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MORPHLOGICAL IDENTIFICATION OF *TRICHODERMA* SPECIES ISOLATED FROM RHIZOSPHERE OF COCOA (*THEOBROMA CACAO*)

Suhaida, S.[1], Muhammad Farid, A.R.[2], Nellysa Nursyafira A.[3] and Shari Fuddin, S.[1]
[1]Malaysian Cocoa Board, 5-7th Floor, Wisma SEDCO, 88999 Kota Kinabalu, Sabah, Malaysia
[2]Kulliyyah of Science, International Islamic University Malaysia (IIUM), Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia
[3]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (Melaka), Jasin Campus, 77300 Merlimau, Melaka, Malaysia
E-mail: suhaida@koko.gov.my

Abstract: The fungi of genus *Trichoderma* can be found nearly in all types of temperate and tropical soils. They can also be found on decaying wood, bark and other kind of plant organic materials. In the present investigation, 24 isolates of *Trichoderma* sp. were obtained from the rhizosphere soils of cocoa planted at Raub, Pahang. Taxonomic identification of isolates was done up to species level based on their macro and micro-morphological characteristics. The macro-morphological characteristics included linear growth, colony features, pigmentation and sporulation pattern. On the other hand, micro-morphological characteristics that were examined were shape and arrangement of conidiophores, phialides, conidia and the presence of chlamydospore. On the basis of macro and micro-morphological characteristics, *Trichoderma* isolates were classified into three species, namely *T. harzianum*, *T. koningii* and *T. hamatum.* The most abundant species in cocoa rhizosphere is *T. harzianum* (58%) followed by *T. koningii* (21%) and *T. hamatum* (21%).
Keywords: *Trichoderma* sp., Cocoa Rhizosphere, Identification, Morphological

INTRODUCTION

Cocoa (*Theobroma cacao*) is one of the most important agricultural commodities in Malaysia. Its seeds, cocoa beans, are used to make chocolate, cocoa butter, cocoa solids and chocolate liquor. Although the world demand for cocoa products is increasing, the cocoa tree can be damaged by several diseases that may affect the sustainability of this crop. Approximately 40% of the annual cocoa production is lost to disease infection (Nembot *et al.*, 2018).

Several practices have been applied in managing cocoa diseases such as by using fungicides, resistant planting materials, quarantine method and cultural practices. However, these methods has led to several problems such as fungicides resistance development, environmental pollution, labor-intensive and uneconomical. One of the potential nonchemical alternative methods for managing crop diseases is by the using of microorganisms as biological control

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agents. This method is environmental friendly, cost-effective and sustainable management of plant diseases (Kulkarni et al., 2007).

Trichoderma spp. is a well-known biological control agent against wide range of plant pathogenic fungi. They are classified as Fungi Imperfecti due to the absence of sexual reproduction and only produce asexual spores i.e. conidia (Singh *et al*., 2006). They occur worldwide nearly in all types of temperate and tropical soils. They can also be found on decaying wood, bark and other kind of plant organic materials (Howell, 2003). The success of *Trichoderma* species as a biological control agent is due to their special characteristics such as extensively distributed, high reproductive capacity, easy to isolate and culture, rapid growth, can compete for food and space, avirulent plant symbionts and producers of antibiotics, antifungal and enzymes (Pandya *et al.*, 2011).

Monte (2003) reported the capability of *Trichoderma* spp. in controlling soil-borne and air-borne pathogens of Ascomycetous, Deuteromycetous and Basidiomycetous fungi. According to Benítez *et al*. (2004) the most common species of *Trichoderma* used as biocontrol agents are *T. harzianum* followed by *T. virens* and *T. viride.* However, the application of *Trichoderma* species in managing cocoa diseases in Malaysia was not really well documented. Therefore, this study was executed to isolate and identify *Trichoderma* spp. from cocoa rhizosphere. The isolated *Trichoderma* can be further tested for their biocontrol potential against cocoa diseases in the next study.

MATERIAL & METHODS

Collection of Soil Samples

The soil samples were collected from two local cocoa plantations in Raub, Pahang, Malaysia. From each location, the samples were obtained from three different cocoa trees. Three subsamples were then taken randomly at a depth of 10 cm away from the surface at the rhizosphere area under the canopy of cocoa tree. The soil samples were then kept into an ice-box and transported to the laboratory. All subsamples from one site were mixed to get a composite sample representing the location (Ru and Di, 2012).

