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Review Article

The Impact of *Trichoderma* spp. on Agriculture and Their Identification

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ABSTRACT

Fungi belonging to the genus *Trichoderma* were discovered in the late 18th century and they have been utilized ever since their biocontrol potential was uncovered. *Trichoderma* species have greatly assisted the blooming of agricultural industries due to their aggressive characteristics against plant pathogens. Their role as a biocontrol agent is owed to their mode of mechanisms: induction of the plant's defence system, mycoparasitism, the production of secondary metabolites, and rhizosphere competence. Meanwhile, their role as a biofertilizer became evident when studies conducted hitherto showed that they could increase plant's nutrient uptake, improve the yield of crops, enhance plant's tolerance to external stresses, and induce the germination of seeds. Since this genus is hyperdiverse, accurate identification of them is indispensable. In the past, *Trichoderma* spp. were identified via their morphological characteristics. However, the emergence of molecular technology has made the identification of *Trichoderma* isolates more precise, explicit and rapid. Hence, this paper briefly reviews the accumulated knowledge in respect of this genus. Nevertheless, an extensive study must be done in order to explore the potential in improving the natural strains of *Trichoderma*.

Key words: Biocontrol agent, biofertilizer, molecular technology, plant pathogens, *Trichoderma*

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INTRODUCTION

Trichoderma spp. are ubiquitous in the soil community and they are found to be adaptable to every ecosystem. This adaptability is ascribed to their diverse metabolic capability and their antagonistic nature. As an endophyte, they colonize plant hosts meanwhile as a saprophyte, they live in soil organic matter. They could also invade mammalian tissues as a pathogen (Gams & Bissett, 1998; Hoyos-Carvajal & Bissett, 2011; Prasun *et al*., 2013).

According to Jaklitsch and Voglmayr (2015), *Trichoderma* asexual morphs are much more common in Mediterranean and sub-Mediterranean climates due to the short periods of moisture, when compared to the sexual morphs. There are a few key differences in the phenotype of sexual and asexual morphs. For an instance, there are ostiole dots and translucent ascospores in sexual morphs, whereas green conidia are observed in asexual morphs (Jaklitsch & Voglmayr, 2015). The bright green conidial pigment that is observed in *Trichoderma* spp. is associated to the *Hypocreaceae* family. In fact, all of the *Trichoderma* spp. were found to be strongly linked to *Hypocrea* and eventually *Trichoderma* spp. were proven to be derived from the *Hypocrea* species (Chaverri & Samuels, 2003a).

In 2015, Jaklitsch and Voglmayr collected *Trichoderma*

isolates from Southern Europe and the Canary Islands. The authors then obtained the *tef1* sequences of the isolates and aligned them with sequences extracted from GenBank in order to increase the coverage of the genus. From that, the authors were able to construct phylogenetic trees that represent major clades of *Trichoderma*. The trees constructed based on the *tef*1 sequences of the isolates were compared with the trees constructed based on the *rpb*2 sequences. Based on the study, the Viride clade was found to have the highest percentage of isolates in the Mediterranean climate, followed by Harzianum and Polysporum.

Although the genus *Trichoderma* was first found in 1794, the evidence of its mycoparasitic and biocontrol aptitude was discovered only by the year 1932 (Weindling, 1932). Moreover, they were found to have a rapid growth characteristic on culture media (Bissett, 1991). On top of that, *Trichoderma* spp. are able to protect plants, enhance vegetative growth, and act as soil amendments. This makes *Trichoderma* spp. an epitome of biocontrol agents and biofertilizers (Woo *et al*., 2014).

ROLE AS A BIOCONTROL AGENT

Over the course of time, many methods have been implemented to shield crops from pests and plant pathogens. These methods include mechanical trunk injection of fungicides and the application of pesticides (Abdul *et al*., 2004). Yet, these approaches have affected the environment and caused pests and pathogens to be resistant to chemicals. According to a study, plant diseases are more likely to be kept under control through biological means (Susanto *et al*., 2005). Therefore, *Trichoderma* spp. are gradually being recognized as an alternative to control pests and plant pathogens in the wake of their aggressive antagonistic characteristics and their ability to induce plant host defense system prior to pathogen invasion.

Today, there are more than 250 *Trichoderma*-based products (biofungicide) available worldwide, accounting for 60% of the whole biofungicide market (Topolovec-Pintarić, 2019). The species *T. harzanium* and *T. viride* are the most mainstream as they are competent in protecting 87 various crops (Sharma *et al*., 2014). In a recent study, *T. harzianum* was reported able to control the severity of *Ganoderma boninense* infection (Japanis *et al*., 2021). Besides the two species, other *Trichoderma* spp. such as *T. asperellum*, *T. koningii*, *T. atroviride*, *T. pseudokoningii*, *T. longibrachiatum*, *T. hamatum*, *T. polysporum, T. virens, T. gamsii,* and *T. reesei* are also famed for their potent ability as a biocontrol agent (Zin & Badaluddin, 2020).

Trichoderma spp. are involved in the interactivity between plant, pathogen and the soil environment. This three-way interaction was evaluated by Yoo and Ting (2017) and it was found that the defence enzymes produced by the host plant restricted the colonization and growth rate of the introduced *Trichoderma* spp. Regardless, it is noteworthy to know that the endophytes still maintained their antagonistic characteristics towards plant pathogens even in a poor growth condition (Yoo & Ting, 2017). Primarily, the mode of biocontrol mechanisms of *Trichoderma* spp. includes (1) mycoparasitism; (2) production of secondary metabolites; (3) competition for nutrients and space; and (4) induction of plant defence responses (Mukhopadhyay & Kumar, 2020) (Figure 1).

