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Inoculation Technology for *Trichoderma harzianum* during Interaction with Oil Palm *Elaeis guineensis* Jacq.

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Plant growth promoting microorganisms are turning their attention to roots to increase yields without causing environmental damage. Those strategies increased need for environmentally friendly agricultural practices to use of fertilizers based on growth promoting microorganisms. The intensive period of research and development started on the interactions between plant and the different growth promoting microorganisms and enhanced agriculture production through the use of bio-fertilizers. The beneficial effect of *Trichoderma* species is based on their potential for plant growth promotion that makes them useful as bio-fertilizer. Here we show that oil palm empty fruit bunch significantly ($P \leq 0.0001$) improved the effectiveness and consistency of *Trichoderma harzianum* inocula during interaction with oil palm. Our inoculation technology for *T. harzianum* production and of the carrier for the interaction with oil palm was successful to their application and enhanced plant growth.

Keywords: Bio-fertilizer, Empty fruit bunches, Plant growth promoting microorganisms.

Oil palm is the most productive and economic oil crop in the world. Palm oil is largest industry sectors contributing to the world's production of oils and fat. The main commercial product of oil palm plantations is oil obtained from the fruit of the palm tree. The major solid wastes from fresh fruit bunches of oil palm after extracting oil are different types of residues including empty fruit bunch (EFB), mesocarp fiber and kernel shell, which are considered to be harmful wastes to the environment if released untreated[1-4]. Approximately 1.07 ton of oil palm EFB waste is generated with every ton of palm oil production[5]. There are many studies going on using these wastes in various industrial enterprises and agricultural activities such as bio-fuels, lumber for wood, feed for animals, shells as activated carbon for water purification. Most of the oil palm mills are used the oil palm residues as fuels in co-generation process of their boiler. Nowadays, the oil palm EFB is used as bio-fertilizer by burning them into ash, which is rich in potassium, and as mulch in the field to control weeds, maintain moisture and prevent soil erosion[1-4,6-8]. The EFB contains about 40–50% cellulose, 20–30% hemicellulose, and 20–30% lignin with moisture content of about 10–50%, depending on plant age and growth conditions, soil conditions, weather effect, and testing methods used. Other components such as pectins and waxes are also present in minor quantities[8-10].

Numerous reports describe how the plant growth promoting fungus, *Trichoderma* produced beneficial effects on plant growth[11,12]. *Trichoderma*

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species are oil palm growth promoters with potential for the bio-control of basal stem rot (BSR)[13,14]. The development of techniques for the production of *Trichoderma* inoculants, with high infectivity potential, is the main issue to be tackled in order to allow a wide use of bio-fertilizers. Lignocellulolytic microrganisms are capable to depolymerizing the lignin barrier in organic materials. Fungi are well known with its ability to metabolize wood and produced an array of cellulolytic and hemicellulolytic activities. Hence, *Trichoderma harzianum* are able to degrade lignocellulose efficiently because they are filamentous and have prolific spore production ability that enable them to attack to woody materials quickly[8,11,14], EFB is easy-to-use and economical carrier material for *Trichoderma* inoculants production. Several types of carriers have been used with success under experimental conditions, for example, clay powder (pyrax) mixed with fermenter biomass, alginate pellets, vermiculite-bran mixtures and manure[15].

The present paper is focusing effects of inoculum carrier on survival of *T. harzianum* during interaction with oil palm *Elaeis guineensis* Jacq., that has improved the effectiveness and consistency of microbial inocula.

MATERIALS AND METHODS

Stocks

Four-month-old of *Elaeis guineensis* Jacq. cv. Tenera (Dura × Pisifera) used in this study were provided by Sime Darby, Golden Hope Plantation, Banting, Malaysia. The culture of *Trichoderma harzianum* FA 1132 (IMI: 375050) isolated from oil palm was obtained from the Mycology Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia.