Isolation of *Trichoderma* spp.

Isolation of *Trichoderma* spp. from collected soil samples was conducted by using serial dilution technique (Arumugam *et al*., 2013). Ten gram of soil samples were added into 90 ml sterile distilled water before agitating in an orbital shaker at 100 rpm for 10 minutes. Dilutions were made up to 10^{-3} and 1 ml of final dilutions was pipetted into a petri dish. About 9 ml of *Trichoderma* selective medium (THSM) was poured into diluted soil, swirled gently and left to solid. The soil plates were examined daily and each colony that exhibited yellowish to greenish colour with lumpy mycelial growth was subcultured onto potato dextrose agar (PDA). Single spore isolation was carried out on new PDA to obtain the pure culture of fungi.

Morphological identification of *Trichoderma* spp.

Trichoderma isolates were identified into species levels based on their macro and micro-morphological characteristics. The pure isolates that were grown on PDA were recorded for their colony features, pigmentation and sporulation pattern and the growth rate was measured according to identification method by Diba *et al*, (2007).

To observe the micro-morphological features of *Trichoderma*, slide culture technique by Hamed *et al*. (2012) was applied. A block (1 cm^2) of PDA was placed on a sterile slide and then cultured with *Trichoderma* on all four sides of the agar block and covered with cover slip. The culture was incubated for three days in a sterile glass petri dish layered with a damp filter paper. The micro-morphological appearances of *Trichoderma* were observed by placing the colonized cover slip on a new slide containing a drop of sterile distilled water. The slide culture was examined using light microscope (Rax Vision, model Y-100). The micro-morphological characteristics were observed as followed: shape and arrangement of conidiophores, phialides, conidia and the presence of chlamydospore.

RESULTS AND DISCUSSION

Trichoderma isolation from soil

A total of 24 isolates of *Trichoderma* have been successfully recovered from cocoa rhizosphere soil collected from Raub, Pahang. They were primarily selected based on the pigmentation and colony features on THSM after three days of incubation. The lumpy growth of colony with yellowish to greenish colour (Figure 1) were subcultured on PDA media to get a pure culture of *Trichoderma* spp. These characteristics were also used by Naher *et al*., (2019) as primary selection for isolation of *Trichoderma* colony from soils.

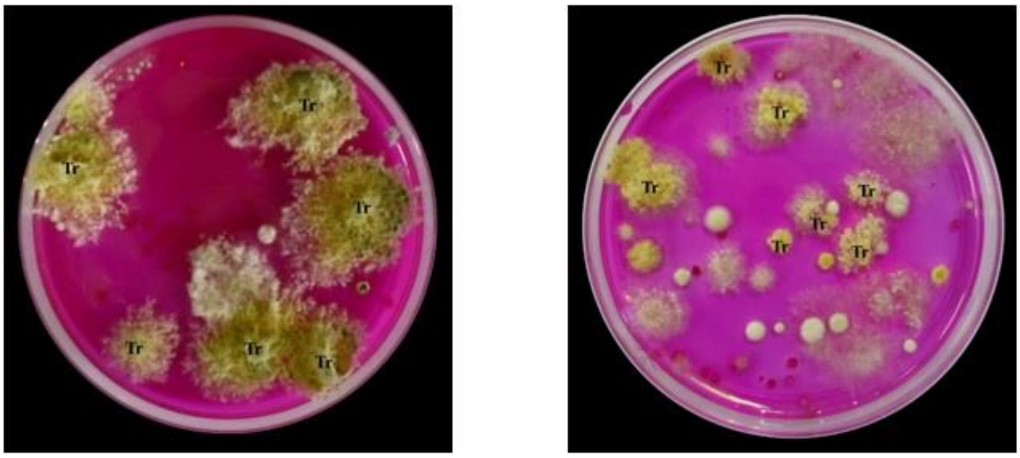

Figure 1: *Trichoderma* colonies (Tr) from cocoa rhizosphere grown on THSM

Macro-morphological characteristics of *Trichoderma*

The pure culture of *Trichoderma* were recorded for their macro-morphological characteristics which were colony colour, pigmentation and sporulation pattern and the growth rate. According to Samuels *et al.*, (2002), these characteristics were regarded as taxonomically useful characteristics for *Trichoderma*. All 24 isolates of *Trichoderma* had different growth rate and diverse colony colour and pigmentation (Table 1). Most of the isolates were fast-growing and fully colonize the media plate in just 72 to 96 hours after inoculation. Only several colonies produced pigmentation on PDA that could be observed on the back of the plates (Figure 2). These pigmentations appeared when the colony are fully matured. The growth formation of *Trichoderma* spp. is irregular with undulate margin and flat elevation. Conidia were formed in two days and turned to light or dark green colour within three days.