Mycoparasitism

One of the main modes of action of *Trichoderma* spp. is mycoparasitism where they act as a parasite toward other fungi. Mycoparasitism takes place following a train of events: (1) growth of *Trichoderma* spp., (2) recognition of prey using diffusible signals, (3) formation of appressoria and coiling, (4) secretion of hydrolytic enzymes, (5) penetration of the hyphae of prey, and (6) the lysis of prey (Mukhopadhyay & Pan, 2012; Singh *et al*., 2018). The mycoparasitic activities encompass the release of extracellular cell wall-degrading enzymes which are required for the penetration of hyphae and this will eventually cause the lysis of the pathogen. Along with that, a high activity of β-1,3 glucanase and chitinase was reported when *Trichoderma* spp. were co-cultured with pathogenic fungi. Both of the said enzymes hydrolyse chitin which is a major component of most fungi. As a result, the co-culturing caused the growth of the pathogenic fungi to be inhibited (Alias *et al*., 2011; Hirpara *et al*., 2017; Baiyee *et al*., 2019).

Production of secondary metabolites

Among the variety of secondary metabolites produced by *Trichoderma* spp., some were found to inhibit the formation of the cell walls of other pathogenic fungi (Table 1). For an instance, research done by Weindling (1934) proved that secondary metabolites that were toxic to other plant pathogens were excreted by *T. lignorum*. Additionally, the release of peptaibols by *Trichoderma* spp. was found to inhibit the activity of β-1,3 glucan synthase, which is an essential enzyme for cell wall construction (Nawrocka & Maloepsza, 2013). Moreover, *Trichoderma* spp. produce gliotoxin, gliovirin, koninginins, trichothecenes, and 6-pentyl-α-pyrone (6-PAP) which are compounds that have antifungal characteristics (Koli & Adusumilli, 2020). The antibiosis mechanism of *Trichoderma* spp., coupled with the excretion of metabolic compounds, resulted in the obstruction of pathogenic fungal

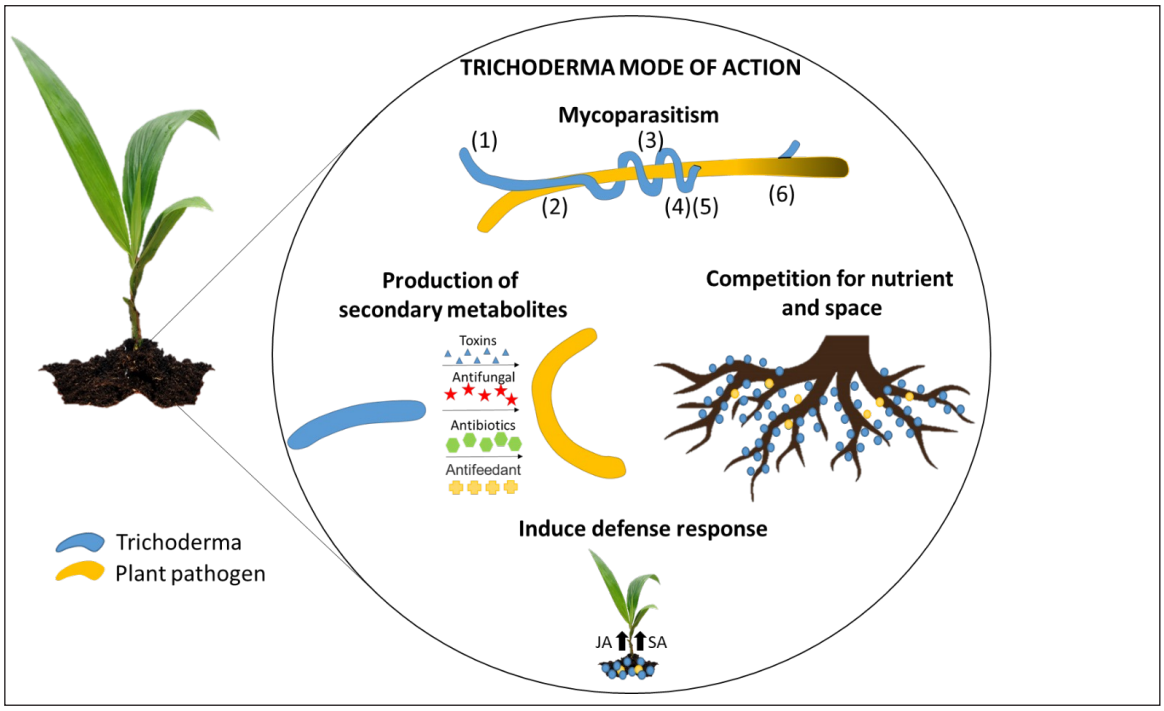

Table 1. Secondary metabolites produced by *Trichoderma* spp.

Secondary Metabolites	Effect	Reference
Peptaibols	Inhibit the activity of β-1,3 glucan synthase	(Nawrocka & Maloepsza, 2013)
Gliotoxin, Gliovirin, Koninginins, Trichothecenes, and 6-Pentyl-A-Pyrone (6-PAP)	Antifungi	(Koli & Adusumilli, 2020)
Trichodermin, Suzukacillin, and Alamethicin	Assist in destroying the cell walls of other pathogenic fungi	(Manibhushanrao *et al*., 1989)
Harzianic Acid, Tricholin, Massoilactone, Viridin, Glisoprenins, and Heptelidic Acid	Antibiotic	(Koli & Adusumilli, 2020)
Jasmonic acid and Terpenes	Repel insects from consuming plant leaves	(Contreras-Corneja *et al*., 2018)

colonization. Antibiotics produced by *Trichoderma* spp. such as trichodermin, suzukacillin, and alamethicin assist in destroying the cell walls of other pathogenic fungi (Manibhushanrao *et al*., 1989). Other reported antibiotics include harzianic acid, tricholin, massoilactone, viridin, glisoprenins, and heptelidic acid (Koli & Adusumilli, 2020). Extensive research done by Contreras-Corneja *et al*. (2018) revealed that the compounds produced by *Trichoderma* spp. such as jasmonic acid and terpenes have modified the feeding habit of insects. As a result, there was a clear cutback in leaf consumption by the insects. Accordingly, the success of *Trichoderma* spp. as a biocontrol agent is attributed to this mechanism.