Fungal treatment and growth conditions

The *T. harzianum* inoculum used in the experiment contained 1.9×10^9 conidia/mL. *T. harzianum* inoculum were prepared by flooding seven-day-old cultures on Potato dextrose agar (Difco Laboratories, Detroit, Michigan, USA) with 100 mg/L streptomycin sulfate with 10 mL of ddH_2O and scraping with a sterilized L-shaped glass rod as described[16]. *T. harzianum* inoculum carrier, the oil palm EFB, were obtained from Seri Ulu Langat Palm Oil Mill in Dengkil, Selangor, Malaysia. The EFB were washed, air dried and 50 g of EFB placed in heat resistant transparent polyethylene bag and sterilized. The EFB carrier was inoculated with *T. harzianum* inoculum and incubated at 28 ± 2 °C for 2 weeks in darkness. Uninoculated EFB carrier was used as a negative control. Inoculation of *T. harzianum* treatments were carried out using the method described by Alizadeh e*t al.*[17]. Briefly, 250 g of colonized *T. harzianum* carrier was applied on the pot surface. To each pot, fresh conidial suspension (1 L) was added every 2 weeks. The seedlings were planted on Munchung series soil in ceramic garden pots under normal temperatures and natural lighting and watered twice a day with 200 mL of tap water. Treatments with uninoculated carrier were used as negative control. A simplified representation of inoculation technology for *T. harzianum* during interaction with oil palm seedling is showed in Fig. 1.

Growth assay

For growth assay, plants were separately washed to remove soil particles adhering on them and oven dried at 80 °C till constant weight was achieved. The plants were weighed and biomass accumulation was expressed as g/plant at 0, 3, 7, 21, 42, 63, 84, 105, 126 and 147 dpi.

Quantitative assessment of the density of EFB microfungal community and isolation frequency of *T. harzianum*

The density of microfungal community was determined via viable pour plate counting technique as described[16]. Ten gram of wet EFB was mixed with 100 mL of sterile ddH_2O and shake for 10 minutes at 100 rpm. The samples were diluted serially, plated out onto streptomycin-rose bengal agar and incubated at 28 °C for 4 and again after 7 days, to allow for the development of slow growing fungal colonies. Average colony forming units (CFU)/g from developed colonies were calculated and expressed on an oven-dry weight basis according to Germida and De Freitas[18]: Number of CFU/g EFB_{DW} = (Mean plate count)(Dilution factor)/(Dry weight EFB, initial dilution)

The fungal colonies that were similar in appearance to *T. harzianum* were subcultured on PDA medium amended with streptomycin sulfate. *T. harzianum* was identified[19] and isolation frequency of *T. harzianum* calculated according to Nesci *et al.*[20]: % Isolation frequency = (Number of *T.harzianum* sample)/ (Total number of samples)×100

Evaluation of EFB moisture content

The mass basis moisture content of EFB was determined according to Topp *et al.*[21].

Experimental design and statistical analysis

The treatments were consisted of *T. harzianum* inoculated (T: + plant + fungi), *T. harzianum* uninoculated (TU: + plant – fungi) plants and *T. harzianum* inoculated carrier without the plant (TPC: + carrier + fungi – plant). The two-factorial experiment of three treatment (T) and three replications of *T. harzianum* inoculum carrier (EFB) were conducted in which the replications were nested within each T × EFB combination. The statistical analysis, i.e., the analysis of variance (ANOVA) was performed by the following liner additive model (LAM):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

wherein Y_{ijk} = ijk$_{th}$ observations; α_i = i$_{th}$ treatment effects; β_j = j$_{th}$*T. harzianum* inoculum carrier effects; $(\alpha\beta)_{ij}$ = *ij*$_{th}$T × EFB interactions effects; and ε_{ijk} = ijk$_{th}$error term.

In the above LAM, ε_{ijk} was pooled error term of experimental and sampling errors. The experimental error was the biological replicate nested to T × EFB combinations, while the sampling error was the technical replicates nested within biological replicate × T × EFB. The protected least-significant difference (LSD) test was used for multiple mean comparisons. Statistical analysis was performed using SAS software (version 9.1; SAS Institute Inc.). All experiments were repeated two times to compensate for possible errors. As the results of analysis obtained in the second repeat were the same as the first, results were only shown for the data of the first experiment. The contrast procedure was used to compare between group means.

The *T. harzianum* inoculum carrier (EFB) were aseptically sampled at the same time points and directly assessed to determine density of microfungal community, isolation frequency of *T. harzianum* and moisture content.

