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**Article**

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# Evaluation of agro-based waste substrates for micropropagule formation in biocontrol fungi, *Trichoderma asperellum* and *T. harzianum*

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**Abstract**

*Trichoderma* species have shown efficiency on biocontrol of phytopathogens. For commercial application, it must be propagated in mass scale using a cost-effective method. As an alternative way to effectively deliver biocontrol fungi inoculum to the field; seven agro-based wastes including rice bran, biochar, empty fruit bunches, coconut fibre, compost, topsoil and mixed soil were used in this study for evaluating mass multiplication of *Trichoderma* species. Based on the evaluation of colony-forming units (cfu) among the agro-based waste media used, coconut fibre is the most suitable in promoting the sporulation of *Trichoderma asperellum* and *T. harzianum*. *Trichoderma asperellum* C1667 showed the higher micropropagule count through incubation period compared to *T. harzianum* C1675. After 120 days on the agro-based waste media, *T. asperellum* C1667 and *T. harzianum* C1675 produced the highest ($7.717\times10^5$ cfu/g and $6.836\pm13.79\times10^5$ cfu/g) coconut fibres, respectively. Meanwhile, the mixed soil appeared with the lowest cfu. Coconut fibres were shown as a great biocomposting medium for both *Trichoderma* species. Findings of the present study are valuable for disease management using agro-based wastes as a cost-effective medium for biocontrol agents like *Trichoderma* species.

**Key words** – Biocontrol agent – mass multiplication – shelf life

## Introduction

The success of bio-controlling soil-borne plant pathogens depends mainly on the ability of introduced microorganisms. One of the mechanisms is to competitively colonise the rhizosphere of host plant, which can influence the availability of nutrients from the substrate or carrier medium through the biocontrol agent applied (Köhl et al. 2019). Several techniques have been employed such as direct application using single strain, mixture of *Trichoderma* strains or combined with thiophanate-methyl for the multiplication of *Trichoderma* species (Stewart & Hill 2014, Marra et al. 2017, Abd-El-Khair et al. 2019). There are studies that introduced *Trichoderma* species to the soil as mycelia preparations growing on agricultural or industrial waste considered as economical media such as compost (Leandro et al. 2007), rice bran (Cavalcante et al. 2008), coconut coir (Kumar & Palakshappa 2009), empty fruit bunch (Siddiquee et al. 2017) and biochar (Graber et al. 2014, Muter & Olga 2017). Although there have been several studies conducted on the use of

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*Trichoderma* spp. as a biocontrol agent in controlling some forms of plant pathogens (Bae et al. 2011, Mandal et al. 2016, Nosir 2016), there are limited studies investigating how *Trichoderma* survive and proliferate in the soil.

*Trichoderma* species grow on different substrates of carbon and nitrogen through the massive secretion of complex mixtures of specific extracellular enzymes (Vinale et al. 2014, Siddiquee et al. 2017, Sala et al. 2019) and able to enhance plant growth performance (Pandya & Saraf 2010). Therefore, the successful biocontrol of pathogen by *Trichoderma* isolates depends on their ability to survive and remain active in the soil for a long period. In this study, seven media were selected as they are inexpensive sources, which were inoculated with *Trichoderma* to obtain higher levels of survival and proliferation to reduce disease. In this context, due to the importance of *Trichoderma* isolates' survival in the environment, the objectives of this study are to distinguish the most suitable agro-based wastes as a natural medium for efficient micropropagule production of *Trichoderma asperellum* and *T. harzianum* based on colony forming units (cfus) counts and to determine the longest survival duration of *Trichoderma* with the highest productivity.