Competition for nutrient and space

Another mechanism of *Trichoderma* spp. is competition via rhizosphere competence. Through this competence, *Trichoderma* spp. are able to engage in fighting for space and nutrients and thus aggressively colonize the roots of a plant (Howell, 2003). The fast germination rate of *Trichoderma* has led to a larger accessible area and more nutrient supply for growth. The species competes for nutrients and space with other plant pathogens, causing the pathogens to be nutrient-deficit. *Trichoderma* spp. especially deprive plant pathogens from iron sources by secreting a siderophore, which is a compound that chelates to iron. This, in turn, will inhibit the growth of the pathogen as iron is an essential nutrient for the growth of other microorganisms (Singh *et al*., 2018). The nutrient uptake by *Trichoderma* spp. is complemented by their relationship with the plant host. Healthy root tissues are penetrated by *Trichoderma* so that they can utilize plant carbohydrates. Simultaneously, the penetrated root tissues enabled the host plant to uptake macro- and micronutrients in a greater amount.

Induction of plant defence responses

Aside from fighting plant pathogens, *Trichoderma* spp. work in such a way that could induce the

defence mechanism of the host plant. As soon as the plant roots are penetrated by the hyphae of *Trichoderma* spp., localized and systemic resistance in the plant will be triggered. As stated by Harman (2006), the plant defence responses are induced as the elicitors excreted by *Trichoderma* spp. and the receptors of the plant host interact with each other. The systemic acquired resistance (SAR) stimulates the production of salicylic acid, which is a defence signal molecule, for further signalling. Afterwards, when the plant is challenged by a pathogen, this priming effect will be expressed (Harman, 2006). Along with that, *Trichoderma* spp. are able to induce the production of regulatory proteins in plants and these proteins would activate the plant's defence system (Ali Nusaibah & Musa, 2019). In 2011, Contreras-Cornejo *et al*. investigated the effect of root colonization by *Trichoderma* on the production of salicylic acid and jasmonic acid, which are defence signal molecules. The end result of the study proved that the colonized roots showed an increase in both of the molecules (Contreras-Cornejo *et al*., 2011).

ROLE AS BIOFERTILIZER

Severe environmental conditions are one of the contributing factors in the decline of the growth and yield of crops. Abiotic stresses due to climatic changes such as an increase in temperature, carbon dioxide, prolonged drought, and hurricanes will cause adverse effects to crop productivity (Zhang *et al*., 2022). Moreover, under these conditions, the population and dispersal of plant pests and pathogens are affected, imposing additional biotic stresses on crops (Kashyap *et al*., 2017). Genetically uniform improved crop varieties are usually susceptible to infestation caused by non-native pests and pathogens (Govindaraj *et al*., 2015, Bebber, 2015). In addition to that, the irrigation practice in arid lands impacts the soil nutrients and increases salinization (Kashyap *et al*., 2017). Salinization is associated with reduced soil microbial activity and it also affects the physical properties of soil, such as causing soil compaction. Root growth is affected in compacted soil with low oxygen content, limiting nutrient and water uptake, and this negatively impacts the productivity of plants (Colombi & Keller, 2019, Zhang *et al*., 2020). Consequently, reports indicate up to 50% of losses in the average yield of major crops (Kashyap *et al*., 2017). This is critical as a steady increase in global food production is vital to sustaining the growing world population, predicted to reach nearly 10 billion people in the year 2050 (Jaggard et al., 2010). As a solution, *Trichoderma* spp. application as biofertilizer offers a sustainable and eco-friendly alternative for maintaining crop yield and productivity under different environmental stresses (Kashyap *et al*., 2017). Besides playing a significant role as a biocontrol agent that protects plants against various pathogens attacks (Shoresh *et al*., 2010), the interaction of *Trichoderma spp.* with plants improves root growth, aids the nutrient uptake, and enhances plants' ability to withstand abiotic stresses of which overall improves the crop productivity (Kashyap *et al*., 2017, Contreras-Cornejo *et al*., 2016). Previous studies indicate that upon colonizing roots, *Trichoderma* was able to induce changes in the epigenetic modifications, transcriptome and proteome of plants, enhancing the plant's ability to tolerate external stresses (Brotman *et al*., 2013, De Palma *et al*., 2019). *Trichoderma* spp. application provides beneficial effects to plants in the absence and presence of abiotic stress factors (Table 2 and Table 3).

Plant growth and yield improvement

Applications of *Trichoderma* spp. have improved growth in several crops such as mustard, wheat, tomato, cabbage, and rice (Table 2) (Doni *et al*., 2017; Haque *et al*., 2011; Mahato *et al*., 2018; Molla *et al*., 2012; Topolovec-Pintaric *et al*., 2013). *Trichoderma* spp. treatment also increases seed germination rate (Shoresh *et al*., 2010). Bezuindenhout *et al*. (2012) reported that *T. harzianum* produced gliotoxin which was most likely perceived as gibberellic acid by the plant system and in turn, seed germination was induced. Enhanced growth, yield, and nutritional quality are mainly due to a more efficient nutrient uptake and enhanced photosynthesis ability by plants colonized by *Trichoderma spp.* (Kashyap *et al*., 2017, Shoresh *et al*., 2010). The presence of *Trichoderma* spp. in roots aids the nutrient uptake of the plant because the fungi can either directly release molecules on plants or indirectly modify the surrounding environment. Tandon *et al*., (2020) deciphered the mechanism of Phosphate (P) solubilisation by a selected *T. koningiopsis* strain under high pH and drought stress *in-vitro*. Release of organic oxalic acid and alkaline phosphatase enzyme were detected in alkaline and drought conditions respectively. These compounds mediate acidification and hydrolysis process which aids in the conversion of P from the unavailable form into HPO_4^{-2} and $H_2PO_4^{-1}$ that can be readily taken up by plant roots (Tandon *et al*., 2020). Additionally, other microelements such as Cu are solubilised through chelation, meanwhile, Fe is solubilised via several mechanisms such as redox, chelation and reduction reactions in presence of *T. harzianum* (Li *et al*., 2015).

