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Development of Multifunctional Biofertilizer Formulation from Indigenous Microorganisms and Evaluation of Their N_2-Fixing Capabilities on Chinese Cabbage Using ^{15}N Tracer Technique

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ABSTRACT

Biofertilizer is an alternative to chemical fertilizers to increase soil fertility and crop production in sustainable farming. Most biofertilizer products consist of a single function micro-organism such as N_2 fixing bacteria. This paper discusses the development of multifunctional biofertilizer products, based on indigenous micro-organisms that have all the desired characteristics, including plant growth promoting, phosphate solubilising and antagonistic towards pathogens, and optimisation of the micro-organisms present in the modified "Natural Farming" compost. Composting through the "Natural Farming" method is a simple and cheap method to turn empty fruit bunches (EFB) of oil palm into compost. Indigenous micro-organisms in each stage of composting were isolated and screened for the abilities to solubilise phosphate and produce indole-3-acetic acid (IAA). These indigenous micro-organisms were developed into biofertilizer products. Effects of these products on plant growth of Chinese cabbage and contribution of N_2 to the plants were evaluated using the ^{15}N isotopic tracer technique in a greenhouse trial. Fertilizer treatment using a combination of microbial strains (T7) was found to significantly enhance the growth of Chinese cabbage. All the plants receiving biofertilizer microorganisms showed N_2-fixing effects as compared to the control (T9). The isolated indigenous micro-organisms may enhance plant growth through N_2 fixation, solubilising insoluble inorganic phosphate compounds or hydrolyse organic phosphate to inorganic P or stimulation of plant growth through hormonal action such as produce IAA. Combination of microbial strains could be a good multifunctional biofertilizer for sustainable agriculture.

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INTRODUCTION

Responding to global warming and global challenges in crop production, Malaysia is steadily adopting sustainable agriculture. Agro-waste management and enhancement of biodiversity are the approaches towards sustainability (Shukor, 2009; Ong, 2009). Empty fruit bunches (EFB) of oil palm are one of the agricultural wastes that are building up at alarming rates at palm oil factories in Malaysia. This particular material is difficult to manage if not treated or turned into valuable products like compost. Meanwhile, micro-organisms are an important component of world biodiversity (Sadi *et al*., 2006). These micro-organisms include phosphate solubilisers, plant growth promoters and nitrogen fixing bacteria (Umi Kalsom & Sariah, 2006). Composting through the modified "Natural Farming" method is simple and cheap at turning EFB into compost. Moreover, it is a natural agro-management method, utilising agricultural waste and indigenous soil micro-organisms. This method was developed in Korea (Cho & Kayoma, 1997), and has been gaining acceptance in several countries and in Malaysia, by the Department of Agriculture. This method involves five stages of composting processes producing inoculants of indigenous microorganisms (IMO). Biofertilizer is a substance containing living micro-organisms, which are applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant, and promote growth by increasing the supply or the availability of primary nutrients to host plants (Vessey, 2003). The objectives of the present study were to isolate and utilise the indigenous micro-organisms from each stage of the composting process to produce bacterial isolates that could be developed as multifunctional biofertilizer micro-organisms. The abilities of the indigenous micro-organisms to solubilise phosphate and produce indole-3-acetic acid (IAA) were evaluated. The potential micro-organisms were selected for development of biofertilizer. The micro-organisms were further evaluated on their ability to fix N_2. The effects of these products on the plant growth of Chinese cabbage and the contribution of N_2 using the ^{15}N isotopic tracer technique in a greenhouse trial were particularly studied.

MATERIALS AND METHODS

IMO Preparation

Composting of EFB, through the modified "Natural Farming" method (Wahid, 2005; Cho and Kayoma, 1997), was conducted as follows: EFB was dried, ground and mixed with composting agents - rice, bran and sugarcane molasses through five stages of composting processes producing inoculates of indigenous microorganisms (IMO). Rice was packed and fermented for 2 days (IMO 1). IMO 1 was then mixed with sugarcane molasses and fermented for a week (IMO 2). IMO 2 was mixed with 1 L of water and 8 kg bran, and incubated for 5 days (IMO 3). After 5 days, IMO 3 was mixed with soil and incubated for 5 days (IMO4). Finally, 200 kg griddled EFB (60% moisture content) was mixed with IMO 4 and fermented for 2 weeks. In each stage, indigenous micro-organisms were isolated by using ten-fold serial dilution technique.

