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Reserch and development of enzymatic producing fungi as biofertilizer for tea and arabica coffee production in Northern Vietnam

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Abstract Tea *(Camellia sinensis)* and Arabica coffee *(Coffea arabica)* are main economic crops in the northern region of Vietnam, contributing for improving income of local farmers. However, the production activities of these crops in Vietnam are depended on agrochemical to maintain crop productivity that has led to many unexpected consequences on the environment, ecosystem, and human health. The potent enzymatic producing fungi were found, screened and developed to be biofertilizer product and used for tea and arabica coffee cultivation. *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghamella* SL2, *Penicillum* TN1, *Aspergillus*PT were selected and all tested isolates produced cellulase, xylanase, and ligninase to degrade organic materials. Those isolates were developed to be organic biofertilizer product (BFP). *In vivo* trial, results in a greenhouse experiments showed that the bio-fertilizer product not only gave significant effects on growth of tea and coffee plants but also increased the organic matter in soil. The bio-fertilizer product could provide a good choice to apply on tea and coffee plants in Vietnam to decrease the application of chemical fertilizers.

Keywords: arabica coffee, tea, soil microorganisms, enzymes, biofertilizers

Introduction

Tea *(Camellia sinensis)* and Arabica coffee (*Coffea arabica*) are the key industrial crops in the northern mountainous region of Vietnam, bringing important jobs and income to the people in the region. Inorganic fertilizers and chemical pesticides have been widely used to increase crop yields. However, the abuse of these materials in tea and coffee production makes the land degenerate, discolored, soil microorganisms become poor (Jayanta *et al.*, 2016; Renla and Ajungla, 2017; Debojyoti *et al.*, 2017).The microbial system in the soil in the tea field is very important, with many

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microorganisms acting as biological fertilizers, they decompose organic and inorganic substances, release enzymes, promote growth and enhance resistant of tea. In soil in the tea rhizosphere, there are various types of potassium-degrading bacteria and phosphorus degradation that convert inorganic insoluble phosphate into soluble forms such as *Bacillus amyloliquefaciens*, *Bacillus pumilus* and *Serratia marcescens*. Many fungal species also help tea roots better absorb phosphate and other nutrients such as fungi belonging to *Glomus*, *Gigaspora*, *Acaulospora* and *Scutellospora* (Renla and Ajungla, 2017). Mycorrhizal fungi are the most common symbiotic species. The main benefit of root fungi is to enhance plant nutrient uptake, especially phosphorus. Many authors have considered the positive effects of mycorrhizal fungi on coffee plants (Diriba, 2007; Al-Areqi *et al*., 2013). Kaewchai *et al*. (2009) reported that fungal biofertilizers play an important role in promoting plant growth, health, productivity and improving soil fertility. Ha, *et al.* (2008) and Vo and Cao (2011) found cellulose-degrading microorganisms in their reserrch finding and could be used for cellulose fermentation to produce compost. Tran (2012) reported that *Trichoderma* spp. could be developed to produce organic fertilizer to promote plant growth in Vietnam. The objectives of reserch findings were to isolate the potent enzymatic fungi and developed to be organic biofertilizer product for the growth tea and coffee.

Materials and Methods

Isolation of enzymatic degrading fungi

Soil samples were taken from tea and Arabica coffee fields, at 0-20 cm soil depth from each location in provinces of Phu Tho (PT), Yen Bai (YB), Thai Nguyen (TN), Son La (SL), Dien Bien (DB) and Lam Dong (LD). A total of 54 soil samples were used for isolation of the enzymatic degrading fungi and selection to develop a bio-fertilizer.

Isolation, selection of fungi was done using the method of Lee *et al.* (2002) with slightly modified. Sterilized filter paper, 1x1cm in size, placed in soil surface in Petri dishes, sprayed with distilled water on paper and soil to keep moist and daily observed. The isolates were made into pure culture on potato dextrose agar (PDA) medium.

Determination of cellulose degradability

All isolates were tested for cellulase production by transferring to peptone yeast glucose (PYG) medium which consisted of peptone (1.25 g), yeast extract (1.25 g), dextrose (3.0 g), agar (20.0 g), water (1000 ml) which modified from Bermejo *et al*. (2012), and incubated at room temperature until fungus grew about 1/2 of Petri dishes, then transferred to cellulolytic

basal medium (CBM) which follow the instruction of Padmavathi *et al*. (2012). The fungus grew on CBM medium about 50-60%, added red congo (2%) to fill up the fungus and leave for 15 minutes, then removed red congo, washed out with distilled water, and soaked in NaCl solution (ratio and of NaCl in water is 1:1). Data were collected by observing on the medium in Petri dishes, if it shows a transparent area meaning the fungus capable of producing enzyme cellulase. Discoloration was observed after 15 minutes to 24 hours according to Pointing (1999), and measured the diameter of the resolution ring according to Pham (2012), Tolan and Foody (1999). The control was done without transferred fungus into those media.