## Materials & Methods

### *Trichoderma* culture

*Trichoderma asperellum* and *T. harzianum* were chosen in this study because both isolates showed a very high antagonistic activity based on inhibition of radial growth (PIRG) as reported by Zainap et al. (2018). The origin and PIRG are tabulated in Table 1 based on dual culture assay against *Fusarium oxysporum* f. sp. *lycopersici* B713T, a causal agent of fruit rot and Fusarium wilt diseases of tomato. Both isolates were cultured on potatoes dextrose agar (PDA) and incubated at (28±2$^{o}$C) for 7 days. Fungal conidia were harvested from 7-day culture. The concentration of conidia was adjusted to 1x10$^{6}$ conidia/ml using a haemocytometer.

**Table 1** Origin and inhibitory activity of the three best antagonistic *Trichoderma* isolates on mycelial growth of *F. oxysporum* f. sp. *lycopersici* B713T, a causal agent of Fusarium wilt disease of tomato in dual culture (Zainap et al. 2018)

| Isolate no. | Locality | Species | PIRG ± SD | [a]Antagonistic activity |
|---|---|---|---|---|
| C1667 | Maran, Pahang | *T. asperellum* | 80.27 ± 0.54 | very high |
| C1675 | Maran, Pahang | *T. harzianum* | 78.45 ± 0.88 | very high |

[a] >75 PIRG = very high antagonist activity, 61-75 PIRG = high antagonist activity, 51-60 PIRG = moderate antagonist activity, <50 PIRG = low antagonist

### Preparation of media and fungal inoculation

Agro-based wastes used in this study were mixed soil, topsoil, rice-bran, compost, coconut fibre, oil palm empty fruit bunch and biochar (Fig. 1). All the agro-based wastes were obtained from different suppliers due to the limited resources. Topsoil, mixed soil, rice-bran, compost and coconut fibre were purchased from D Syira Enterprise, Selangor, Malaysia. Oil palm empty fruit bunch (EFB) was obtained from Felda Jengka 21 Palm Oil Factory, Pahang, Malaysia while biochar was purchased from YMWOO Corporation Sdn Bhd, Kuala Lumpur, Malaysia. Each medium (200 g) was put in a polybag and inoculated with 1x10$^{6}$ conidia/ml, five replicates per treatment. The inoculated media were incubated at plant house condition. Sangle et al. (2004) reported that 50% of moisture is essential for sporulation; therefore, the media were adjusted to 50% moisture daily using distilled water where sufficient water was added to adjust the moisture intensity in all media and incubated in a polybag for 120 days. The experiments were repeated for three times.

### Measurement of colony-forming unit (cfu) of *Trichoderma*

Colony forming unit (cfu) of *Trichoderma* isolates that survived in each medium was counted using soil dilution isolation technique. The cfu data were recorded at day 15, 30, 45, 60, 75, 90, 105

and 120 after inoculation.

Evaluation of cfu of *Trichoderma* was conducted by mixing 10 g of the inoculated medium sample with 100 ml sterile distilled water followed by agitation in an environmental orbital shaker at 100 rpm for 10 minutes. The serial dilutions at $10^{-3}$ were used for cfu estimation. 1 ml of soil solution was pipetted out and seeded into each Petri dish followed by pouring 9 ml of sterilised *Trichoderma* selective medium (TSM) (Elad et al. 1981). The culture plates were incubated for 4 days at room temperature, 28±2°C. The plates were examined daily and the cfu of *Trichoderma* was calculated.

