

**MORPHOLOGICAL CHARACTERIZATION OF *Trichoderma* spp. AND
THEIR EFFICACY AS BIOCONTROL AGENTS AGAINST *Ganoderma*
boninense *In Vitro***

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SUMMARY

Trichoderma species act as biological control agents against fungal plant pathogens either directly or indirectly. In this study, dual culture was used to assess the potential of *Trichoderma asperellum*, *T. harzianum*, *T. brevicompatum* and *T. viren* to control *Ganoderma boninense*. These species were morphologically and Potato dextrose agar (PDA) and malt extract agar (MEA) supported the highest mycelia growth. provides useful information on the sporulation. Antagonistic effects of *Trichoderma* spp. against *G. boninense* mycelia growth were evaluated using dual culture test. The results demonstrated that the percentage inhibition of radial growth (PIRG) of *G. boninense* was >75% in in-vitro assay. The highest percentage inhibition of radial (PIRG) values was observed in *T. asperellum* (84.1%), while the minimum colony overgrowth was observed in *T. brevicompatum* (76%). This study showed that *Trichoderma* spp. have a good antagonistic effect on *G. boninense* mycelia growth and *T. asperellum* has

the best potential to control the basal stem rot disease pathogen.

Keywords: *Trichoderma* spp., *G. boninense*, Dual culture, PIGR

Trichoderma is a filamentous ascomycete saprophytic fungus with a worldwide distribution [3]. They are cosmopolitan fungi found both in the tropics and temperate climates; growing on the bark of dead woods, building materials, other fungi and animals; demonstrating its high adaptability to different ecological conditions [1] [19]. They are successful colonizers of their habitats, due to their efficient utilization of the substrates available in the habitat and production of antibiotic metabolites and enzymes [18]. Their capacity to produce antibiotics, parasitize other fungi and compete with deleterious plant microorganisms have been widely studied [2]. These fungi produce a number of secondary metabolites such as non-ribosomal peptides, terpenoids, pyrones and indolic-derived compounds [2]. Many strains of *Trichoderma* are antagonistic to other fungi and have shown promising potentials as biological control agents of soil-borne diseases [8]. The biological control potential of *Trichoderma* spp. has been demonstrated on a wide spectrum of plant diseases both in *in vitro* and *in vivo* [11]. Species of *Trichoderma*

are recognized for their antagonist ability towards various pathogens: *Rhizoctonia solani*, *Sclerotium rofsii*, *Alternaria alternata*, *Botrytis cinerea*, *Fusarium solani*, *F. oxysporum* [10]. This characteristic has promoted an increase in the interest on these fungi. *T. harzianum* is most widely used in biological control of many pathogenic fungi such as *A. alternata*, *B. cinerea*, *Cochliobolus heterostrophus*, *F. oxysporum*, *R. solani*, *S. rofsii* [17]. The concern surrounding food safety and the environmental impacts on agriculture, caused by the use of classic fungicides, biological control agents have received increased recognition. The biological control agents are being used more often as a complement or replacement of fungicides [13].

Due to the needs for large scale production of *Trichoderma* spp. as reliable alternative for the control plant diseases, there is need to properly characterize. Characterization of *Trichoderma* was based on the morphological and cultural behaviors: growth pattern and speed as well as colony's colour and odour. The other characters studied with the aid of light

microscope include conidial size, shape and colour; conidiophores apex elongation, shape and branching pattern [18]. The objectives of this study were to morphologically characterize selected *Trichoderma* spp. and to determine their *in vitro* antagonistic activities against *Ganoderma boninense* which causes basal stem rot disease in oil palm [9].

MATERIALS AND METHODS

Culture of *Trichoderma* species

Three *Trichoderma* spp (*T. asperellum*, *T. harzianum*, *T. virens* and *T. brevicompactum*) isolated from different oil palm plantation soils in Malaka, Malaysia, using dilution plate method onto *Trichoderma* selective medium (TSM) and maintained on potato dextrose agar (PDA) at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ were studied.