Determination of lignin degradability

All isolates were transferred from PYG into corn meal agar (CMA), incubated at room temperature until growing 50-60% in Petri dishes, the made the holes at equal distance near the colony edge, dropped hygallic reagent, and followed by hydrogen. Discoloration was observed after 15 minutes to 24 hours. The control was done without transferred fungus into those media.

Determination of xylan degradability

All isolates were cultured on xylan medium, incubated at room temperature until 50-60%, poured 1% iodine reagent solution on the surface, after 15 minutes, the iodine was washed out by rinsing with distilled water. Discoloration was observed after of 15 minutes to 24 hours. The control was done without transferred fungus into those media.

Evaluation of bio-fertilizer product for the growth of Tea and Arabica coffee plants in greenhouse

All active enzymatic producing fungi were selected and made to be bio-fertilizer product (BFP). Each active isolate was used at the cocenration off 1.5 X 10^7 cfu/g of raw materials (china clay). Tea and coffee seedlings were grown in pots containing sterilized soil with mixture with compost at a ratio of 3: 1.

The experimental pots were separately planted tea and Arabica coffee seedlings and maintained in greenhouse for each experiment. Treatments were set up as follows:- T1 = 1g bio-fertilizer product (BFP), T2 = 1.5 g bio-fertilizer product(BPF), T3 = 2.0 g bio-fertilizer product(BFP) and T4 = non-treated control. BPF was applied directly into the soil after planting tea or coffee seedlings in each experimewnt. All tested plants in each experient was maintained by watering to keep soil moist for six months.

Data were collected as fresh plant height, stem & leaf weight, root weight, and fungal density. Soil quality was determined according to

Vietnam national standards. Fungal density was determined. The soil sample was taken in each treatment, crushed, and mix, then poured 0.1 grams of soil in 1ml of water to be suspended. The soil solution was diluted to 10^{-2}, poured into Czapek-dox medium and incubated at room temperature forv 3-5 days. Data were colleted by counting colony forming unit (cfu) in each tratement. The cfu.g /g of soil in each treatemt for fungal density was calculated as follows:-

$$N = \frac{\sum c}{d.V.n}$$

Where N = total cfu/g), $\sum c$ = The total number of colonies counted on petri dishes, n = Number of Petri dish, d = dilution rate and
V = volume of diluted solution

Experimental designs and data analysis

The experiments for determination of enzymatic degradability were set up as Completely Randomized Design (CRD) with four replications. The experiments for evaluation of bio-fertilizer product in greenhouse werec set up into tea or coffee experiment by using Randomized Completely Block Design (RCBD) with four replications. All data were subjected to analysis of variance (ANOVA) using Microsoft excel and IRRISTAT. Means were compared using Duncan's multiple range tests (DMRT) at P = 0.05 and P = 0.01.

Results

Isolation of enzymatic degrading fungi

Result showed that 31 potential degrading fungal isolates were found from 54 soil samples (Table 1) by baiting method (Fig. 1). Based on their morphological characteristics, they were identified as *Penicillium* sp. (6 isolates), *Cunninghamella* sp. (5 isolates), *Chaetomium* sp. (4 isolates), *Aspergillus* sp. (5 isolates), *Mucor* sp. (6 isolates) and *Trichoderma* sp. (5 isolates). All isolates were screened for enzymatic degradability.

Figure 1. baiting technique using fileter paper pieces to isolate fungi from soil samples, A= baiting tyechnique, B = appearance of degrading fungi, C= fungi growing on filter papers

Table1. Fungal isolates obtained from tea and arabica coffee soil samples

Fungi \ Locations	Thai Nguyen(TN)	Phu Tho(PH)	Yen Bai(YB)	Son La(SL)	Dien Bien(DB)	Lam Don(LD)g	Total
Penicillium	1	1	1	1	1	1	6
Cunninghamella	0	2	0	2	1		5
Chaetomium	0	2	0	1	1		4
Aspergillus	1	1	1	1	0	1	5
Mucor	2	1	1	1	1		6
Trichoderma	0	2	1	1	1		5
Total	4	9	4	7	5	2	31

Determination of enzymatic degradability

Cellulase degrading ability: As result, *Cunninghamella* SL2 and *Mucor* PT1 gave highest significantly produced cellulase which clear zone were 22.5 mm., and followed by other species eg *Chaetomium*, *Penicillium* and *Aspergillus* which the clear zone varied, except for *Trichoderma* PT1, *Mucor* SL1 were not produced cellulase.