RESULTS

Koch's postulates were fulfilled for infection analysis with *T. harzianum*. Growth of *T. harzianum* was investigated quantitatively and qualitatively by percent root colonization and slide culture techniques, respectively. The oil palm root

Table 1. EFB moisture content (%) during interaction of oil palm with *T. harzianum* at 0-147 dpi

Treatments/ Sampling period (dpi)	0	3	7	21	42	63	84	105	126	147	Mean
T	50.16±0.30^a	50.51±0.31^a	50.37±0.31^a	50.07±0.47^a	50.66±0.35^a	49.16±0.34^a	50.29±0.30^a	49.91±0.23^a	50.39±0.43^a	50.39±0.29^a	50.19±0.33
TU	50.25±0.27^a	50.47±0.34^a	50.41±0.37^a	50.09±0.27^a	50.40±0.29^a	49.00±0.33^b	50.31±0.38^a	50.22±0.47^a	50.34±0.19^a	50.29±0.25^a	50.18±0.32
TPC	50.23±0.17^a	49.99±0.21^a	50.02±0.38^a	50.21±0.21^a	50.45±0.32^a	50.12±0.30^b	49.93±0.34^a	49.50±0.33^a	49.35±0.36^b	49.14±0.37^b	49.89±0.30

dpi, days post-inoculation; T, *T. harzianum* inoculated; TU, *T. harzianum* uninoculated; TPC, *T. harzianum* positive control
Data presented as mean ± S.E of three biological replicates and three technical replicates
Different letters indicate significant differences in a column at $P \leq 0.0001$

colonization achieved by *T. harzianum* was 68.33% ± 9.39 at 3 dpi and continued to increase to 90% ± 3.27 at 7 dpi and 100% at 21 until 147 dpi. On the other hand, the percentage of plant root colonization by *T. harzianum* was increased gradually at 0 to 147 dpi from 3.33 ± 1.78 to 28.33 ± 1.09 in *T. harzianum* uninoculated treatment[16].

In order to determine the effect of *T. harzianum* on plant growth, dry and fresh biomass of oil palm seedlings were determined at 0, 63 and 147 dpi. Our data revealed that *T. harzianum* inoculated treatment significantly ($P \leq 0.0001$) increased dry and fresh biomass of oil palm at 63 and 147dpi in compared to uninoculated treatment (Fig. 2).

Our findings indicate that the densities of EFB microfungal community displayed significant variation in different treatments over a time course of 147 days (Fig. 3). Under *T. harzianum* inoculated treatment, density of microfungal community at 147 dpi exhibited the highest value of $15.51 \pm 0.61 \times 10^9$ CFU/g dry weight of EFB. The density in terms of microfungal community in *T. harzianum* inoculated plants increased at 3 dpi, reached a maximum at 42 dpi and thereafter increased gradually between 63 to 147 dpi. The *T. harzianum* uninoculated treatment caused a significant ($P \leq 0.0001$) increase in the density of microfungal community. The *T. harzianum* uninoculated treatment showed a clear increase in the density of the microfungal community from 3 to 147 dpi. Quantification of microfungal community also indicated that the density of microfungal community were maintained