Culture morphology

Seven days old *Trichoderma* spp cultures on PDA at $27 \pm 2^{\circ}\text{C}$ were observed under light microscope (Nikon Model Eclipse E200, Japan) at 400 magnification where conidia size (length and width) was measured as well as conidia shape and colony colour observed [4]. A minimum of 50 conidia was measured per replicate for each species. Culture slides of each species were prepared using a

modified method for fungal slide culture [8]. Sterile distilled PDA (seven to eight milliliters) was poured into each sterile 90 mm plastic petri dish and allowed to solidify. A sterile 22 mm² cover glass was centered on the agar. The PDA medium (10 ml) was poured into a second 90 mm petri dish, allowed to solidify, and cut with a sterile stainless steel spatula into blocks approximately five to eight millimeters. One block was aseptically removed and placed on the cover glass. Inoculation of the agar block on one or more sides with fungal hyphae or conidia was followed by placement of a second sterile cover glass on top of it. Then the petri dish lid was replaced. The completed modified slide cultures were incubated at the $27 \pm 2^{\circ}\text{C}$ for three days. Using sterile forceps, the top cover slide was gently lifted onto a glass slide with a drop of lactophenol cotton blue and viewed microscopically at 400 magnifications.

Cultural characteristics of *Trichoderma* species

The colony growth rate of each specie was studied on four different types of artificial media (Potato dextrose agar (PDA), Czapek- dox agar (CDA), cornmeal agar (CMA) and malt extract agar (MEA)); PDA

at varying pH levels (5, 6, 7 & 9) and on PDA at varying temperatures (20, 25, 30 & 35°C). The pH of levels were adjusted with potassium hydroxide (KOH) and set using pH meter prior to autoclaving at 121°C and 100 kPa (15 psi) above atmospheric pressure for 20 min. Mycelia plugs of six millimeters in diameter from each species were taken from actively growing margin of a three days old cultures, placed onto petri dishes with media for the various treatments and incubated at 27±2°C for five days.

In vitro* antagonistic activity of *Trichoderma* spp. against *G. boninense

An agar disc (6 mm) was taken from four days old PDA plate cultures of each *Trichoderma* spp. and placed on the periphery of the PDA plates (9 cm). Another agar disc of the same size of *G. boninense* was placed opposite end the same plate, while another plate inoculated with just a

$$\text{PIRG} = \frac{(R1 - R2)}{(R1)} \times 100$$

Where R1 – radius of the pathogen colony (mm) in the control plate, R2 – radius of the pathogen colony (mm) in the dual cultures plate.

G. boninense plug served as a positive control. Each treatment was replicated five times and incubated at 27±2°C. Antagonistic activity was determined as from four days after incubation (DAI) by measuring the radius of the *G. boninense* colony growth in the direction of the antagonist (*Trichoderma*) colony (R2) and the radius of the *G. boninense* colony in the positive control plate (R1). The experiment was repeated twice. The percentage (%) inhibition of *G. boninense* radial growth was measured at 5 days after inoculation by measuring the radial growth (mm) of the developing colony toward the antagonist until the plant pathogen (*G. boninense*) colony was completely surrounded by the antagonist (*Trichoderma* spp.). The percentage growth rate inhibition (PIRG) of pathogen was calculated according to the formula below developed by Skidmore and Dickinson [23].

Experimental design and statistical analysis

The experimental design used in this study was completely randomized design (CRD) in five treatments (*Trichoderma asperellum* + *Ganoderma boninense*; *T. harzianum*

+ *G. boninense*; *T. brevicompactum* + *G. boninense*; *T. virens* + *G. boninense*; *G. boninense* alone as positive control), each treatment was replicated six times for the laboratory experiments. The data collected were analyzed using with SAS software [SAS 9.4 Version Institute Inc. Cary, NC, USA], and statistical means were separated using Duncan's multiple range test (DMRT) at 5% significant level.