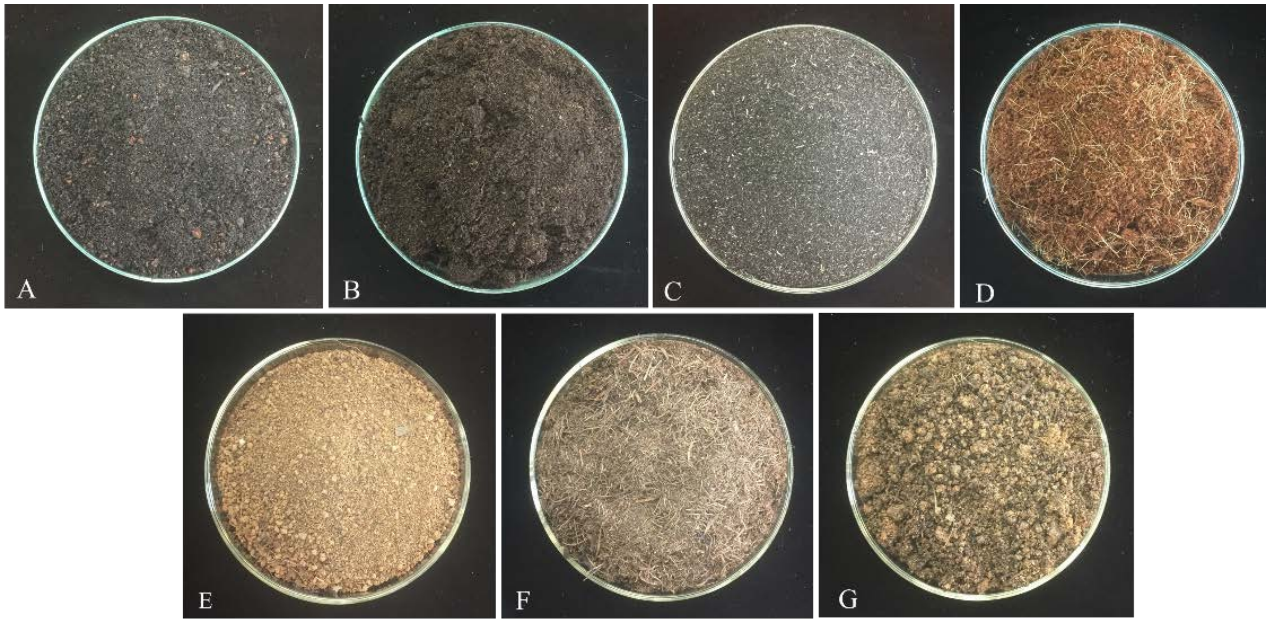


**Fig. 1** – Seven media used in this study. A Biochar. B Compost. C Rice-bran. D Coconut fibre. E Topsoil. F Oil palm empty fruit bunch. G Mixed soil.

**Data analysis**

The cfu of *Trichoderma* was recorded and analysed by One-way ANOVA followed by Least Significant Difference (LSD) in which all data were employed in SPSS software v.22.0 (Armonk, NY: IBM Corp).

**Results**

The presented data revealed that the population of *Trichoderma* isolated from the inoculated agro-based wastes media significantly differed from mixed soil at 15, 30, 45, 60, 75, 90, 105 and 120 days of inoculation. The population of inoculated-*Trichoderma* was varied according to the isolates of *Trichoderma* as tabulated in Table 2. Based on the cfu evaluation among the media, coconut fibre was the most suitable in promoting the sporulation of *Trichoderma*. The finding showed *T. asperellum* isolate C1667 gave the greater population in all time periods compared to *T. harzianum* isolate C1675 as shown in Figs 2-3.

After 15 days of inoculation, all media recorded a very high concentration of cfu. These include topsoil, mixed soil, rice bran, compost, coconut fibre, oil palm empty fruit bunch and biochar. Media inoculated with *T. asperellum* C1667 at different periods at 60 days after inoculation gave a greater population density than that of other ages and in 120 days old. They recorded the least population in all media by $0.8\times10^3$, $0.2\times10^3$, $77\times10^3$, $94\times10^3$, $7.717\times10^5$, $5.984\times10^5$, $0.37\times10^5$ cfu/g for top soil, mixed soil, rice bran, compost, coconut fibre, oil palm empty fruit bunch and biochar, respectively. As for inoculation, the media by *T. harzianum* C1675 recorded the highest cfu at day 60 after inoculation and continued to decline until at the end of experiment (Fig. 4).