Xylanase degrading ability: *Trichoderma* DB and *Cunninghamella* SL2 expressed xylanase activity which highest clear zones of 23.5 and 24.4 mm., respectively. All isolates of tested *Chaetomium*, *Cunninghamella*, *Mucor*, *Penicillium* and *Aspergillus* produced xylanase except for *Mucor* SL1.

Ligninase degrading ability: *Chaetomium* PT2 and *Penicillium* TN1 showed significantly highest clear zones of 24.7 and 23.6 mm., respectively. Trichoderma YB1 *Cunninghamella* SL2, *Penicillium* LD1, *Mucor* TN, *Aspergillus* LD1were not produced ligninase. It noted that all tested isolates of *Chaetomium* produced ligninase.

It concluded that 6 isolates, *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghamella* SL2, *Penicillum* TN1, *Aspergillus*PT were selected and all tested isolates produced cellulase, xylanase, and ligninase to degrade organic materials (Fig. 2, 3 and 4). Those isolates were proved for synergistic action to each other and mixed to sterilized china clay to be bio-decomposer and used to produce organic biofertilizer product (BFP).

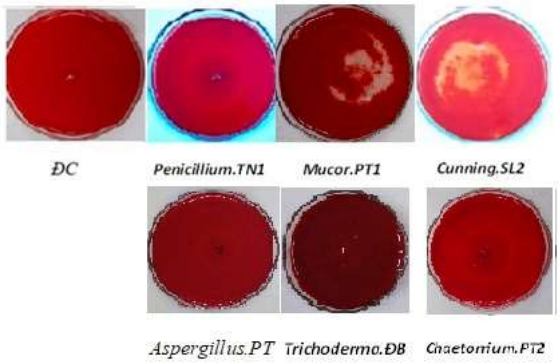

Figure 2. Cellulase degrading activities of fungal isolates, DC = Control

Table 2. Enzymatic activities testing for different saprophytic fungi

Enzyme / Isolates	Cellulase (mm)	Xylanase (mm)	Ligninase (mm)
*Chaetomium*PT2	10.5^{i*}	21.2^{d}	24.7^{a}
*Chaetomium*PT1	10.2^{i}	18.1^{h}	21.3^{ab}
*Chaetomium*SL1	9.8^{ij}	18.5^{fg}	21.4^{ab}
*Chaetomium*DB1	$10,1^{ij}$	18.3^{gh}	21.4^{ab}
*Trichoderma*DB	14.5^{e}	23.5^{a}	19.6^{bc}
Trichoderma PT1	0^{p}	13.2^{kl}	12.3^{fgh}
Trichoderma PT2	13.5^{g}	13.5^{k}	12.4^{fgh}
*Trichoderma*YB1	17.5^{b}	15.8^{i}	0^{j}
Trichoderma SL1	13.5^{f}	15.9^{i}	10.5^{h}
Cunninghamella SL2	22.5^{a}	24.4^{a}	0^{j}
Cunninghamella SL1	18.5^{c}	3.5^{q}	3.5^{i}
*Cunninghamella*PT1	16.5^{e}	2.7^{r}	0^{j}
Cunninghamella PT2	19.5^{b}	2.8^{r}	3.2^{ij}
Cunninghamella DB1	17.5^{b}	2.9^{r}	4.8^{i}
Penicillium TN1	12.5^{h}	18.7^{f}	23.6^{a}
Penicillium PT1	7.5^{l}	4.1^{p}	4.7^{i}
Penicillium YB1	3.5^{n}	4.6^{p}	14.5^{fgh}
Penicillium SL1	7.5^{l}	5.4^{n}	6.2^{i}
Penicillium DB1	9.5^{ij}	12.5^{l}	18.7^{bcd}
*Penicillium*LD1	4.5^{n}	13.6^{j}	0^{j}
Mucor PT1	22.5^{a}	23.5^{b}	21.25^{b}
Mucor TN1	7.5^{l}	3.98^{q}	0^{j}
Mucor TN2	7.5^{l}	3.54^{r}	11.5^{gh}
Mucor YB1	9.5^{j}	4.67^{o}	10.7^{h}
Mucor SL1	0^{p}	0^{t}	15.6^{def}
Mucor DB1	7.5^{l}	20.5^{e}	14.3^{efg}
Aspergillus TN1	7.5^{l}	15.7^{i}	14.2^{efg}
Aspergillus PT	5.2^{m}	22.3^{c}	23.5^{a}
Aspergillus YB1	10.5^{i}	15.7^{i}	18.7^{bc}
Aspergillus SL1	7.4^{l}	14.2^{j}	16.6^{cde}
Aspergillus LD1	7.9^{k}	11.5^{m}	0^{j}
CV(%)	33.68	21.81	20.45