RESULTS

Morphological characteristics of *Trichoderma* species

Seven days old *Trichoderma* spp. cultures on PDA showed variations in colony, conidia size and shape

(Table 1). *T. harzianum* had pale green colony while all the other species were dark green (Plate 1) colour from green, pale green, to dark green. Morphological colour changed from pale green to dark green for *Trichoderma* spp (Plate 2). Conidia of *T. asperellum* and *T. harzianum* were subglobose; *T. brevicompactum* obovoid and *T. virens* ellipsoidal (Table 1 and Plate 2). *T. asperellum* and *T. brevicompactum* produced longest and shortest conidia which ranged between 3.0 – 4.5 and 2.5 -2.5 μm , respectively (Table1). Conidia width range for *T. virens* (3.0 -3.0 μm) was the highest while *T. brevicompactum* recorded the least (2.0-2.4 μm).

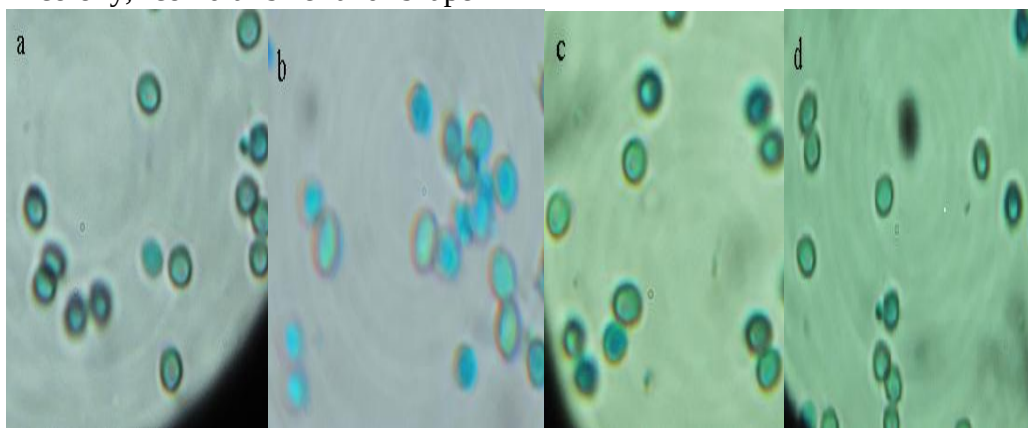


Plate 1: Conidia shape of *T. asperellum* (a), *T. harzianum* (b), *T. brevicompactum* (c), and *T. virens* (d)

Table 1: Morphological and cultural characteristics of *Trichoderma* species

Fungal species	Conidia size range (µm)		Conidia shape	Colony colour
	Length	Width		
<i>T. harzianum</i>	2.8 - 3.4	2.0 - 2.9	subglobose	pale green
<i>T. asperellum</i>	3.0 - 4.5	2.2 - 3.0	subglobose	dark green
<i>T. brevicompactum</i>	2.5 - 2.7	2.0 - 2.4	obovoid	dark green
<i>T. virens</i>	2.9 - 4.3	3.0 - 4.0	ellipsoidal	dark green