Maize roots colonized by *Trichoderma spp.* have shown some physical changes including improved branching, increased length, increase in secondary root size, and a greater area of root hair (Harman, 2006, Shoresh *et al*., 2010). A well-developed root system contributes to plant growth

Table 2. Beneficial role of *Trichoderma spp.* to the host plants

Trichoderma species	Crop	*Effects on growth/yield/nutritional quality*	Reference
T. harzianum	Mustard	Improved plant growth and seed yield	(Haque *et al*., 2011)
T. viride	Wheat	Increase plant growth and grain yield	(Mahato *et al*., 2018)
T. harzianum and *T. virens*	Scotch pine	Increase seedling biomass and rhizosphere soil nutrient content	(Halifu *et al*., 2019)
T. harzianum	Tomato	Increase production and nutritional quality	(Molla *et al*., 2012)
T. asperellum	Rice	Enhance growth, physiological traits, and yield	(Doni *et al*., 2017)
T. viride	Cabbage and red beet	Improve yield	(Topolovec-Pintaric *et al*., 2013)
T.harzianum	Crack willow	Increase shoot and root growth	(Adams *et al*., 2007)
Trichoderma spp.	Bean	Strain-specific enhancement of early stage of seedling growth	(Hoyos-Carvajal *et al*., 2009)

Table 3. Effects of *Trichoderma spp.* in enhancing host plants tolerance against abiotic stresses

Trichoderma species	Abiotic Stress	Crop	Enhancement of abiotic stress tolerance	Reference
T. hamatum	Drought	Cacao	Delay onset of drought	(Bae *et al*., 2009)
T. asperelloides	Salinity	Arabidopsis and cucumber	Improve seed germination and upregulation of osmo-protection genes	(Brotman *et al*., 2013)
T. harzianum	Salinity	Rice	Enhance photosynthetic performance, higher antioxidant activities and proline content	(Yasmeen & Siddiqui, 2017)
T. harzianum	Salinity	Mustard	Increase in biomass, pigment, proline and oil content	(Ahmad *et al*., 2015)
T. virens	Heavy metal, hydrocarbon	Tobacco	Enhance tolerance to cadmium and anthracene	(Dixit *et al*., 2011a, Dixit *et al*., 2011b)
T. harzianum	Drought	Tomato	Enhance antioxidative machinery activities and improve seedling growth	(Mastouri *et al*., 2012)
T. harzianum	Heat	*Arabidopsis*	Improve tolerance to heat, salt and osmotic stresses	(Montero-Barrientos *et al*., 2010)
T. asperellum	Heavy metal	Onion	Reduce copper accumulation in shoots and increased proline content	(Téllez Vargas *et al*., 2017)
T. logibrachiatum	Heavy metal	Sunflower	Increase antioxidant enzyme levels in lead oxidative stress	(Devi *et al*., 2017b)

by enabling better soil exploitation (Shoresh *et al*., 2010). *Trichoderma harzianum* enriched biofertilizer (BioF/compost) application in both mustard and tomato plants has been reported to increase the seed and fruit yield, branching capacity, root and shoot biomass (Haque *et al*., 2011, Molla *et al*., 2012). On top of that, the higher nutritional content of protein and minerals in tomatoes fertilized with BioF/compost was observed (Molla *et al*., 2012). It has been proven that the application of a 1:1 ratio of BioF/compost:NPK yielded a comparable yield when compared to NPK only application in mustard. The former ratio provides an environmentally friendly and cost effective fertilizer option for crop cultivation (Haque *et al*., 2011). Topolovec-Pintarić *et al*. (2013) inoculated *T. viride* into cabbage and red beet that were planted in sterilised soil. By doing so, the authors were able to assess the growth promoting ability of the fungus in the absence of any pathogens. The result obtained has indicated an amount of 27% and 29% of growth enhancement in terms of root weight in red beet and cabbage respectively (Topolovec-Pintaric *et al*., 2013). Similarly, root-dipping treatment of crack willow tree saplings with *T. harzianum* displayed a significant increase in shoot and root length as well as in their biomass (Adams *et al*., 2007).

Enhancing tolerance to abiotic stresses

Numerous times, *Trichoderma* spp. were proven to effectively improve plant's tolerance to abiotic stresses in mustard, tobacco, cacao and sunflower (Table 3) (Bae *et al*., 2009; Dixit *et al*., 2011a; Ahmad *et al*., 2015; Devi *et al*., 2017b). The ability of *Trichoderma* spp. to ameliorate the abiotic stress-induced plant growth retardation was reported to be contributed by different mechanisms (Shoresh

et al., 2010, Kashyap *et al*., 2017). Besides that, the seeds treated with *T. harzianum* have better germination rates and seedling vigour compared to non-treated seeds when exposed to abiotic stresses such as heat and cold temperatures as well as salt and osmotic stresses (Björkman *et al*., 1998; Harman *et al*., 2004; Shoresh *et al*., 2010, Mastouri *et al*., 2010).

A common stress that plants are exposed to in the field is drought condition (Shoresh *et al*., 2010). Detrimental effects caused by drought include a reduction in stomatal conductance, impaired photosynthesis, and cessation of the shoot and root growth (Bae *et al*., 2009). In water deficit conditions, an improved germination rate of tomato and *Arabidopsis* was observed when the seeds were inoculated with *T. harzianum* (Björkman *et al*., 1998; Mastouri *et al*., 2012). Maize plant which had undergone seed treatment with *T. harzianum* was able to withstand water deficit conditions better in the field and this attributes to the deep root formed in the soil (Harman, 2000). Starch content in the leaves of maize inoculated with *T. harzianum* was found to be elevated than un-inoculated ones. This is beneficial in prolonged drought conditions where carbon starvation may occur (Shoresh *et al*., 2010). Additionally, delayed responses to drought stress were observed in *T. hamatum* colonised cacao seedlings, where reduction in photosynthesis rate and stomatal conductance was less by 21% and 30% respectively, compared to non-treated seedlings (Bae *et al*., 2009).