IAA Production

The isolates were tested for indole-3-acetic acid (IAA) production by culturing on TSA amended with 1-tryptophan, followed by overlying them with 82 mm diameter nitrate cellulose membrane and incubating at 28 °C for 3 days. The membranes were overlaid on a filter paper saturated with Salkowsky's reagent (Gordon & Webber, 1950; Alvarez *et al*., 1995). Isolates producing IAA showed pink to red colour after 0.5 to 3 hours. The isolates were also tested for their ability to solubilise phosphate.

Phosphate Solubilising Test

In the phosphate solubilising test, the isolates were cultured on phosphate agar plate (Freitas *et al.*, 1997) and incubated for 14 days. The isolates which produced clear zones were selected and developed into biofertilizer products.

Greenhouse Study of Biofertilizer for N_2-fixing Capabilities and Plant Growth

Biofertilizers were prepared by culturing three selected isolates viz. AP1, AP2 and AP3 on tryptic soy broth for 24 hours. These isolates were individually mixed and in combination with the sterile carrier irradiated by gamma process (Phua *et al*., 2009). The effectiveness study of these products on the growth of Chinese cabbages was carried out in the greenhouse (Table 1). The N_2-fixing activity assessment was carried out using ^{15}N dilution method. A week before transplanting, 0.1 g of ^{15}N labelled ammonium sulphate (10.18 % atom excess) was mixed with 1 kg of soil (FNCA, 2006). Two-week-old seedlings were transplanted into pots containing 1 kg of soil mixture containing soil, peat and sand in the ratio of 2:1:1. Crops were harvested after two months, and their dry weights were also determined. The abundance of ^{15}N in the samples was determined by emission spectrometry after Kjeldahl digestion and titration of digests. The percentages of N derived from labelled fertilizer (%Ndff), atmosphere (%Ndfa) and soil (%Ndfs) were calculated by using the following equations:

$$\% \text{ Ndff} = \{^{15}\text{nae}/^{15}\text{N } (10.18)\} \times 100 \%$$

$$\% \text{ Ndfa} = \{1- (\text{ndff treatment}/\text{ndff control})\} \times 100\%$$

$$\% \text{ Ndfs} = 100 - \% \text{ ndff} - \% \text{ ndfa}$$

Data were analyzed by ANOVA, with the means separated by Duncan's test ($P \leq 0.05$).

TABLE 1
Treatments for greenhouse experiment

Treatments	
T1	AP1
T2	AP2
T3	AP3
T4	AP1 + AP2
T5	AP1+AP3
T6	AP2+AP3
T7	AP1+AP2+AP3
T8	NF
T9	Control

Key:
AP1 = Phosphate solubilise and antagonistic micro-organisms against Bacterial Wilt
AP2 = Plant growth promoter and phosphate solubilise
AP3 = Phosphate solubilise
NF = Natural Farming Compost
Control = Receiving ^{15}N only

RESULTS AND DISCUSSION

"Natural Farming" composting method is a simple and cheap method for the production of EFB compost. The compost became matured within one and a half months, while other composting methods take three to four months. A total of 56 indigenous micro-organisms were isolated from the five stages of IMO. There were 8, 11, 13, 13 and 11 indigenous micro-organisms isolated from IMO 1, IMO 2, IMO 3, IMO 4 and IMO 5, respectively. Sixteen of the bacterial isolates were Gram positive and others were Gram negative. The result derived from the IAA test showed that two isolates were IAA producers and six isolates produced clear zones on phosphate agar plates indicating phosphate solubilising activity. These isolates had been developed into biofertilizers.

The greenhouse experiment for the evaluation of the effect of biofertilizers on plant growth showed that all the treated plants significantly increased ($p < 0.05$) the dry weights of the test plants compared to the control (T9). Meanwhile, the treatment using a combination of microbial strains (T7) was found to have significantly enhanced the growth of Chinese cabbage (Fig.1). The combination treatments showed better results as compared to the single treatments. Han *et al.* (2006) also showed that the combined treatment of *Bacillus megaterium* var. *phosphaticum* and *Bacillus mucilaginosus* increased the availability phosphorus and potassium in soil, and thus, increasing the uptake and plant growth of pepper and cucumber. Sarma *et al*. (2009) reported a combination bio-inoculation, namely, two *Fluorescent pseudomonas* strains, increased *Vigna mungo* yield by 300% in comparison to the control crop. These results indicated that a combination of beneficial micro-organisms might increase the nutritional assimilation of plant and total N in soil.