**Table 2** Colony forming unit (cfu) of *T. asperellum* C1667 and *T. harzianum* C1675 in seven types of media inoculated with fungal conidia.

| Days of inoculation | *T. asperellum* C1667 (*mean x10$^3$ **cfu/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Topsoil** | **Mixed soil** | **Rice bran** | **Compost** | **Coconut fibre** | **EFB** | **Biochar** |
| 15 | 95.80±11.19$^{c}$ | 68.90±6.29$^{d}$ | 246.00±16.87$^{b}$ | 133.60±14.79$^{c}$ | 996.90±13.12$^{a}$ | 1041.50±16.30$^{a}$ | 234.80±15.83$^{b}$ |
| 30 | 85.20±8.43$^{d}$ | 63.50±6.69$^{d}$ | 114.30±12.02$^{c}$ | 125.90±10.43$^{c}$ | 899.30±15.18$^{a}$ | 906.50±10.46$^{a}$ | 312.10±10.86$^{b}$ |
| 45 | 155.50±12.72$^{d}$ | 63.90±5.05$^{e}$ | 94.70±12.80$^{e}$ | 42.20±6.75$^{e}$ | 1043.10±13.37$^{a}$ | 991.30±11.31$^{b}$ | 324.60±11.79$^{c}$ |
| 60 | 117.00±1.29$^{c}$ | 31.00±4.44$^{d}$ | 117.40±2.44$^{c}$ | 141.70±16.24$^{c}$ | 1064.30±14.06$^{a}$ | 1099.40±14.05$^{a}$ | 345.90±12.63$^{b}$ |
| 75 | 82.10±2.65$^{d}$ | 73.60±7.04$^{d}$ | 423.80±9.44$^{b}$ | 116.50±9.04$^{c}$ | 1061.90±11.11$^{a}$ | 1081.70±13.38$^{a}$ | 260.10±12.37$^{c}$ |
| 90 | 36.70±6.00$^{e}$ | 0.90±0.17$^{e}$ | 436.00±10.29$^{c}$ | 273.60±16.75$^{d}$ | 1010.00±15.88$^{a}$ | 807.90±15.02 $^{b}$ | 296.60±14.61$^{d}$ |
| 105 | 21.50±1.43$^{e}$ | 0.50±0.16$^{e}$ | 140.20±11.32$^{d}$ | 242.00±11.21$^{c}$ | 834.50±9.40$^{a}$ | 657.10±11.49$^{b}$ | 25.80±3.90$^{e}$ |
| 120 | 0.80±0.35$^{e}$ | 0.20±0.13$^{e}$ | 77.00±4.55$^{d}$ | 94.00±6.56$^{c}$ | 771.70±13.58$^{a}$ | 598.40±13.87$^{b}$ | 37.00±4.53$^{e}$ |

| Days of inoculation | *T. harzianum* C1675 (*mean x10$^3$ **cfu/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Top soil** | **Mixed soil** | **Rice bran** | **Compost** | **Coconut fibre** | **EFB** | **Biochar** |
| 15 | 86.40±7.23$^{d}$ | 76.90±5.64$^{d}$ | 204.80±16.94$^{c}$ | 210.80±14.58$^{c}$ | 950.70±13.30$^{b}$ | 1018.10±15.52$^{a}$ | 158.00±9.35$^{c}$ |
| 30 | 95.60±11.04$^{c}$ | 59.20±9.65$^{d}$ | 204.10±15.63$^{b}$ | 119.90±16.96$^{c}$ | 860.50±13.12$^{a}$ | 956.40±12.75$^{a}$ | 325.10±12.35$^{b}$ |
| 45 | 110.20±11.72$^{c}$ | 48.10±9.50$^{d}$ | 99.10±12.97$^{c}$ | 129.30±14.40$^{c}$ | 906.30±13.95$^{a}$ | 961.80±13.51$^{a}$ | 367.60±14.59$^{b}$ |
| 60 | 127.20±8.70$^{e}$ | 28.10±4.15$^{f}$ | 382.00±9.37$^{d}$ | 127.40±12.73$^{e}$ | 913.90±13.95$^{b}$ | 1071.10±14.78$^{a}$ | 362.00±8.62$^{d}$ |
| 75 | 91.50±3.00$^{d}$ | 55.20±1.99$^{d}$ | 305.70±15.95$^{c}$ | 60.60±6.45$^{d}$ | 904.40±11.64$^{b}$ | 1010.10±9.57$^{a}$ | 305.60±10.47$^{c}$ |
| 90 | 8.20±0.41$^{e}$ | 0.80±0.20$^{e}$ | 114.00±3.25$^{d}$ | 213.20±12.83$^{c}$ | 914.30±14.37$^{a}$ | 766.10±11.85$^{b}$ | 192.20±14.69$^{c}$ |
| 105 | 4.90±1.00$^{d}$ | 0.20±0.13$^{d}$ | 116.90±3.38$^{c}$ | 266.50±11.20$^{c}$ | 806.50±15.60$^{a}$ | 679.10±13.84$^{b}$ | 17.40±1.46$^{d}$ |
| 120 | 0.90±0.48$^{e}$ | 0.20±0.13$^{e}$ | 65.60±4.36$^{d}$ | 104.50±8.33$^{c}$ | 683.60±13.79$^{a}$ | 548.30±12.92$^{b}$ | 45.20±3.29$^{d}$ |