Table 1: Macro-morphological characteristics of *Trichoderma* isolates

No.	Isolate	Growth rate (mm/day)	Fully colonize plate after (hrs)	Colony Colour	Pigmentation	Species
1.	TC4	32.08	72	Yellowish green	Brownish yellow	*T. hamatum*
2.	TC6	32.05	72	Dark green	-	*T. hamatum*
3.	TC9	27.83	72	Whitish green	-	*T. hamatum*
4.	TC10	31.28	72	Whitish green	-	*T. hamatum*
5.	TC12	32.58	72	Yellowish green	Light yellow	*T. hamatum*
6.	TC5	28.05	72	Yellowish green	-	*T. koningii*

7.	TC8	29.10	72	Whitish green	-	*T. koningii*
8.	TC20	18.70	96	Whitish green	Light yellow	*T. koningii*
9.	TC21	21.70	96	Whitish green	-	*T. koningii*
10.	TC27	24.43	72	Whitish green	-	*T. koningii*
11.	TC7	31.08	72	Dark green	-	*T. harzianum*
12.	TC11	32.53	72	Whitish green	-	*T. harzianum*
13.	TC13	32.01	72	Yellowish green	Light yellow	*T. harzianum*
14.	TC14	30.61	72	Yellowish green	-	*T. harzianum*
15.	TC15	31.26	72	Yellowish green	-	*T. harzianum*
16.	TC16	31.03	72	Whitish green	-	*T. harzianum*
17.	TC17	28.91	72	Whitish green	-	*T. harzianum*
18.	TC18	31.68	72	Whitish green	Brownish yellow	*T.harzianum*
19.	TC19	30.46	72	Whitish green	Light yellow	*T.harzianum*
20.	TC22	29.15	72	Whitish green	-	*T. harzianum*
21.	TC23	31.70	72	Whitish green	-	*T. harzianum*
22.	TC24	29.45	72	Yellowish green	-	*T. harzianum*
23.	TC25	30.25	72	Yellowish green	Light yellow	*T. harzianum*
24.	TC26	30.80	72	Whitish green	Light yellow	*T. harzianum*