Although irrigation practices have enabled the cultivation of crops in semi-arid areas, the prolonged effect of this practice is soil and ground-water salinization, which limits the crop yield in the long run (Fita *et al*., 2015). Salinity causes the accumulation of toxic ions in shoots to occur and this retards the plant growth, reduces the plant water potential, and causes hormonal and nutritional disproportion (Negrão *et al*., 2016, Yasmeen & Siddiqui, 2017). *Trichoderma harzianum* treated rice seedlings exhibited positive physiological responses including enhanced photosynthetic ability, stomatal conductance, and elevated proline content in a salt stress environment. Proline contributes to intracellular osmotic adjustment, thus protecting plant cells from damage (Yasmeen & Siddiqui, 2017). *Trichoderma harzianum* biofertilizer inoculated *Medicago sativa* seedlings grown in alkaline-saline soil displayed an increase in the shoot and root biomass. This showed that the application of biofertilizer was able to alter soil properties while increasing the microbial and fungal population, which contributed to plant growth (Zhang *et al*., 2020).

Anthropogenic activities and modern agricultural practices are infamous for being the sole contributor to heavy metal contamination in soils, which is toxic to plant growth and affects the crop productivity. In particular, the harmful effects of heavy metal contamination to plants consist of lower germination and nutrient uptake rate, photosynthesis impairment, chlorosis, phytotoxicity, and senescence (Singh *et al*., 2016, Devi *et al*., 2017b). Nonetheless, the application of *T. asperellum* to onion plant was able to relieve the heavy metal stress caused by copper toxicity. This is due to the fact that inoculated plants were found to exhibit an increase in growth and chlorophyll contents when compared to non-treated plants (Téllez Vargas *et al*., 2017). Furthermore, sunflower plants treated with *T. logibrachiatum* demonstrated an increased tolerance to lead toxicity. The elevated levels of antioxidant enzymes have minimised the oxidative damage due to stress-induced accumulation of reactive oxygen species in these plants (Devi *et al*., 2017b). All these findings point out that the advent of molecular technology and studies have enabled a better understanding of *Trichoderma*-plant interaction. Thus, continuous efforts are being made to exploit the beneficial characteristics of the microbe for crop improvement and climate resilient agriculture.

IDENTIFICATION OF *Trichoderma* spp.

Morphological Identification

The identification of *Trichoderma* spp. is crucial because not all of them are potential antagonists. Conventionally, a *Trichoderma* species is identified by using morphological characterization. They have key characteristics that are regularly observed in order to identify them up to species level such as the colour of the colony, growth pattern, growth rate, odour, formation of chlamydospores, conidiophores and phialides, and the shape of the conidia (Gams & Bisset, 1998; Samuel *et al*., 2002). For an instance, *T. harzianum* was reported to form some concentric rings with green conidial on Potato Dextrose Agar (PDA), whereas no concentric rings were produced by *T.viride* and *T. pseudokoningii* (Shah *et al*., 2012). Figure 2 shows an example of the morphology of different *Trichoderma* species with a different patterns of concentric rings.

Since *Trichoderma* spp. produce numerous spores (conidia), microscopic observations on the arrangement of their spores, conidiophores, conidia, phialide, and chlamydospore are crucial in classifying them into their own respective species (Chaverri *et al*., 2003b). Figure 3 illustrates the typical conidiophore branching patterns of *Trichoderma*.

Nevertheless, the isolates could not be distinguished up to the species level by morphological and cultural characteristics alone, especially in a hyperdiverse genus like