*Averagec of four replications, Means followed by common letters are not significantly differed by DMRT at P=0.01.

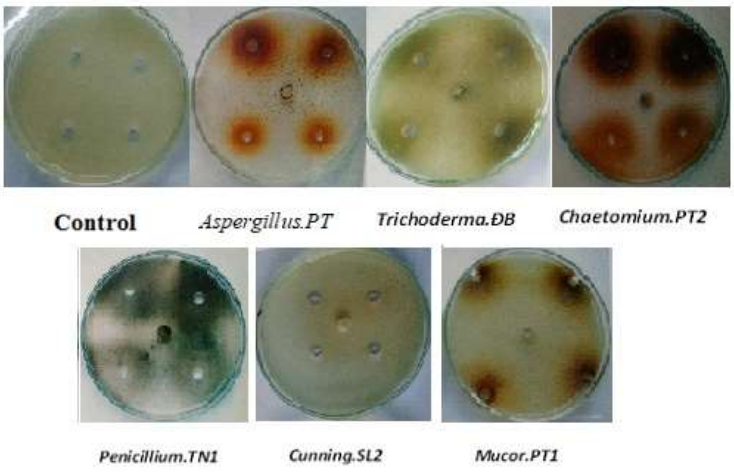

Figure 3. Cellulase degrading activities of fungal isolates

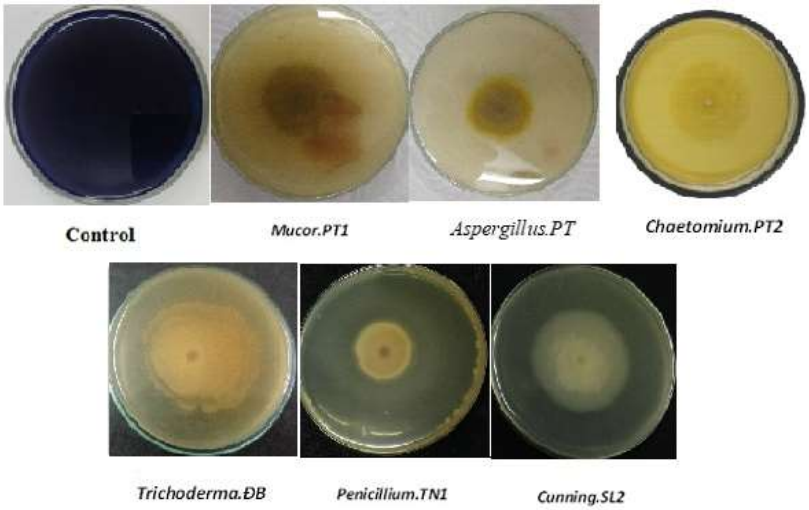

Figure 4. Xylanase degrading activities of fungal isolates

Application of biofertilizer product for the growth of tea and arabica coffee in greenhouse

The biofertilizer product developed from six effective isolates of enzymatic producing fungi, *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghameela* SL2, *Penicillum* TN1, and *Aspergillus* PT) were tested in tea and Arabica coffee for six months.

The coffee plants increased significantly for their growth parameters as compared to the non-treated control. The developed biofertiliozer product sifnificantly affected in the plant heigh, root weigh and plant weigh of Arabica coffee plants which were ranged from 50.67 to 55.83 cm/plant, 7.8 – 9.2 g/plant and 75.5 – 100.9 g/plant, when compared to the non-treated control which were 45.7cm/plant, 5.6 g/plant and 68.9 g/plant, respectively. In addition, there was significantly highest in the growth parameters of Arabica coffee plants at application rates of 1.5 and 2.0 g/plant when compared to the non-treated control. Furthermore, the biofertilizer product also showed good effects on soil when it increased significantly the soil organic meter from averaged 2 % to 4 % as compared to the control. The fungal density in soil planted tea was not cleary resulted due to many factors in the planted soil (Table 3).