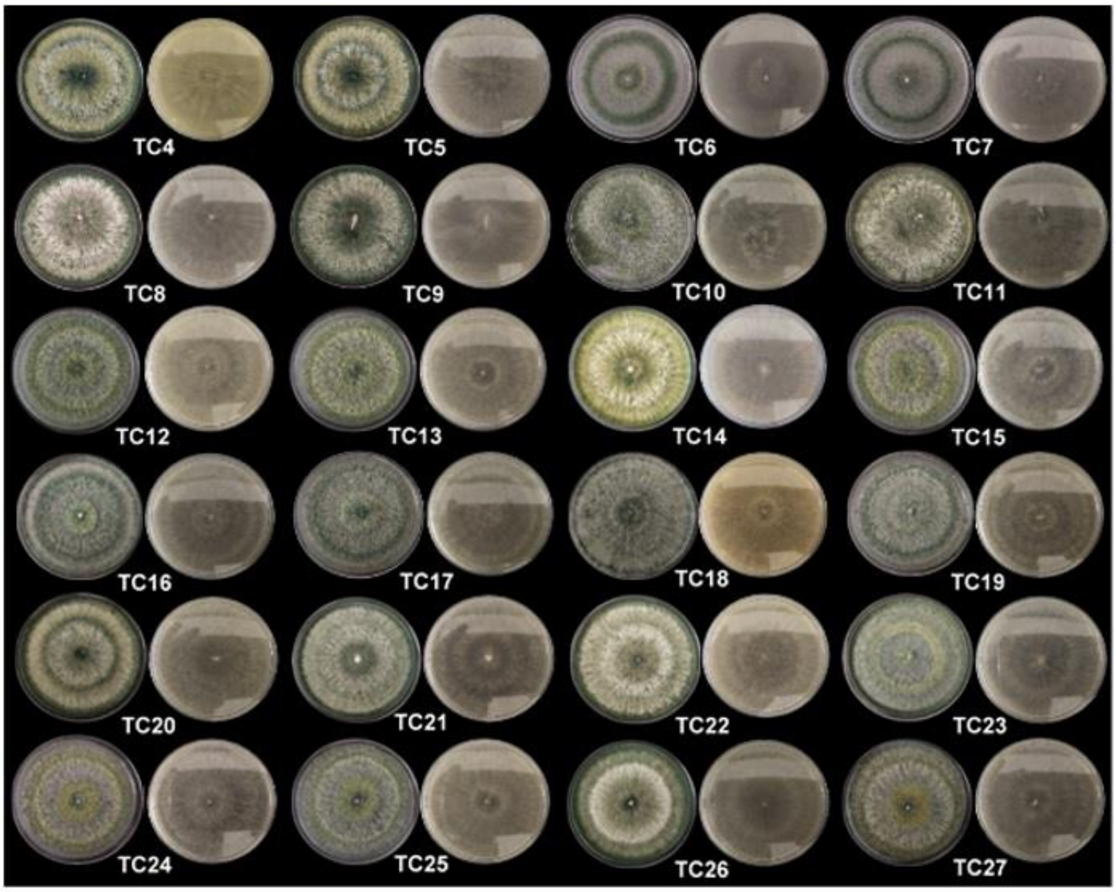

Figure 2: Colony features and pigmentations of 24 isolates of *Trichoderma* on PDA medium at 5th day incubation

The growth rate of *T. hamatum* isolates were ranged from 27.83 – 32.58 mm/day and all isolates were fully colonized the media plate after 72 hours. Their colony colours were varied (dark green, whitish green and yellowish green) and some isolates produced brownish yellow and light yellow pigments on PDA. The slowest growth rate of *Trichoderma* was recorded on *T. koningii,* isolate TC20 followed by TC21 with rate of 1.70 mm/day and 21.70 mm/day, respectively. These isolates took 96 hours to fully colonize the media. Most of the *T. koningii* isolates colony colour were whitish green and only TC20 produced pigmentation on PDA which was light yellow in colour. Meanwhile, *T. harzianum* isolates produced two pigmentation colours which were light yellow and brownish brown. The growth rate of *T. harzianum* isolates were ranged from 28.91 - 32.53 mm/day. Sekhar *et al.,* (2017) also found the similar growth rate of *Trichoderma* spp. ranged from 24.33 – 30.00 mm/day. They isolated them from groundnut and identified them as *T. koningii, T. reeseii, T. harzianum and T. aureoviride.*

The above characteristics are macro-morphological features of *Trichoderma* spp. However, these characteristics are not sufficient to identify them into species level. Therefore, the observation of micro-morphological characteristics is crucial in order to confirm the species of *Trichoderma.*

Micro-morphological characteristics of *Trichoderma*

Micro-morphological observation was carried out by doing slide cultures to examine the microscopic characteristics of *Trichoderma.* The observed characteristics are shape and arrangement of conidiophores, phialides, conidia and the presence of chlamydospore (Gams and Bisset, 1998). Out of 24 isolates, the most abundance species in cocoa rhizosphere is *T. harzianum* (58%) followed by *T. hamatum* (21%) and *T. koningii* (21%) (Table 2). This result is supported with the finding by Sharma and Singh (2014) which managed to obtain 80% isolates of *T. harzianum* from rhizosphere of different crops in India. The abundance of this species in the soil is because *T. harzianum* is the predominant species cluster in that habitat (Jaklitsch and Voglmayr, 2015).