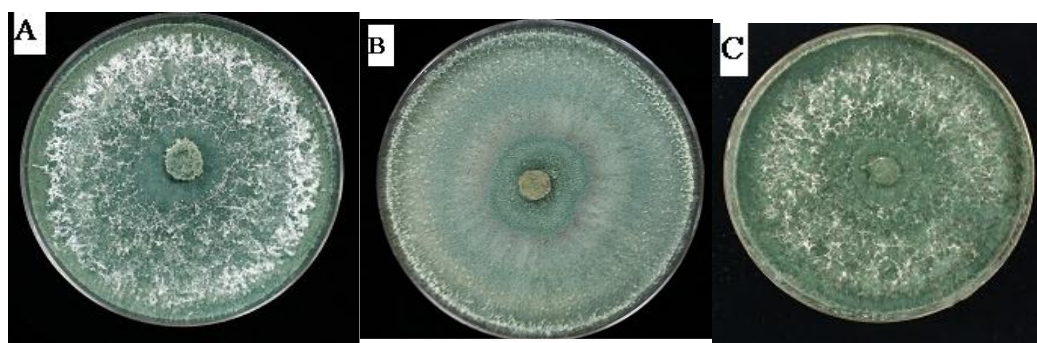


Plate 2: Morphological growth and sporulation appearance of *T. asperellum* (A), *T. harzianum* (B), *T. virens* (C) and *T. brevicompactum* (D) on PDA after five days at 27±2°C

***Trichoderma* species colony growth on different media**

Trichoderma species grew on all nutrient media tested with the fastest growth recorded in PDA and MEA (Table 2). On PDA, the mycelial growth of *Trichoderma* spp significantly varied from each other with *T. asperellum* exhibiting the highest mycelial growth (8.57 cm), while *T. brevicompactum* had the least (6.50 cm). However, there were

significant differences in colony growth between the species on each medium (PDA, MEA, Czapek and CMA). Effect of the media tested showed that the nature of media affected the mycelial growth. They were found to be capable of growing on almost all the media tested with different growth. Potato dextrose agar proved to be the most suitable medium for the growth of *Trichoderma* spp. On PDA, the

growth pattern showed slight differences in the nature of the colony growth and sporulation. The colony colour changed from light green shade to green and dark green with the production of conidia. The

conidia wall patterns and shape were rough, subglobose, and smooth. These observations were in consistent with the previous reports on different growing media for *Trichoderma*.

Table 2: *Trichoderma* species colony growth on different fungi growing media at 5 days after inoculation

Species	Colony diameter (cm) on:			
	PDA	CMA	Czapek agar	MEA
<i>T. harzianum</i>	8.17±0.32 ^b	7.43±0.25 ^a	5.52±0.40 ^{ab}	7.80±0.45 ^a
<i>T. asperellum</i>	8.57±0.58 ^a	6.80±0.34 ^{ab}	6.03±0.21 ^a	7.90±0.20 ^a
<i>T. virens</i>	7.10±0.20 ^c	7.70±0.11 ^a	5.16±0.04 ^b	7.52±0.36 ^a
<i>T. brevicompactum</i>	6.50±0.25 ^d	5.60±0.78 ^b	4.31±0.10 ^c	6.11±0.20 ^b

± Standard deviation (SD). The values followed with the same superscript letter within a column were not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Effect of temperature on the colony growth of *Trichoderma* species

All the four *Trichoderma* species produced colony growth at different temperatures, with the maximum colony growth recorded at 25 and 30 °C. The colony growth was generally increasing with an increase in temperature but drastically decreased

at 35°C (Fig. 1). At 15°C, *T. harzianum* and *T. viren* recorded significantly higher mycelia growth than *T. asperellum* and *T. brevicompactum*. However, at 20°C, *T. asperellum* and *T. viren* which did not differ from each other recorded the highest colony growth as shown in figure 1.