* Mean of colony forming unit (cfu) /g soil on *Trichoderma* selective medium (TSM)
** Value followed by the different letter within a row are significantly different ($p<0.05$) according to Duncan's two-way ANOVA

Nevertheless, the high cfu of coconut and oil palm fibre could hold them after four months up to the standard level. From the observation, the isolate can survive for a longer period. Regarding this issue and advantages of organic materials, coconut fibre can be the perfect solution with the use of *Trichoderma* in the biocontrol of plant disease. The result provided implies that micropropagules from *Trichoderma* ssp. can be efficiently produced with coconut fibre and oil palm fibre, which are inexpensive and abundant agricultural by-products. It is also significant to state that all the cfu production processes were carried without adding extra nutrients to the production medium.

## Discussion

Coconut fibre is a great medium for micropropagule of *Trichoderma*, suggesting that this medium can be used as a new way to save and keep fungi for a long period. Asiah et al. (2004) reported that coconut coir dust has higher nutrient contents (P, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) than in empty fruit

bunches. *Trichoderma* could produce chlamydospores and maintain long periods of vigorous vegetative growth during use (Fravel 2005). Ali et al. (2012) found a decrease in cfu of *T. harzianum* during the period of incubation than the initial count at the beginning of their experiment.

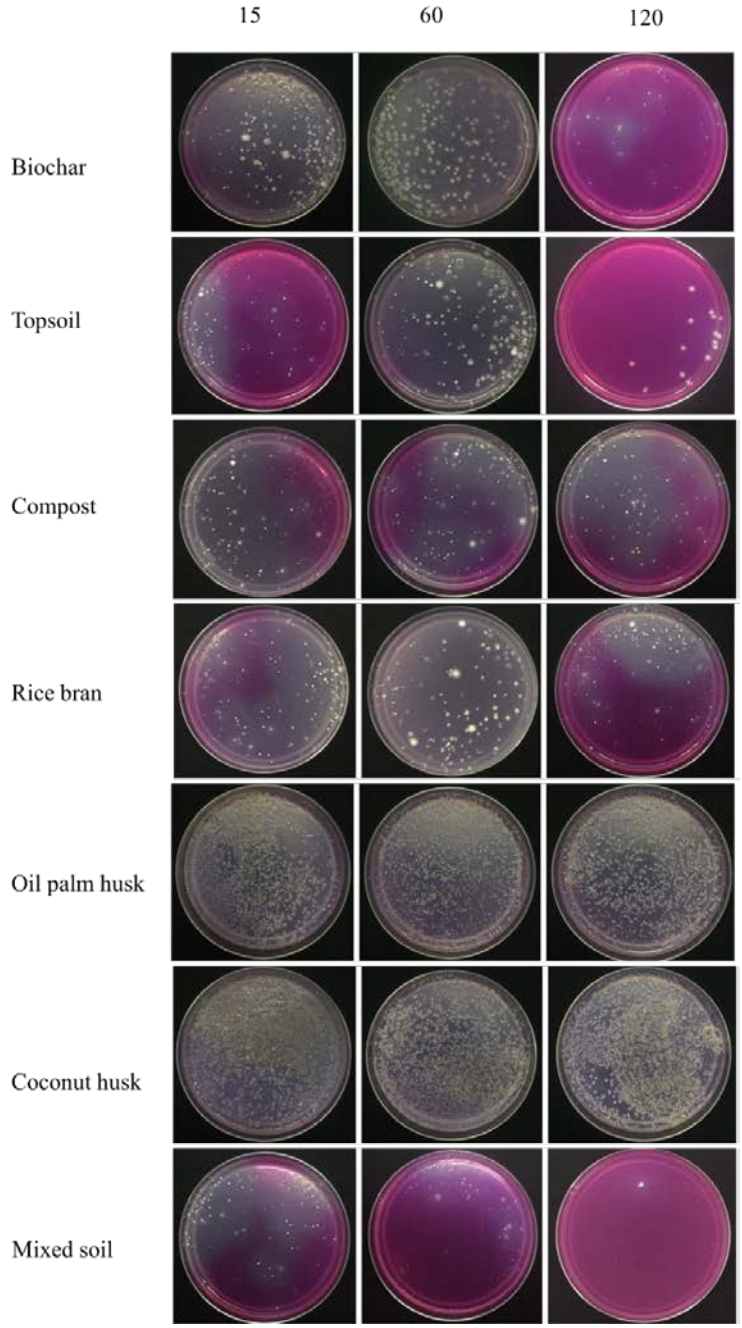