Table 2: Micro-morphological characteristics of *Trichoderma* isolates

Isolate	Characters				
	Conidio-phore	**Phialides**	**Conidia**	**Chlamy-dospore**	**Species**
TC4, TC6, TC9, TC10, TC12	Long; branched; verticillate	Short; thick; ampulliform,	Globose to ellipsoidal	Present	*T. hamatum*
TC5, TC8, TC20, TC21, TC27	Branched; erect,	Pair phialides, lageniform	Ellipsoidal s	Present	*T. koningii*
TC7, TC11, TC13, TC14, TC15, TC16, TC17, TC18, TC19, TC22, TC23, TC24, TC25, TC26	Frequent branching; erect; verticillate	Frequently paired, lageniform	Globose to subglobose	Present	*T. harzianum*

Micro-morphological features observed on TC4, TC6, TC9, TC10 and TC12 were showed them as *T. hamatum.* The features on conidiophores were shown as long, branched and verticillate (Figure 3A-B). Phialides were characteristically short, thick and ampulliform in shapes (Figure 3C). In this study, there is a character that has been observed only on *T. hamatum* which is the occurrence of sterile or fertile elongations of conidiophores (Figure 3D). According to Chaverri and Samuels (2003), majority of this species have pachybasium-like morphology with irregular branching patterns. These fertile branches develop near the base of the extension or along the length of the main axis in cases when no extensions are formed. Conidia of *T. hamatum* isolates were formed with a shape of globose to ellipsoidal (Figure 3E) and chlamydosporos were formed in the middle of the hyphae (Figure 3F).

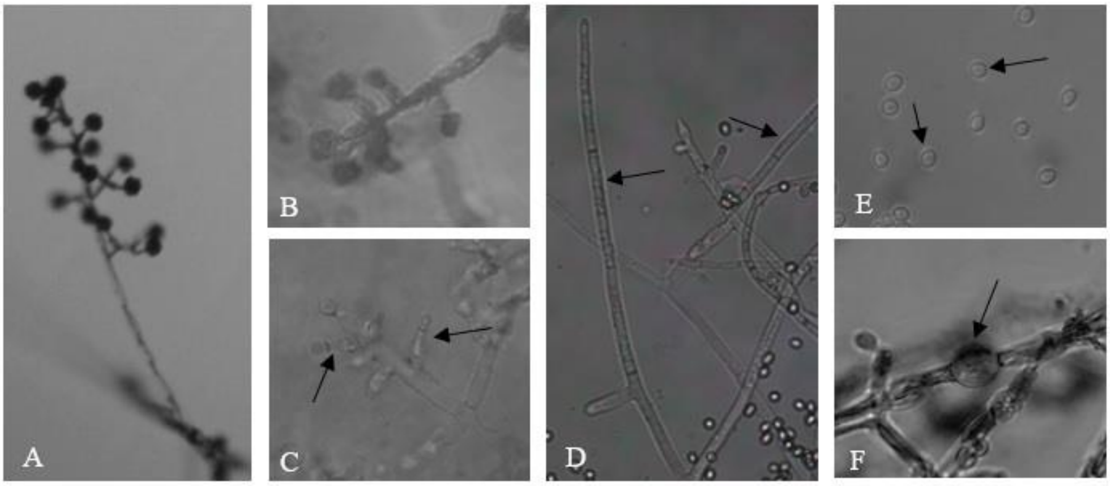

Figure 3. Micro-morphological observation of *Trichoderma hamatum* (TC6). A-B: Spore masses with conidiophores; C: Phialides (arrows); D: Sterile elongation of conidiophore (arrows); E: Phialospores (arrows); F: Chlamydospores (arrow)

The isolates TC5, TC8, TC20, TC21 and TC27 features were *T. koningii* similarity. The micro-morphological observation on conidiophores formed were branches and usually formed in nearly 90^{o} to the main axis (Figure 4A). Phialides tend to be paired and lageniform in shapes (Figure 4B-C). The conidia were shown as ellipsoidal (Figure 4D) and most of the chlamydospores in all isolates were formed on the hyphal tips (Figure 4E). The above features are also similar to the morphological observation of *T. koningii* isolated from rhizosphere of oil palm in Malaysia (Naher *et al*., 2019). Beside *T. koningii*, they also managed to isolate *T. harzianum* from rhizosphere of paddy, scallion, long bean, banana and tomato. Meanwhile, the other species which are *T. asperellum, T. pararesei, T. virens* and *T. viride* were isolated from rhizosphere of eggplant, rubber and pineapple.