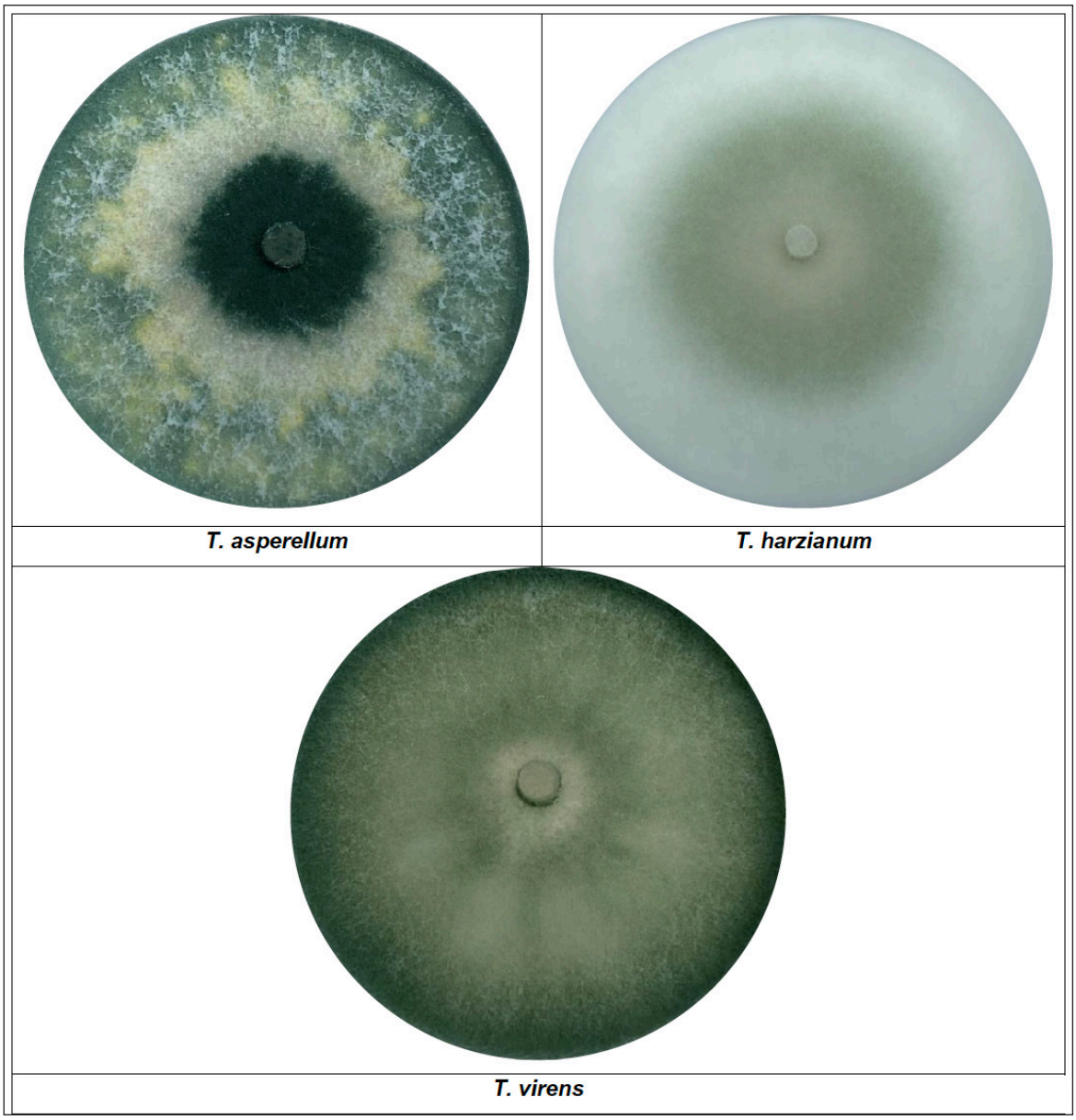

Fig. 2. The morphology of *T. asperellum, T. harzianum*, and *T. virens* on PDA after 7 days of incubation. The morphology of each *Trichoderma* species shows different density of conidia and different pattern of concentric rings.

Trichoderma. Since there is a limited number of morphological characters and variations, there is a high probability in isolate misidentification. Besides, the morphological characteristics of *Trichoderma* isolates are influenced by culture conditions and they can be misleading due to the occurrences of hybridization, cryptic speciation, and convergent evolution. On top of that, a lack of certainty arose when different names are assigned to a fungus that has both asexual and sexual stages (Fahmi *et al*., 2016). Therefore, the application of molecular tools is the key element in studying and exploiting *Trichoderma* to our benefit (Gherbawy & Voigt, 2010). In fact, molecular tools have long been coupled with morphological characterization in fungal identification. For an instance, *Ganoderma* Selective Media was used by Chong *et al*. (2011) together with molecular tools in order to identify *Ganoderma boninense*.

Molecular tools used in identification

Over the past decades, molecular techniques have blossomed the *Trichoderma* research through the development of molecular markers. A molecular marker is usually constructed based on the mutations that occur in an organism's nucleotide sequence. Up to this date, various molecular markers have been employed in the identification of *Trichoderma*: namely, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequence-characterized amplified region (SCAR), internal transcribed spacer (ITS) region, translation elongation factor alpha 1 (*tef*1) gene, simple

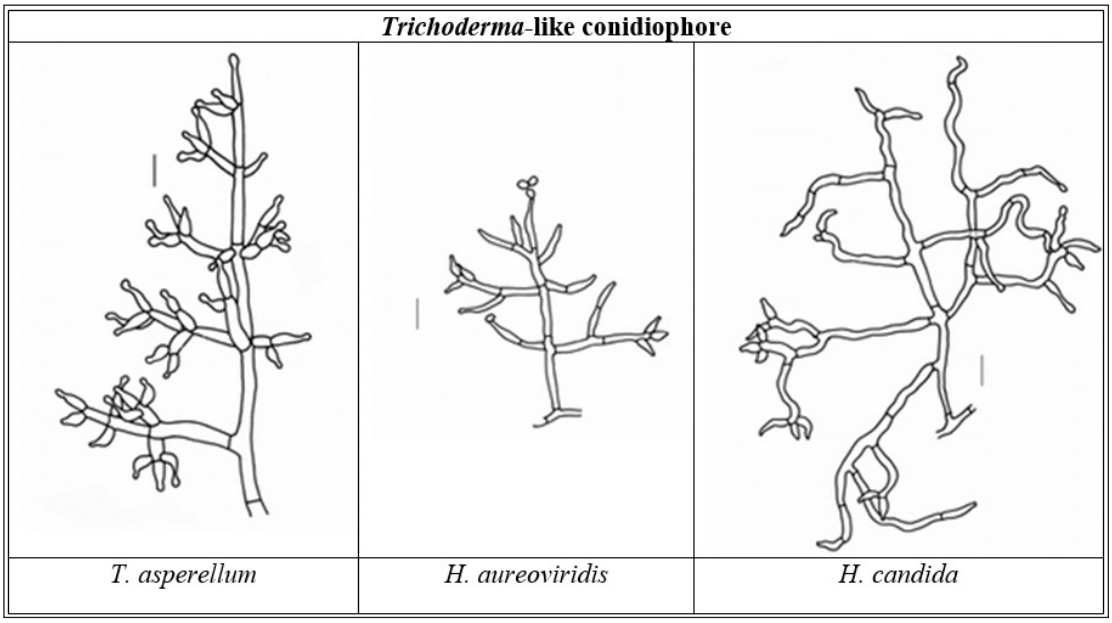

Fig. 3. *Trichoderma*-like conidiophore branching patterns (Adapted from: Chaverri & Samuels, 2003a).

sequence repeat (SSR), and single nucleotide polymorphism (SNP) (Skoneczny *et al*., 2015; Hassan *et al*., 2019) (Table 4).

Restriction Fragment Length Polymorphism (RFLP)

In 1974, Grodzicker *et al*. ventured to find where the mutation sites on a genome are located. Consequently, a method called restriction fragment length polymorphism (RFLP) was founded. In general, there are three steps in RFLP: (1) the DNA is digested into different sizes of fragments by using known restrictive enzymes, (2) the fragments are separated via electrophoretic analysis, (3) the separated fragments are hybridized with probes and made visible under autoradiography (Yang *et al*., 2013). Bowen *et al*. (1996) made use of this method to develop a molecular marker that could specifically detect *T. atroviride* C65 (formerly *T. harzianum* C65) and thus differentiate the target species from other closely related *Trichoderma* isolates. Later on, this marker was delved into by Dodd *et al.* (2004) with a dot-blot assay to assist in screening *T. atroviride* C65 in a large sample size. RFLP markers are reliable as they are generated from specific sites that are constant over time. However, this limits their ability to identify a species from a hyperdiverse genus. Although RFLP markers are co-dominant, which makes them an ideal molecular marker, their level of polymorphism is low (Yang *et al*., 2013).