**Fig. 2** – Growth of *Trichoderma asperellum* C1667 at 15, 60 and 120 days after inoculation in different types of media.

Amira et al. (2011) revealed that adding *T. virens* to oil palm empty fruit bunches and palm oil mill discharge shortened the composting time due to enzymatic activity that was at a greater level. Lewis & Papavizas (1984) also showed the potential of different *Trichoderma* sp. collections to develop chlamydospores easily and in abundance in natural soil as well as in parts of organic matter. When fungus was introduced to the soil as conidia, isolates were included as they can aggressively colonise and position themselves in organic matter within the natural environment. The addition of *Trichoderma* to soil as dry formulation seemed to multiply significantly (Lewis &

Papavizas 1984), demonstrating an increase from the original amount of $5\times10^3$ to a peak of 6-$7\times10^6$ conidia/g of the soil with different organic matter content. Tewari & Bhanu (2004) carried out solid-state fermentation of *T. harzianum* on various substrates and found that rice straw and wheat straw resulted in high conidial yield of $4.95\times10^8$ cfu/g and $4.86\times10^8$ cfu/g, respectively. Media containing paper waste, rice bran and chickpea flour were utilized for mass production of *T. harzianum* and reported a cfu of $11.73\times10^9$ cfu/g following 10 days of incubation (Tewari & Bhanu 2004). The use of cow dung, beem cake, coir pith, sorghum grains, sawdust and rice bran in mass production of *T. harzianum* and *T. viride* improved conidial yield from $23.66\times10^8$ to $34.00\times10^8$ and $45.6\times10^8$ cfu/ g, respectively (Rini & Sulochana 2006). These studies proved that *Trichoderma* can be cultured and maintained in various media with different organic matter content. For instance, in many species of *Trichoderma*, biotic factors particularly C/N ratio influenced the formation of conidiospore (Gao et al. 2007).