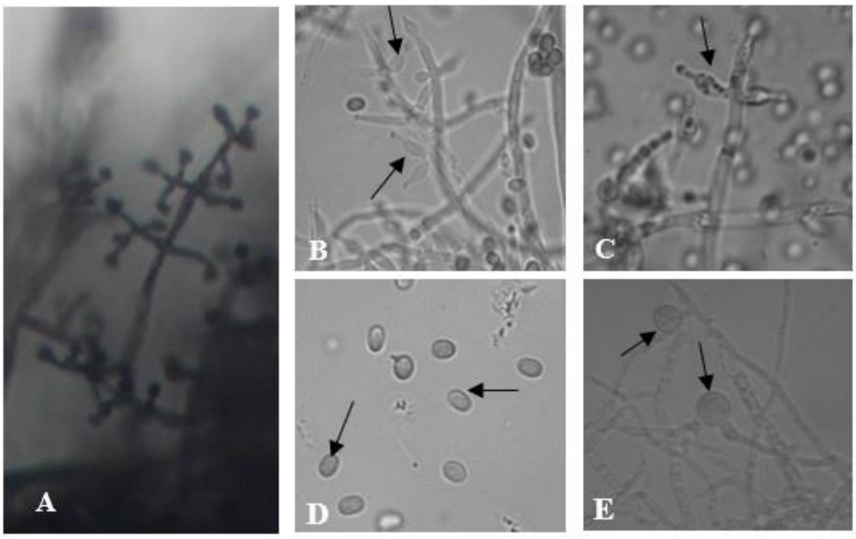

Figure 4. Micro-morphological observation of *Trichoderma koningii* (TC5). A: Spore masses with conidiophores; B-C: Phialides (arrows); D: Phialospores (arrows); E: Chlamydospores (arrows)

Trichoderma harzianum is the most abundant isolated species during this study. All isolated colonies showed more or less similar in their micro-morphological features (Figure 5). Conidiophores of *T. harzianum* were frequently branched nearly at 90^{o} in verticillate pattern (Figure 5A). Phialides were frequently paired and lageniform in shapes (Figure 5B-C). The conidia were globose to subglobose in shape (Figure 5D) and the formation of chlamydospores were observed in the middle of hyphae on most isolates (Figure 5E).

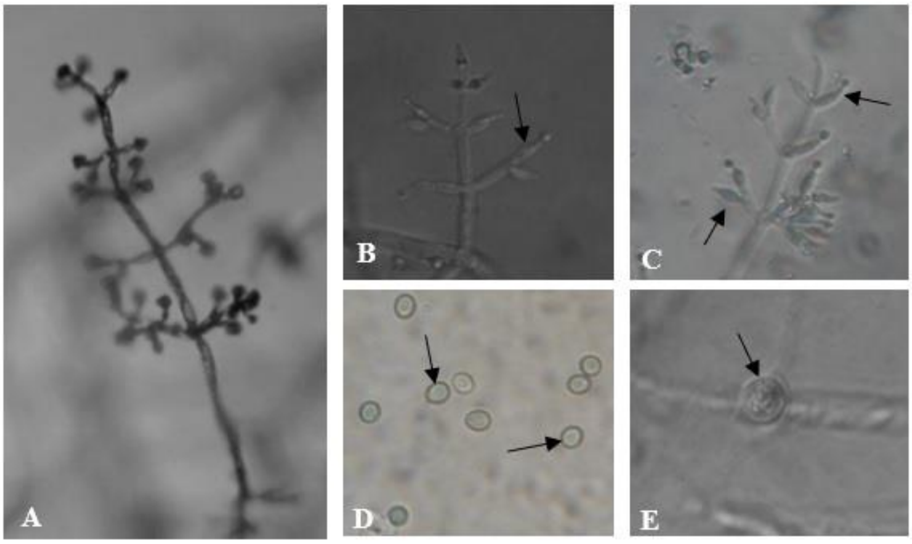

Figure 5. Micro-morphological observation of *Trichoderma harzianum* (TC25). A: Spore masses with conidiophores; B-C: Phialides (arrows); D: Phialospores (arrows); E: Chlamydospores (arrow)

CONCLUSION

Our study concluded that out of 24 isolates, the most abundant species in cocoa rhizosphere is *T. harzianum* (58%) followed by *T. koningii* (21%) and *T. hamatum* (21%). The cultural characteristics were not highly variable. These isolates will be further tested for their potential on controlling cocoa diseases.

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