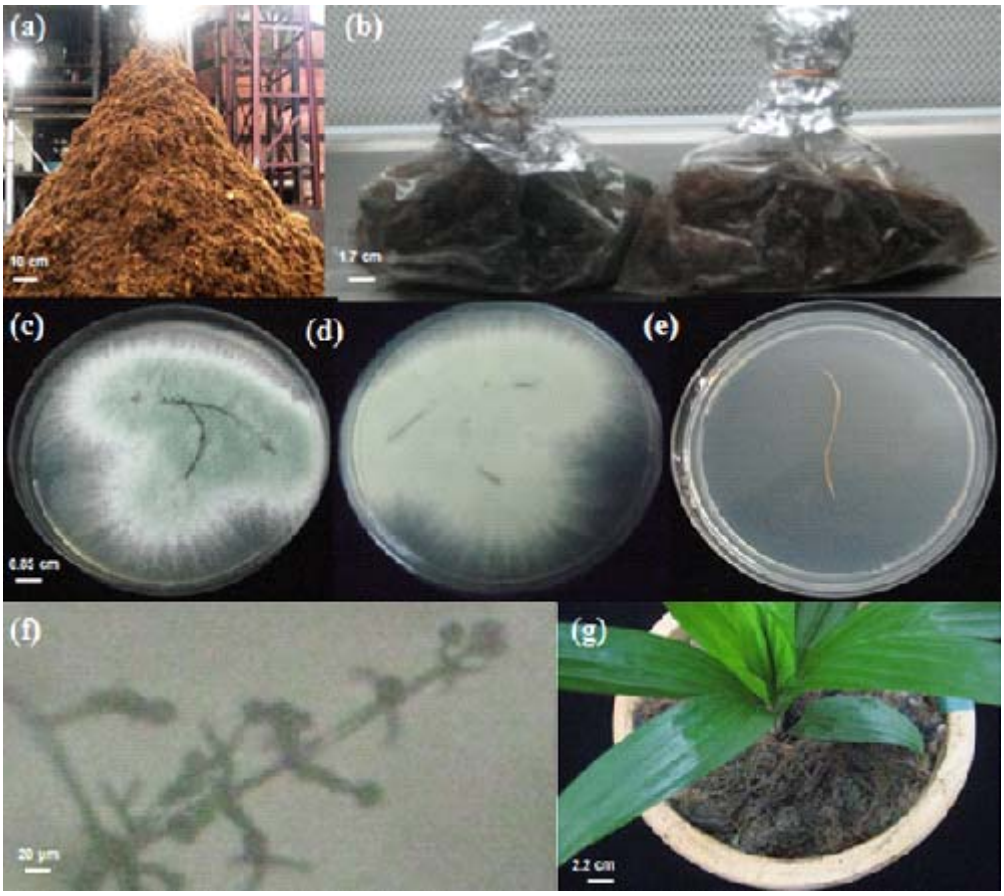

Fig. 1. Simplified representation of inoculation technology for *T. harzianum* during interaction with oil palm seedling. (a) empty fruit bunch (EFB) from Seri Ulu Langat Palm Oil Mill; (b) inoculated EFB carrier with *T. harzianum* and uninoculated EFB carrier; (c, d) *T. harzianum* on PDA amended with streptomycin sulfate isolated from inoculated EFB; (e) uninoculated EFB on PDA; (f) mycelium and spores of *T. harzianum* observed under light microscope; (g) applied *T. harzianum* inoculum on oil palm seedling.

constant at 0 to 84 dpi and decreased significantly ($P \leq 0.0001$) thereafter at 105 to 147 dpi in the *T. harzianum* positive control. Interestingly, frequency of *T. harzianum* remained 100% at 0 to 147 dpi in *T. harzianum* inoculated treatment. Isolation frequency of *T. harzianum* significantly ($P \leq 0.0001$) increased to approximately 20-60% in *T. harzianum* uninoculated treatments at 3 to 147 dpi. The frequency of *T. harzianum* was decreased to approximately 90% in *T. harzianum* positive control at 3 to 147 dpi (Fig. 4). Our analyses for EFB moisture content indicated that there was no significant difference among *T. harzianum* treatments. The average EFB moisture content was approximately 50% (Table 1).

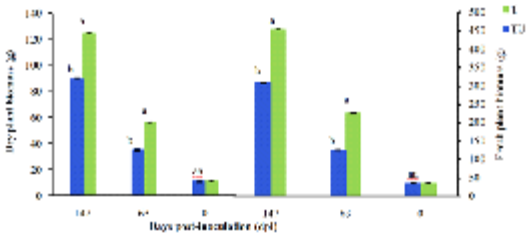

Fig. 2. Quantitative assessment of oil palm (a) dry and (b) fresh biomass during interaction with *T. harzianum* at 0, 63 and 147 dpi. T, *T. harzianum* inoculated; TU, *T. harzianum* uninoculated. Data presented as mean ± S.E of three biological replicates and three technical replicates. Different letters indicate significant differences in each time points at $P \leq 0.0001$.

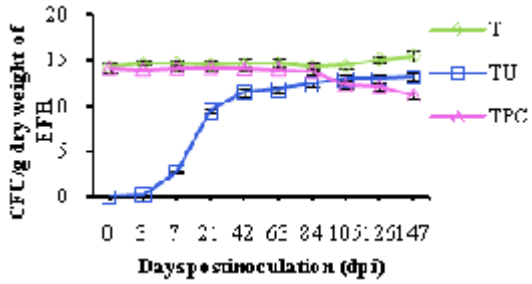

Fig. 3. Mean of CFU/g dry weight of EFB × 10^9 of microfungi during interaction of oil palm with *T. harzianum* at 0 to 147 dpi. T, *T. harzianum* inoculated; TU, *T. harzianum* uninoculated; TPC, *T. harzianum* positive control. Data presented as mean ± S.E of three biological replicates and three technical replicates.