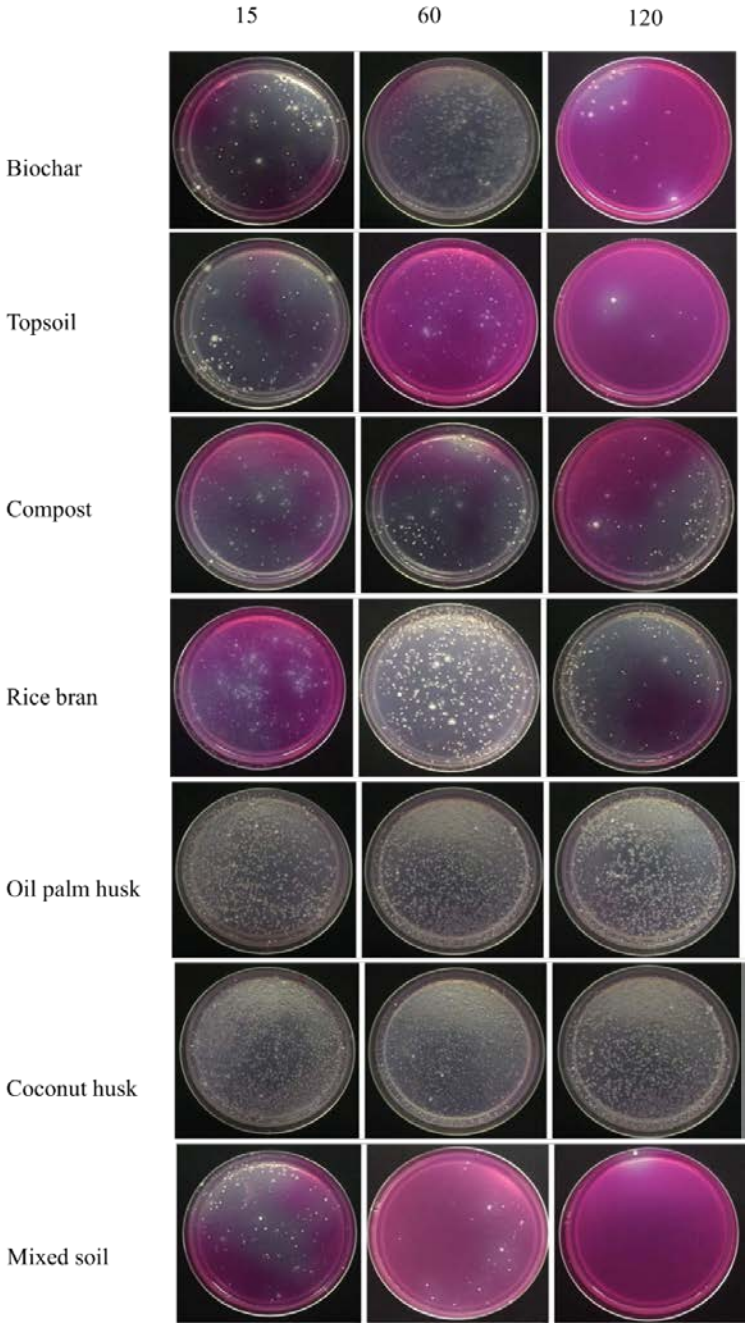


**Fig. 3** – Growth of *Trichoderma harzianum* C1675 at 15, 60 and 120 days after inoculation in different types of media.