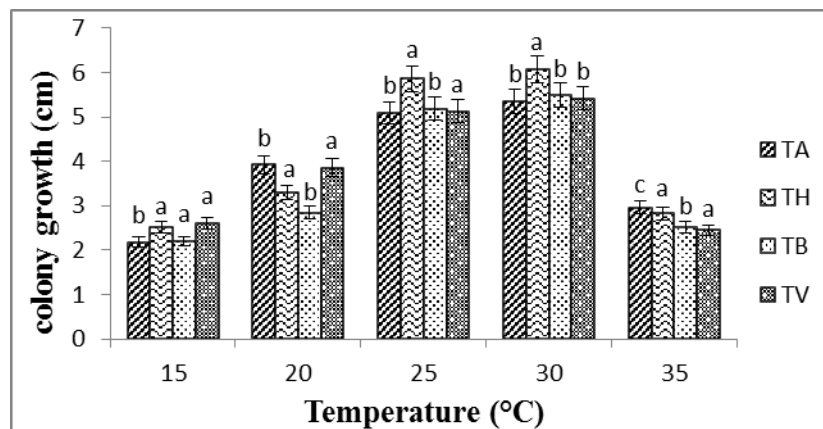


Figure 1: Effect of temperature on colony growth of *Trichoderma* spp on PDA. Error bars represents the Standard Error (SE) of the mean. Bars with the same letter are not significantly different according to a to Duncan's multiple range test at $P \leq 0.05$. TH: *Trichoderma harzianum*; TA: *Trichoderma asperellum*; TV: *Trichoderma virens* and TB: *Trichoderma brevicompactum*

Effect of pH on the colony growth of *Trichoderma* species

The pH was observed to affect colony growth of all *Trichoderma*

species studied with significant variation amongst the species at various acidity levels (Fig.2). and mean growth for species grown at different pH levels 4, 5, 7, 8 and 9 were significantly different ($P \leq 0.01$). Colony growth for all *Trichoderma* spp were at pH 6, but there were significant difference ($P \leq 0.01$) between *T.brevicompactum* and three others species at pH6. The minimum colony growth for *Trichoderma* species were recorded at pH 9.

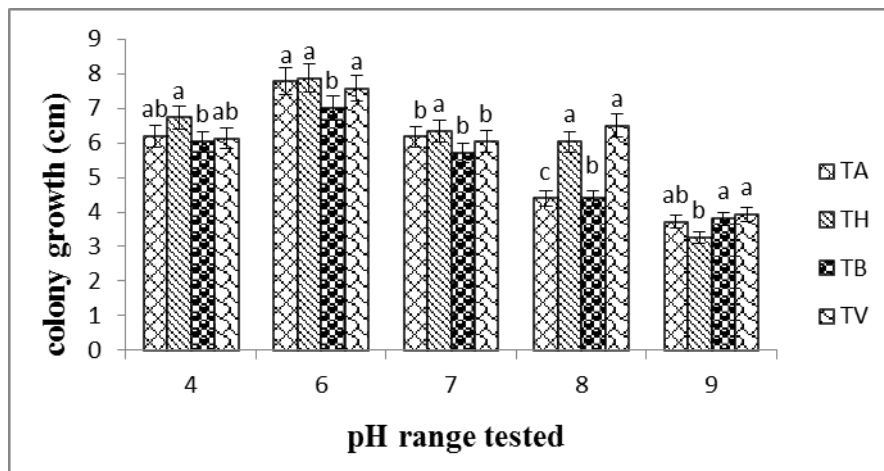


Figure 2: Effect of pH on colony growth of *Trichoderma* spp on PDA. Error bars represents Standard Error (SE) of the mean. Bars with same letter are not significantly different according to a Duncan's multiple range tests at $P \leq 0.05$. TH: *Trichoderma harzianum*; TA: *Trichoderma asperellum*; TV: *Trichoderma virens* and TB: *Trichoderma brevicompactum*

In vitro screening of *Trichoderma* spp. against *Ganoderma boninense*

The dual plate assays performed with four *Trichoderma* species against *G.*

boninense showed varying levels of antagonistic responses against the pathogen (Fig.3 and plate 4). There were significant differences in percent growth inhibition (PIGR) between the four *Trichoderma* species studied. The PIRG values ranged from 76.0 to 84.6%, with *T. asperellum* recording the maximum (84.6%) and *T. brevicompactum* the least (76.0%) as shown in Figure 3 and Plate 4. All the *Trichoderma* spp tested clearly demonstrated high (above 50%) antifungal activities, and they may be used as antagonist against *G. boninense*.