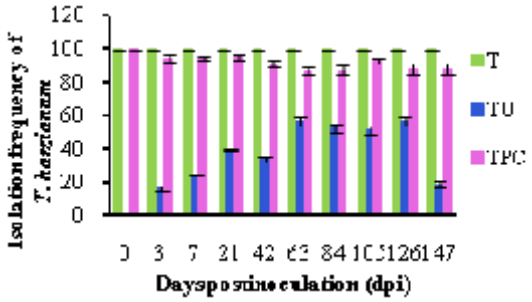

Fig. 4. Isolation frequency of *T. harzianum* in EFB during interaction of oil palm with *T. harzianum* at 0 to 147 dpi.T: *T. harzianum* inoculated; TU, *T. harzianum* uninoculated; TPC, *T. harzianum* positive control. Data presented as mean ± S.E of three biological replicates and three technical replicates. Different letters indicate significant differences in each time points at $P \leq 0.0001$.

DISCUSSION

Bio-fertilizers will be important to improve crop yields without compromising ecological integrity and public health. High-quality inoculants of plant growth promoting microorganisms play crucial role in crop productivity. *Trichoderma* species are best known to confer fitness benefits to oil palm and play a crucial role in improving crop yields[22]. *Trichoderma* species have been used as bio-control agent of BSR diseases in oil palm field because of their ability to mycoparasitism and higher production yield. Preparing inoculants with suitable carrier materials, and distributing viable inoculants to application in field is essential. In the present study we developed inoculant production for *T. harzianum* and effects of inoculum carrier on survival of *T.harzianum* during interaction with oil palm seedlings were investigated.

It is of interest that the *T. harzianum* was the only isolate in inoculated EFB during interaction of oil palm with *T. harzianum*. *Trichoderma* species have potential to produce lignin-degrading enzymes, hemicellulases and cellulases that lead to rapid degradation of lignin, hemicelluloses and cellulose in woody material. Due to their ability to colonize woody material, therefore application of *T. harzianum* on EFB as carrier demonstrated a great potential of *T. harzianum* in colonizing lignocellulosic carbon source[11,14,23]. The rapid growth of *T. harzianum* strongly increased its ability to use EFB in competition with other fungi. *Trichoderma* species can produce or release a series of bioactive metabolites that enhance their colonization and rapid development[11,12,14]. The density of microfungi increased significantly in uninoculated EFB with

approximately 20-60% frequency of *T. harzianum*. The positive effect of media constituents was influential on the microfungal community in uninoculated *T. harzianum* treatments[24]. The density of microfungal community found in the *T. harzianum* positive control also show decline in compare to *T. harzianum* inoculated seedling. Plant root exudates are a source of nutrients and signaling molecules, and also appear to be an important driving force for microbial colonization that stimulates growth[25]. Interaction between soil and oil palm and secondary metabolism of the associated oil palm, suggesting that provide favorable condition for *T. harzianum* in inoculated seedling. The mean EFB moisture content observed in the present study favored fungal growth particularly *T. harzianum*[9].

The quantification analysis of oil palm root for assessment of the *T. harzianum* demonstrates high level of *T. harzianum* with 100% potency to colonize the roots[16]. Inoculation with *T. harzianum* stimulated growth and biomass production in oil palm. *Trichoderma* hyphae attach and colonize the root surface or cortex and penetrate into the epidermis and a few cells below this level. They produce or release a series of bioactive metabolites that induce walling off and biochemical mechanisms in the host plant. *Trichoderma* metabolite mixtures may have a role in both elicitation of defense reactions and growth regulation of plants plant[11,14,26]. *Trichoderma* species enhance plant growth development and productivity. The increased growth response induced by *Trichoderma* species has been reported for many kinds of crops. They increase the uptake and concentration of soil nutrients that resulted in an improvement of plant active-uptake mechanism. The results of these responses have direct effects on plants, decreased activity of deleterious root microflora and inactivated toxic compounds in the root area[11,27].

This work provides insight into the successful technology for *T.harzianum* inoculation production and of the carrier for the interaction with oil palm to their application and enhanced plant growth. As the oil palm is the most commercial agriculture in the production of palm oil, technology for high-quality *T.harzianum* inoculation production, could lead to the design of appropriate strategies to produce palms resistant to *G. boninense* for improved disease control and enhance plant growth and oil levels.

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