Random Amplified Polymorphic DNA (RAPD)

In 1990, Welsh and McClelland published a method called arbitrarily primed PCR (AP-PCR) in which they used to generate genomic fingerprints. The genomic fingerprints were then utilized in order to set apart different strains of a species by comparing their polymorphic regions. Today, this method is known as random amplified polymorphic DNA (RAPD). Skoneczny *et al*. (2015) used RAPD in designing primers that are specific to certain species. Based on the sequence analysis of twenty RAPD markers and *T. atroviride* genome, three species-specific markers for *T. atroviride* were successfully developed by the authors.

What makes RAPD markers desirable is that they are suitable for unknown genomes, require a low cost, and are satisfyingly efficient. The technique does not require an elaborate method and is able to be applied to DNA templates that are in a limited quantity. Despite that, RAPD markers are not co-dominant and this causes the inability to distinguish between homozygotes and heterozygotes. Furthermore, the result of the amplified products will remain ambiguous unless aided by pedigree data (Kumari & Takhur, 2014).

Sequence-Characterized Amplified Region (SCAR)

Sequence-characterized amplified region (SCAR) marker is derived from the primer pairs that were developed based on RAPD amplicons. In turn, SCAR becomes the key that could solve the sensitivity and specificity issues of RAPD (Frías De León *et al*., 2011). Previously Hermosa *et al.* (2001) have successfully developed a strain-specific marker for *T. atroviride* 11 based on SCAR markers derived from RAPD analysis of 16 biocontrol agent isolates of *Trichoderma* spp.

Table 4. Molecular tools used in the identification of *Trichoderma* spp.

Species	Target region/ Method	Marker Specificity-Level	Reference
T. atroviride C65	RFLP	Species	(Bowen *et al*., 1996, Dodd *et al*., 2004)
T. atroviride	RAPD	Species	(Skoneczny *et al*., 2015)
T. atroviride 11	SCAR	Strain	(Hermosa *et al*., 2001)
T. guizhouense NJAU 4742	2-step sequence comparison	Strain	(Zhang *et al*., 2019)
Trichoderma spp	ITS	Genus	(Kim & Knudsen, 2008)
T. pleurotum, T. pleuroticola	tef1	Species	(Kredics *et al.,* 2009)
T. asperellum	tef1	Species	(Devi *et al*., 2017a)
T. harzianum	rbp2	Species	(Devi *et al*., 2017a)
T. atroviride, T. harzianum, T. reesei, T. virens, T. asperellum	SSR	Species	(Rai *et al.,* 2016)
T. cf. atroviride LU132, T. cf. atroviride LU140	SNP	Strain	(Lange *et al*., 2016)

Howbeit, the authors (Hermosa *et al*., 2001) claimed that the procedure was time-consuming and not advisable for a large number of screenings. Despite the advantages of SCAR markers in being highly reliable, highly reproducible and highly sensitive, the fact that they are time-consuming makes them less appealing (Zheng *et al*., 2021).

To overcome the long process of developing a SCAR marker, Zhang *et al.* (2019) suggested a two-step sequence comparison technique in developing strain-specific markers. The authors designed 10 strain-specific primers for a mutant *T. guizhouense* NJAU 4742 tagged with *gfp* gene based on the two-step sequence comparison technique: (1) whole genome against NCBI and Joint Genome Institute (JGI) databases, and (2) against local BLAST database of closely related strain. As a result, the authors managed to report three strain-specific primer pairs for *T. guizhouense* NJAU 4742.

Internal Transcribed Spacer (ITS) Region

In the late-nineties, the morphology-based classification of the genus *Trichoderma* was re-confirmed with the combination of molecular markers designed based on the internal transcribed spacer (ITS) region of the 18S-5.8S-28S nuclear ribosomal cistron (Figure 4) (Hermosa *et al*., 2001; Druzhinina & Kubicek, 2005). ITS regions have the fastest rate of evolution and are highly variable due to spontaneous mutations, deletions, duplications, insertions, and single nucleotide substitutions that occur in these regions. Hence, mycologists chose ITS regions as the official barcode for fungi because they are easy to amplify, well-established, and generally have a wide barcode gap (Raja *et al*., 2017).

Even so, it is important to note that Druzhinina and Kubicek (2005) mentioned the low reliability of the sequences deposited in the Genbank database. There is a high chance for the *Trichoderma* isolates to be falsely identified and named. Therefore, a DNA-barcode database for *Trichoderma* was launched by Druzhinina *et al*. (2005). The authors achieved genus and species level identification through their DNA-barcode database by aligning the invariable and variable regions of ITS1 and ITS2 from over 900 isolates with the sequences of established *Trichoderma* species. In addition, the authors developed TrichOKEY, an identification program based on the DNA-barcodes database built. After the implementation of the DNA-barcoding system, the number of molecularly characterized *Trichoderma* spp. increased exponentially, whereby in 2014-2017 more than 50 species of *Trichoderma* were registered every year into its taxonomy (Cai & Druzhinina, 2021). Since then, TrichOKEY has been used as a supporting tool used to assess the reliability of BLAST results (Hageskal *et al*., 2008). Alas, species identification via the ITS region does not work well in the *Trichoderma* genus as it is highly diverse (Raja *et al*., 2017).

In search for markers that could detect *Trichoderma* spp., Kim and Knudsen (2008) developed PCR primer sets that are specific for this particular fungi by utilizing the ITS region. Over 100 ITS sequences from different *Trichoderma* spp. were aligned and the primer sets were designed based on the conserved regions of the consensus sequence of the alignment. Out of 6 candidate primer sets, a primer set named TGP4 was reported to have successfully amplified the genomic DNA of *Trichoderma* isolates only. This primer set also showed high specificity towards *Trichoderma* spp. (Kim & Knudsen, 2008).