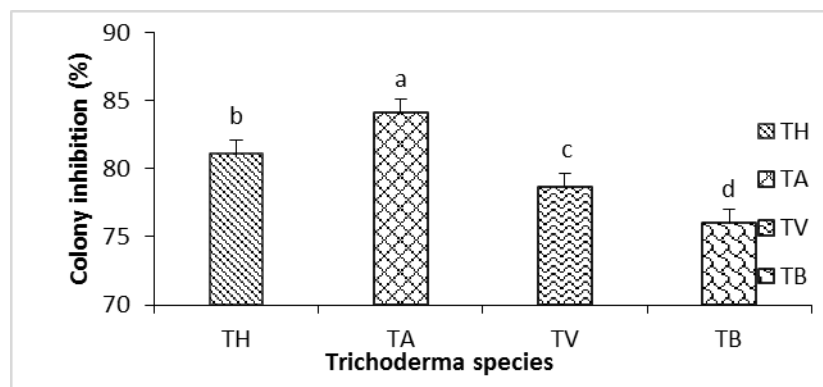


Figure 3: *In vitro* antagonistic activity of *Trichoderma* species against *G. boninense*. Bars with same letter are not significantly different according to a Duncan's

multiple range tests at $P \leq 0.05$. TH: *Trichoderma harzianum*; TA: *Trichoderma asperellum*; TV: *Trichoderma virens* and TB: *Trichoderma brevicompactum*

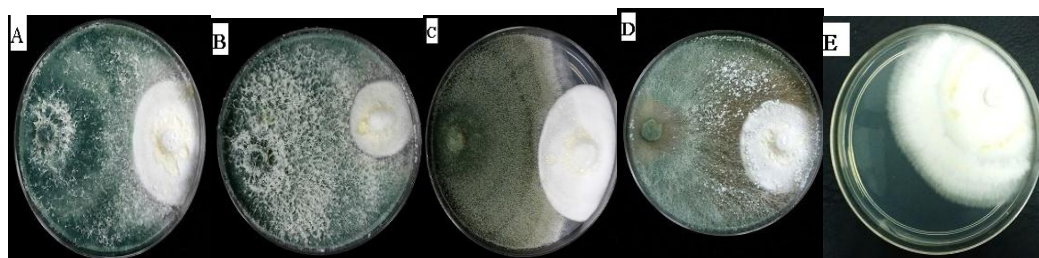


Plate 4: *In-vitro* growth and inhibition of *G. boninense* on PDA by *T. virens* (A), *T. asperellum* (B), *T. brevicompactum* (C), and *T. harzianum* (D) and control (E) at seven days of incubation

DISCUSSION

Our results showed that morphological characters of *Trichoderma* spp. studied showed close similarity in their

conidia shape, colour and conidia size. Thus, making it difficult to characterize at species level based on the microscopic features. The shapes of conidia were not useful in characters for characterization of *Trichoderma* spp. because of the confusion caused by the use of different terms in different literatures to describe the shapes of the conidia. Furthermore, no systematic rule was established in defining the shapes of

the conidia. The description of the shapes of conidia may be subjective, and thus it may not be precise for characterization of *Trichoderma* species.

It was reported that different growing media used for *Trichoderma* spp. characterization based on their morphological characteristics, confirmed that PDA provides useful information on the pigment production while MEA is an appropriate medium for conidium production and observation of conidiophore branching [20]. *Trichoderma* spp. colony growth was affected by medium pH and temperature. The results showed that pH and temperature influenced the mycelial growth which clearly demonstrated the ability of *Trichoderma* spp: (*Trichoderma asperellum*; *T. harzianum*; *T. brevicompactum* and *T. virens*) to grow on a wide range of pH and temperature, with an optimal pH and temperature of 4 - 7 and 25-30°C respectively. The results are in conformity with Limon *et al.*, [12], who reported that acidic pH favoured the fungal growth than alkaline pH. Singh *et al.*, [21] also confirmed that *T. atroviride* optimum soil pH is 4 -7.5 for growth and sporulation. The optimum pH for *Trichoderma* spp. growth varied between 4.6 and 5.6 depending on the strains [22].