Similarly, the biofertilizer showed good effects on tea plants. All tea plants treated with the biofertilizer gave significantly greater plant heigh, root and plant weigh as compared to the non-treated plants. The biofertilizer product significantly increased organic matter averaged from 2.19 to 4.56 %. and microorganism density of soil. The fungal density in soil planted tea was not cleary demonstrated due to many factors in the planted soil and also technical investigation (Table 3 and 4).

Table 3. Effects of biofertilzer product on Arabica coffee plants for 6 mionths in greenhouse

Parameters / Treatments	Plant heigh (cm/plant)	Root weigh (g/ plant)	Plant weigh (g/ plant)	Organic meter of soil (%)	Fungal density (CFU/g soil)
1 g BFP/plant	50.67^{b*}	7.80^{b}	75.50^{b}	4.21^{a}	8.60×10^{3c}
1.5 g BFP/plant	55.16^{a}	8.60^{a}	98.20^{a}	4.36^{a}	1.33×10^{4b}
2.0 g BFP/plant	55.83^{a}	9.20^{a}	100.90^{a}	4.42^{a}	3.63×10^{4a}
control	45.66^{c}	5.60^{c}	68.93^{c}	2.15^{b}	5.26×10^{3d}
CV(%)	11.32	19.10	14.35	22.36	13.19

*Averagec of four replications, Means followed by common letters are not significantly differed by DMRT at P=0.01.

Table 4. Effects of the biofertilzer product on tea plants in greenhouse after treated for six months

Parameters / Treatments	Plant heigh (cm/plant)	Root weigh(g/plant)	Plant weigh (g/plant)	Organic meter of soil (%)	fungal density (CFU/g soil)
1 g BFP/plant	50.66^{b*}	4.5^{b}	18.5^{b}	4.54^{a}	8.13×10^{3c}
1.5 g BFP/plant	54.5^{a}	5.1^{ab}	21.5^{ab}	4.61^{a}	1.23×10^{4b}
2.0 g BFP/plant	54.66^{a}	5.9^{a}	22.3^{a}	$4,56^{a}$	2.56×10^{4a}
control	37.55	2.3^{c}	8.5^{c}	2.19^{b}	4.5×10^{3d}
CV(%)	14.82	22.69	15.58	26.75	28.78

*Averagec of four replications, Means followed by common letters are not significantly differed by DMRT at P=0.01.

Discussion

As results, *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghamella* SL2, *Penicillum* TN1, *Aspergillus* PT were proved to produce cellulase, xylanase, and ligninase to degrade organic materials. The biofetilizer product based on enzymatic fungi investigation was produced to be an organic biofertilizer product (BFP). Some research findings were also reported to screened the effective microorganism to degrade organic materials and produced biofertilizers. Zhang, *et al.* (2013) found that *Trichoderma reesei* Rut C-30 had ability to produce cellulase and xylanase and indicated that the cultures degraded corn stoves. Our results are similar to previous investigation as reported by Herman, *et al.* (2019) that *Chaetomium globosum* was highly effective organic-degrading enzyme. *Cunninghamella* spp. could also produce exoglucanase, endoglucanase and *β*-glucosidase with strong bioactivities. Moreover, Kumar *et al.* (2018)

stated that some strains of *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. could degrade strongly compositons of wood such as cellulose, lignin, xylan which similar to the work of Yeoh, *et al.* (1984) who demonstrated cellulolytic enzymes production from several fungi isolated from wood materials anf gave positive result. The reserch finding in this project revcealed that *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghamella* SL2, *Penicillum* TN1, *Aspergillus* PT gave high positive potential to degrade organic materials and developed to be biofertilizer to improve soil planted to tea and arabica coffee plants. Withthis, Glory, *et al.* (1998) found that the biofertilizer containing beneficial microorganisms can be increased in plant heigh, root and plant weigh of coffee plants under greenhouse conditions. As similar to‒ Nguyen, *et al.* (2016) demonstrated that the application of biofertilizer product could strongly increase in organic meter and microorganism density in cultivated soil.

The research finding demonstrated that biofertilizer product which *Chaetomium* PT2, *Trichoderma* DB, *Mucor* PT1, *Cunninghamella* SL2, *Penicillum* TN1, *Aspergillus* PT showed good effects on the growth of tea and coffee plants and also improved soil fertility with high organic matter. The biofertilizer product may appropriate to produce in mass production and extension to the tea and coffee growers in Vietnam.

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