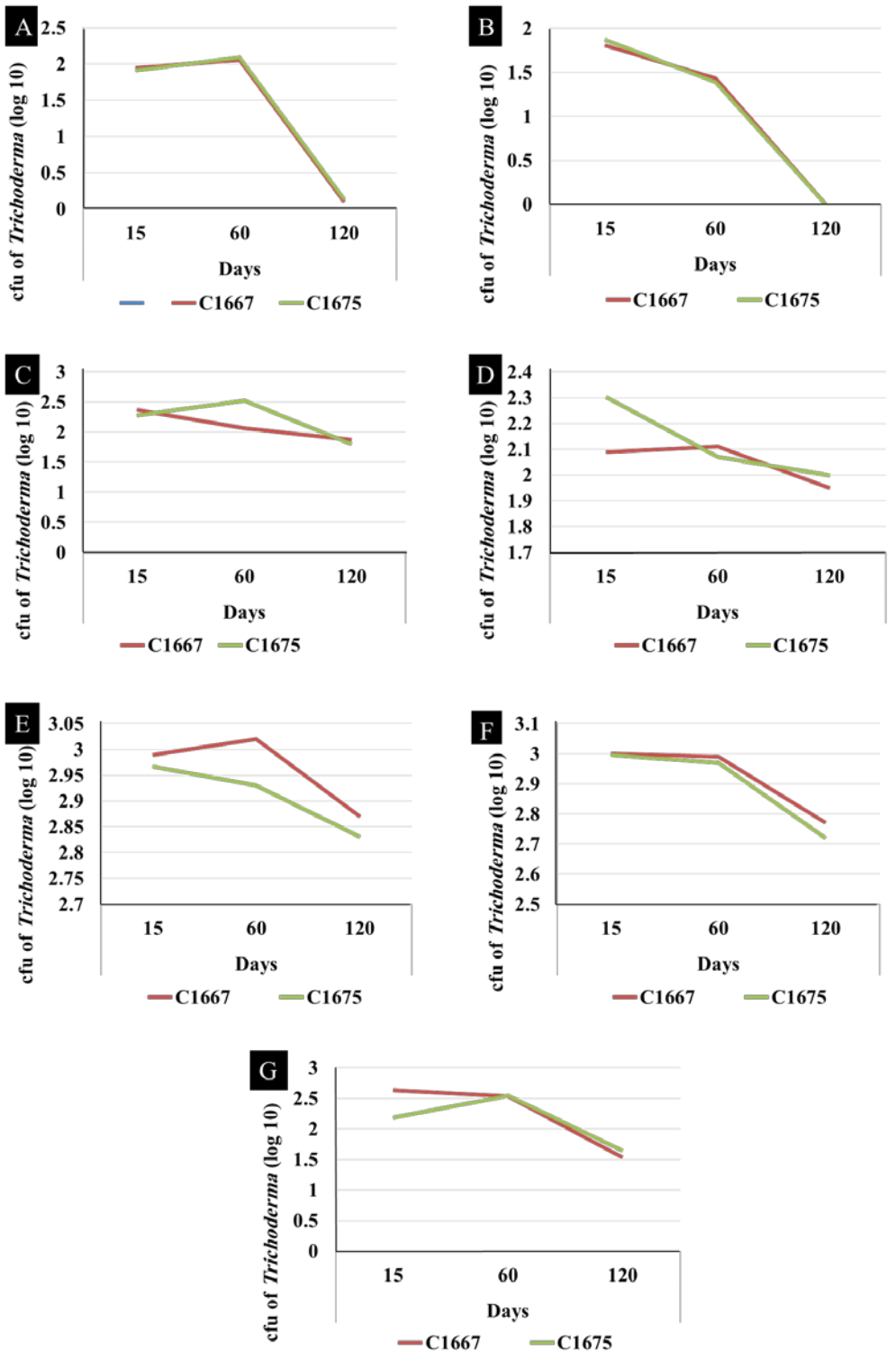


**Fig. 4** – Survival of *Trichoderma* spp. in seven types of media in 15, 60 and 120 days of incubation. Where A) Topsoil B) Mixed soil C) Rice bran D) Compost E) Coconut fibre F) oil palm empty fruit bunch (EFB) and G) Biochar.

The composting of organic wastes with the help of *Trichoderma* does not only helps in recycling waste literally, but also give good results in the preparation of economic and environmentally friendly organic biofertilisers. Coconut fibre followed by oil palm empty fruit bunch was more favorable as a medium used for the survival and proliferation of the tested fungus. This approach could provide benefits to agriculture crops since it gave the highest biomass and

number of cfu/g as observed on *T. asperellum* C1667 and *T. harzianum* C1675 using cost-effective media in this study.

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