The most favourable temperature for growth and sporulation of *T. harzianum* and *T. viride* was found in between 25- 40°C, whereas for *T. asperellum* and *T. hamatum*, it was 25-35°C. Similarly, the most favourable range of pH was in between 4.6-7.6 for all the four spp. of *Trichoderma* [26].

Trichoderma spp. tested have the potential as a biological control agent of *G. boninense* with all of them inhibiting colony growth by over 70%. The selection of *Trichoderma* spp. as biological control agents depends largely on their effectiveness in the *in vitro* results [5] the biological control potential of *Trichoderma* spp. could be due to production of antibiotic, secondary metabolite compounds or lytic enzymes, which contributed to direct antagonistic effect on the *G. boninense* cell wall and mycoparasitic activities [14]. The potential of *Trichoderma* in producing antibiotic and lytic enzymes capable of hydrolyzing the fungal pathogens cell wall contents [24] [13]. Many *Trichoderma* spp. are also capable of improving plant growth and disease resistance [6] [25].

Due to variable antagonistic ability of individual *Trichoderma* species, it is important that they be screened

first to select for the most active antagonist against *G. boninense*, thus *T. asperellum* can be considered as a best biocontrol agent.

REFERENCES

1. **Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L.G., Héraud, C., and Steinberg, C. 2010.** Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biology*.114: 691–701.
2. **Contreras-Cornejo, H.A., Macias-Rodriguez, L., Ek del Val and Larsen, J. 2016.** Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: interactions with plants. *Federation of European Microbiological Societies, Microbiology Ecology*, 92, 2016.
3. **Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V. and Kubicek, C.P. 2011.** *Trichoderma*: the genomics of opportunistic success. *Natural Review Microbiology*, 9: 749–759.
4. **Gams, W. and J. Bissett, 1998.** Morphology and Identification of *Trichoderma*. In: *Trichoderma and Gliocladium: Basic Biology, Taxonomy and Genetics*, Harman, G.E. and C.P. Kubicek (Eds.). Taylor and Francis, London, UK.,1: 3-34.
5. **Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M. 2004.** *Trichoderma* plant symbionts species-opportunistic avirulent, *Natural Resource*, 2: 43-56.
6. **Hermosa, R., Belén, Rubio M., Cardoza, R. E., Nicolás, C., Monte E. and Gutiérrez S. 2012.** The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *International Microbiology*, 12: 23-46.
7. **Kacprzak, M. J., Rosikon, K., Fijalkowski, K. and Grobelak, A. 2014.** The Effect of *Trichoderma* on Heavy Metal Mobility and Uptake by *Miscanthus giganteus*, *Salix* sp., *Phalaris arundinacea*, and *Panicum virgatum*. *Applied and Environmental Soil Science*, 10; 1- 10.

