic fungi through complex mechanisms. Trichokonins, a type of peptaibol from *Trichoderma pseudokoningii* SMF2, produced a molecular biocontrol mechanism which efficiently induces apoptosis in fungal cells. Apoptotic hallmarks such as the deposition of cytoplasmic vacuoles, presence of reactive oxygen species, breakage of DNA molecule, and exposure of phosphatidylserine were observed in *Fusarium oxysporum* cells treated with Trichokonins [44]. Mycoparasitism has been reported in *Trichoderma* species antagonist to Anthracnose disease of strawberry.

Microscopic examination revealed that the hyphae of *Trichoderma* “grew alongside and coiled compactly around the hyphae of the fungal pathogen isolates” [5].

Recent development has further shown the significance of *Trichoderma* to induce systemic/localized resistance in plant by colonizing the root epidermis and subsequent release of bioactive metabolites, to transform the transcriptome and proteome of resultant plants [11, 30]. It further revealed that metabolites/enzymes produced in *Trichoderma* such as chitinase and β -1,3 glucanase as recorded in *T. viride* and *T. harzianum*, respectively [37]; cellulases and hemicellulases in *Trichoderma* spp. [12, 45]; and proteases and lipases [46] are responsible for breaking down the component of the fungal cell wall to reduce the integrity of the pathogen cell. Relevant biocontrol processes such as production of hydrolytic enzymes and antifungal metabolites and the formation of infection structures are controlled by heterotrimeric G proteins and mitogen-activated protein (MAP) kinases. Similarly, induction of plant systemic resistance in *Trichoderma virens* and hyperosmotic stress response in *Trichoderma harzianum* are related to MAPK signaling [47], while biocontrol associated with coiling and chitinase production in *Trichoderma* is regulated by internal cAMP level.

7. *Trichoderma* in human health

“*Trichoderma* species are possible source of important antimicrobial agents against gram negative, positive, fungi and yeast” [48]. Earlier in 1995, isolated peptides from *Trichoderma* strains showed antibacterial activity against *S. aureus* [49]; *T. harzianum* produced 44.06 $\mu\text{g/mL}$ of the well-known antifungal drug, cyclosporine [50]. Similarly, ‘Trichodermanins C–E (1–3), new diterpenes with a rare fused 6-5-6-6-ring system, have been isolated from a fungus *Trichoderma harzianum*’ detached from a marine sponge *H. okadai*. Cytotoxicity assay using three cancer cell lines showed significant activity in 1 [51]. Broth extracts of *Trichoderma* species (*Trichoderma harzianum*, *Trichoderma longibrachiatum*, and *T. koningii*) showed antifungal and antibacterial activity against *Paecilomyces variotii*, *Penicillium notatum*, *Nematospora coryli*, *Mucor miehei*, *Bacillus brevis*, *Bacillus subtilis*, *Enterobacter dissolvens*, and *Sarcina lutea* using agar disk diffusion method [48].

8. Industrial and environmental applications of *Trichoderma* spp.

Trichoderma are utilized in the production of low-cost enzymes for applications in food, pulp, and paper and textile industries to generate biofuel. The biotechnological workhorse of *Trichoderma* is the production of cellulases [52], in addition to the extracellular laccase [53]. Together with cellulases, endoglucanases (EG1, EG2, EG3, and EG5), and β -glucosidase, these enzymes catalyze the breakdown of ligninolytic biomass to produce an important industrial enzyme for the production of second-generation biofuels and other value-added products such as fermentable sugars, organic acids, solvents, drink softeners, etc. [54]. The production of proteins such as heterogenous proteins from cellobiohydrolase I (cbhI), a strong and inducible promoter of the gene encoding the major cellulose [55], and hydrophobins HFBI and HFBI [56–58] for industrial applications has been reported. Research efforts have focused on increasing enzyme yield and other valuable products from *Trichoderma* spp. through genome sequencing [45]. The highest yield of 1.4 g hydrophobins HFBI and 0.24 g hydrophobins HFBI per liter was obtained from the fungus through genetic manipulation on glucose-containing culture medium [59]; up to 40 mg/l of chymosin was produced from a transformed strain [55]; newly

constructed *Trichoderma* strains designed for specific industrial applications such as biofinishing and biostoning of cotton increased the cellulose CBHI by 1.5-fold and CBHII by fourfold as to the main strain. These were further increased to 1.6-fold CBHI and 3.4-fold CBHII by transformation as compared to the host strain. In addition, CBHII proteins were produced by the gene promoter (*Trichoderma* cellulases).

9. Conclusion

Trichoderma spp. is one of the frequently isolated fungal genera from soil and plant roots that have been extensively studied for their vast metabolites with various applications (agricultural, industrial, health, etc.).

In the field of agriculture, *Trichoderma* are suitable antimicrobial agents against pathogenic bacteria, fungi, and yeast. Similarly, they play a vital role in improving the vegetative growth of plants and nutrient content of soil through decomposition and biodegradation. It is a safe, cost-effective, and environmentally benign technology to attain a sustainable agriculture.

In the field of medicine, different metabolites of medical importance have been reported from *Trichoderma*. Earlier in 1995, isolated peptides from *Trichoderma* strains showed antibacterial activity against *S. aureus* [49]. *T. harzianum* produced 44.06 µg/mL of the well-known antifungal drug, cyclosporine [50].

Cellulases, an important industrial enzyme from *Trichoderma*, are essential in the breakdown of biomass to produce second-generation biofuels and other value-added products such as fermentable sugars, organic acids, solvents, drink softeners, [54] etc., in addition to the laccase production for textile industries. With advances in genetic engineering, efforts are focused on designing new strains of *Trichoderma* spp. through genome sequencing for production of novel metabolites of various applications.

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