Translation Elongation Factor Alpha 1 (tef1) gene

To make up for the limitations of the ITS region

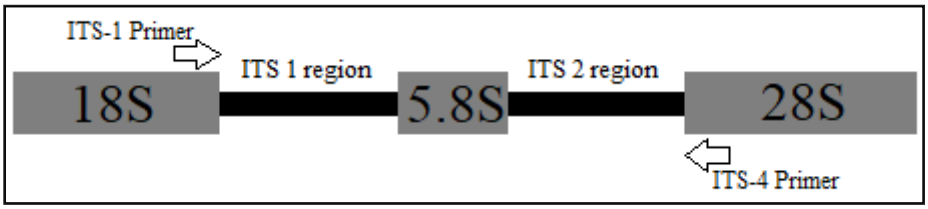

Fig. 4. The structure of 18S-5.8S-28S nuclear ribosomal cistron with the ITS regions.

in species identification, protein-coding genes are usually harnessed. One example of such genes is the translation elongation factor alpha 1 (*tef*1). Former studies proved that the region of *tef*1 intron 4 was able to significantly distinguish different *Trichoderma* spp. when used in combination with *tef*1 intron 5 region (Jaklitsch, 2011; Jaklitsch & Voglmayr, 2015). Early on in the year 2009, Kredics *et al.* utilized the *tef*1 gene in developing species-specific primers for the detection of *T. pleurotum* and *T. pleuroticola*. The obtained result showed that the species-specific primers were proficient in differentiating between *T. pleurotum* and *T. pleuroticola*, and also between the two species with 28 other *Trichoderma* spp. Manzar *et al.* (2021) mentioned that when both ITS region and *tef1* gene are integrated in molecular identification, the taxonomy and classification will become more reliable. Apart from that, the evaluation of the effectiveness of *tef*1 gene marker showed a positive light when it was reported to be good enough in discriminating between various different *Trichoderma* spp. (Mokhtari *et al*., 2017).

Regardless, the primers generated from *tef1* gene are not sufficient for species level identification. In order to identify each *Trichoderma* isolate efficiently, the markers used must be species-specific. Devi *et al*. (2017a) have established two primer pairs based on the *tef*1 gene and *rbp*2 gene. Consequently, the authors aligned the *tef*1 gene sequences of *T. asperellum* isolates and the *rbp*2 gene sequences of *T. harzianum* isolates. The resulting conserved region of each species was then used to design for species-specific primers. As a result, the primer pair T2A was proven to be specific to *T. asperellum*, whereas the primer pair Th1 was specific to *T. harzianum*.

Simple Sequence Repeat (SSR)

Another widely utilized molecular tool for species identification is the simple sequence repeat (SSR). SSRs consist of motifs made up of tandem repeats of nucleotide bases. The repeated motifs are highly polymorphic between different species, meanwhile, the flanking regions of SSRs are mostly conservative. This led to the blooming of species-specific SSR markers (Yang *et al*., 2013). In 2016, simple sequence repeat (SSR) markers were developed by Rai *et al*. in order to investigate the diversity within *Trichoderma* species. The authors used whole genome sequences that are accessible to the public in designing SSR markers for five different *Trichoderma* species (Rai *et al*., 2016).

SSR markers are desirable because not only are they exceptionally polymorphic, consistent over time and co-dominant, but they are also compatible with multiplexing and fluorescent methods. This makes them desirable as molecular tools. On the other side, it requires a high cost in designing species-specific SSR markers (Miah *et al*., 2013).

Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism (SNP) refers to a single polymorphic site at a specific locus of a nucleotide sequence. The single nucleotide variation is associated with either transition, transversion, insertion, or deletion of the nucleotide bases (Yang *et al*., 2013). SNP was initially employed as a molecular marker by Lander (1996) and over the last decades, it has been proven to be one of the most potent molecular tools with profuse applications, especially in genotyping. Hirotsu *et al*. (2010) proposed a simpler method of SNP genotyping through primers that are allele-specific. These primers were designed in such a way that their 3' end nucleotide would complement the targeted allele but would not complement other non-targeted alleles. To improve specificity, artificial mismatches were added at the 3' end of the primers.

Lange *et al*. (2016) successfully implemented SNP molecular technology in developing a strain-specific marker that could discriminate between *T. cf. atroviride* strain LU132 and strain LU140, and also with other *Trichoderma* strains. The authors first did whole genome sequencing for both strains and the SNP-containing regions were identified by referring to the reference genome. Putative SNPs were validated and one non-synonymous SNP between the strains was found. Based on that SNP, an RFLP protocol was designed and tested on 39 other *T. atroviride* and *T. cf. atroviride* strains from New Zealand, Europe and Asia. However, the authors noted that in order to use this method in

microbiological identification, it must be applied together with phenotypic characterisation (Lange *et al*., 2016).

SNP markers are preferable because they are well-distributed, co-dominant, and polymorphic. SNP markers are suitable for allelic variation analysis, QTL mapping, and MAS implementation (Mehboob-ur-Rahman *et al*., 2009). At the same time, SNP markers can be designed to be strain-specific. However, the development of strain-specific SNP markers is time-consuming and requires a high cost.

CONCLUSION

Over the years, *Trichoderma* species have become attractive as biocontrol agents and biofertilizers in the agricultural industry. This is owing to their aggressive qualities against plant pathogens and their ability to ramp up the plant host's defence system. Other than that, they have proven their worth in boosting agricultural productivity by increasing plant's nutrient uptake, improving the yield of crops, enhancing plant's tolerance to external stresses, and inducing seed germination. Since *Trichoderma* species are like diamonds in the rough, it is crucial to identify and distinguish them from other non-*Trichoderma* species. To date, a great deal of molecular tools for *Trichoderma* species identification has been developed and each of them has its own boons and banes. Strain-level identification is obligatory when a *Trichoderma* isolate is being used for industrial purposes. By far there are only 26 genome assemblies of different *Trichoderma* species deposited in the GenBank. For these reasons, further study on *Trichoderma* must be incessantly done in order to acquire more insights into this valuable genus and to enable more efficient exploitation of the beneficial microbe for the agricultural industry.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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