8. **Kalli, A., Srirangaraj S., Pravin Charles, M.V. 2014** A modified fungal slide culture technique. *Indian Journal of Pathology and Microbiology*. 57: 356 – 357
9. **Khairudin, H. 1991.** Pathogenicity of three *Ganoderma* species on oil palm seedlings. *Journal of Perak Planters Association*, 43-49.
10. **Kotasthane, A., Agrawal, T., Kushwah, R. and Rahatkar, O. V. 2015.** *In-vitro* antagonism of *Trichoderma* spp. against *Sclerotium rolfsii* and *Rhizoctonia solani* and their response towards growth of cucumber, bottle gourd and bitter gourd. *European Journal Plant Pathology*, 141: 523–543.
11. **Kotze, C., Van Niekerk, J., Mostert, L., Hallen, F. and Fourie, P. 2011.** Evaluation of biocontrol agent for grapevine running wound protection against trunk pathogen infection, *Phytopathologia Mediterranean*, 50: 247 –263.
12. **Limón M.C., Chacón, M.R., Mejías, R., Delgado-Jarana, J. and Rincón, A.M. 2004.** Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. *Applied Microbiology and Biotechnology*, 64: 675-685.
13. **Martínez-medina, A., Del, M. and Alguacil, M. 2014.** Phytohormone Profiles Induced by *Trichoderma* Isolates Correspond with Their Biocontrol and Plant Growth-Promoting Activity on Melon Plants. *Molecular Plant Pathology*, 40: 804 – 815.
14. **Nusaibah, S.A., G. Saad and T.G. Hun, 2017.** Antagonistic efficacy of *Trichoderma harzianum* and *Bacillus cereus* against *Ganoderma* disease of oil palm via dip, place and drench artificial inoculation method. *International Journal Agriculture and Biology*, 19: 299–306.
15. **Salas-Marina, M.A., Silva-Flores, M.A., Uresti-Rivera, E.E., Castro- Longoria, E., Herrera-Estrella, A. and Casas-Flores, S. 2015.** Colonization of *Arabidopsis* roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonic acid ethylene and salicylic acid pathways. *European Journal of Plant Pathology*. 31: 15–26.
16. **Samuels, G.J. 1996.** *Trichoderma*: a review of

- biology and systematic of the genus. *Mycological Research*.10: 923-935.
17. **Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A. and Petrini, O. 2012.** *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia*. 94: 146-170.
18. **Schmoll, M., Seibel, C., Tisch, D., Dorrer, M. and Kubicek, C. P. 2010.** A Novel Class of Peptide Pheromone Precursors in Ascomycetous Fungi, *Molecular Microbiology*,77, (6), 1483-501.
19. **Schuster, A. and Schmoll, M. 2011.** Biology and biotechnology of *Trichoderma*. *Applied Microbiology and Biotechnology*, 87(3): 787–799.
20. **Shaiesta Shah, Sahera Nasreen and P.A. Sheikh, 2012.** Cultural and Morphological Characterization of *Trichoderma* spp. Associated with Green Mold Disease of *Pleurotus* spp. in Kashmir. *Research Journal of Microbiology*, 7: 139-144.
21. **Singh, B.N., Singh, S.P., Singh, A., and Singh, H.B. 2011.** Reprogramming of oxidant and antioxidant metabolites in root apoplast of sunflower by *Trichoderma harzianum* NBRI-1055 against *Rhizoctonia solani*. *European Journal Plant Pathology*, 131: 121–134.
22. **Singh A, Shahid M, Srivastava M, Pandey S, Sharma A, 2014.** Optimal Physical Parameters for Growth of *Trichoderma* Species at Varying pH, Temperature and Agitation. *Virology and Mycology* 3:127.
23. **Skidmore, A. M. and Dickinson, C. H. 1976.** Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Trans British Mycological Society*,66: 57-64.
24. **Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Woo, S., Nigro, M.L., Marra, R. Lombardi, N., Pascale, A., Ruocco, M., Ianzuise, S., Manganiello, G. and Lorito, M., 2014.** *Trichoderma* secondary metabolites active on plant and fungal pathogens, *the Open Mycology Journal*, 8: 127-139.
22. **Vos, C. M. F., De Cremer K., Cammue, B. P. A. and De Coninck, B. 2015.** The toolbox of *Trichoderma* spp. in biocontrol of *Botrytis cinerea* disease. *Molecular Plant*

- Pathology*, 16: 400–412.
26. **Zehra, A., Dubey, M. K. Meena M. and Upadhyay R. S. 2016.** Effect of different environmental conditions on growth and sporulation of some *Trichoderma* species. *Journal of Environmental Biology*, 38: 197-203.