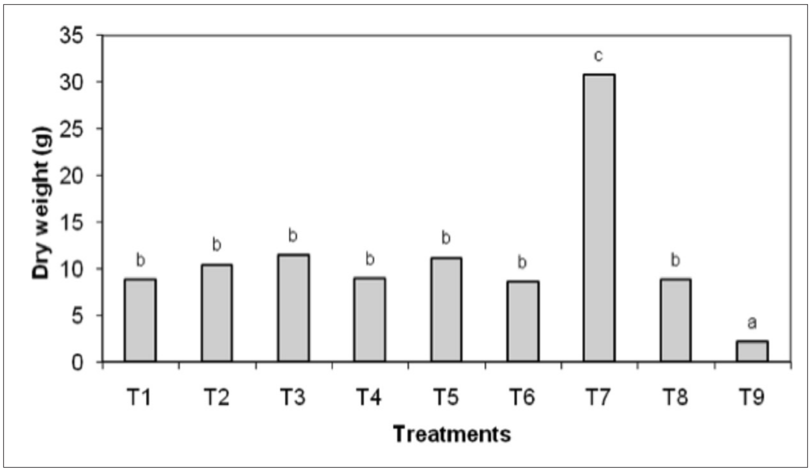
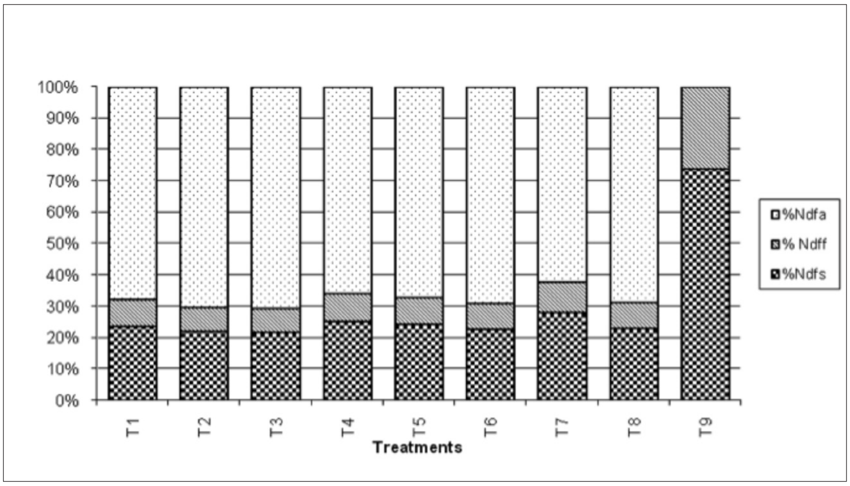


Fig.1: Dry weights (g) of Chinese Cabbage



Keys: % Ndfs = % N derived from the soil
% Ndff = % N derived from the labelled fertilizer
% Ndfa = % N derived from the atmosphere

Fig.2: Effects of biofertilizer treatments in contributing N from N_2 to Chinese cabbage plants by using ^{15}N isotopic tracer

Fig.2 illustrates that all the treated plants have the N_2-fixing effects as compared to the control (T9). It seems probable that there is an influence of these phosphate solibilising bacteria on N uptake. Previous reports have shown the influences of phosphate solubilising micro-organisms on nitrogen uptake and root zone biodiversity. Linu *et al*. (2009) showed that phosphate solubilise improved nodulation, root and shoot biomass, straw and grain yield and phosphorus and nitrogen uptake of cowpea. Similarly, Kuey *et al.* (1989) also reported phosphate solubilise helped increase the ability of accumulated phosphate, efficiency of biological nitrogen fixation and increase the availability of Fe, Zn etc., through production of plant growth promoting substances. Therefore, the isolated indigenous microorganisms may enhance the plant growth through N_2 fixation, solubilising insoluble inorganic phosphate compounds, hydrolyse organic phosphate to inorganic P or stimulation of plant growth through hormonal action such as produce IAA. Combination of microbial strains could be a good multifunctional biofertilizer for sustainable agriculture.

CONCLUSION

The modified “Natural Farming” composting method is a simple, cheap and fast method used to produce EFB compost that contains beneficial micro-organisms with the potential to be developed into biofertilizer. Meanwhile, multifunctional biofertilizer products produced from the combination of indigenous micro-organisms have been shown to enhance the growth of Chinese cabbage and contribute N through fixation of atmospheric N_2 by the micro-organisms in a greenhouse trial.

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