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EFFECT OF MICROBIAL FORMULATION USING PHOSPHATE SOLUBILIZING BACTERIA ON THE FRESH WEIGHT OF COCOA SEEDLINGS AND LEAF NUTRIENTS

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INTRODUCTION

Development of bioinoculant formulation requires evaluation on the effectiveness of the formulation on the crops. The efficacy of formulation using phosphate-solubilizing bacteria has to be evaluated to prove that the formulation has the functionality when applied to soils for phosphorus uptake by the plants. Evaluation of bioinoculant formulation is always carried out either in field trial or pot trial in greenhouse or growth chamber under controlled conditions which the formulation is applied to the soils planted with tested crop (Esitken *et al*., 2003; Han and Lee, 2005; Emine *et al*., 2006; Hameeda *et al*., 2008; Patel *et al*., 2008; Linu *et al*., 2009; Mishra *et al*., 2011; Dastager *et al*., 2010; Castagno *et al*., 2011). Successful formulation of bioinoculant can be proven by the significant growth of crop parameters such as fresh biomass, root, stem, leaf and yield due to the ability of formulation of bioinoculant, which can add, fix, mobilize or solubilize the nutrients in the form which can be easily and quickly absorbed by plants. Besides, bioinoculant formulations can be considered effective on their applications when they are capable of multiplying in soil rhizosphere and benefiting the growing crops. Furthermore, application of the formulation when the soil conditions are favorable, the added bacterial populations are built up in the rhizosphere of plants. They are also able to function in mobilizing nutrients to the crops. In this case, they are capable of solubilizing insoluble phosphate and make it available to the crops. Normally, the successful formulation benefits the crops by increasing up to 20% of germination, improved seedling emergence and growth, increase yield from 10 to 40%, improves the quality of fruit, saving of 25 to 35% inorganic fertilizer, increase the availability and uptake of N and P in plants. Other benefits that formulation can work properly when the population of beneficial microorganisms in the soil increase nutrient retention and availability and leads to improve yield and growth, improve nitrogen and phosphorus fertilizer efficiency. It is safe to handle and easy to apply, leaves no harmful residues in plants or soil. The efficiency of formulation can also be noted when they can suppress detrimental and pathogenic soil microorganism, ability to decompose waste matter and produce organic manure. They are also compatible with organic manures, fertilizers, agrochemicals non-polluting and environmentally friendly. The capability of producing plant growth promoting substances is also one of the features of an efficient formulation. Application of bioinoculants from a wide range of bacterial genera of the PSB demonstrated significant increase in the growth of many crops such as raspberry (Emine *et al*., 2006), eggplant (Han and Lee, 2005), sugarcane

(Patel *et al*., 2008), cowpea (Linu *et al*., 2009; Dastager *et al*., 2010), maize (Mishra *et al*., 2011; Hameeda *et al*., 2008) and chickpea (Castagno *et al*., 2011). The application of bioinoculant formulation increased the population of the bioinoculant in the rhizosphere of crops and increased the availability of nutrients in the soil as well as the nutrient uptake in the plants.

METHODS

The experiment was carried out in a greenhouse at Cocoa Research and Development Centre, Malaysian Cocoa Board, Miles 10, Apas Road, Tawau, Sabah which located at latitude 4°15'43.52"N and longitude 118° 00'47.50"E. Non-sterile of low nutrient soil from the top of 15 cm of a soil from the Table Series was collected from QL Farms Sdn Bhd, Quoin Hill, Tawau, Sabah. This soil is a member of the Table Family which is very fine, oxidic, isohyperthermic, brown Tipik Tempalemoks. It typifies this family and is developed over basalts (basic igneous rocks). Soils of the Table Series are characterized by their deep heavy clay textured oxic horizons with dark yellowish brown colours and an ECEC that is more than 1.5 cmol (+) kg^{-1} clay in all horizons between 25 to 100 cm depth. Structures are weak, medium to fine subangular blocky and consistence is friable. The soil was dried, sieved (2 mm), and used in a pot study. The bulked soil sample was also analyzed for nutrient contents prior to the experiment. Eight kilograms of soil were put into clean plastic pots, which were thoroughly cleaned by rubbing using a clean gauge cloth, which dipped in 70% ethanol. The pots were arranged in the experimental design layout. Cocoa seeds from open-pollinated seed garden from UIT1 x NA33 were surfaced sterilized for 15 min using 70% ethanol. Then, the seeds were put into a double layer of sterilized moist gunnysacks for germination. After two days, three germinated seeds were transferred into the pots. The pots were watered at field capacity during the course of experiment. After one month of sowing, all seedlings were thinned out and one healthy seedling was retained in the pots.

A randomized complete block design (RCBD) consisted of 15 treatments with 4 replications was used as experimental design (Figure 6.1 and Appendice A. 6.1)**.** Each treatment consisted of 6 seedlings in all blocks. The treatments were T1, Control (uninoculated bacteria and no fertilizer application); T2, Standard NPK blue 12:12:17:2 + TE ; T3, NKP; T4, NKP + Formulation of CPH and *Pseudomonas aeruginosa* strain AGKT1; T5, NKP + Formulation of CPH and *Serratia marcescens* strain AGKT4; T6, NKP + Formulation of CPH and *Bacillus amyloliquefaciens*; T7, NKP + Formulation of CBS and *Pseudomonas aeruginosa* strain AGKT1; T8, NKP + Formulation of CBS and *Serratia marcescens* strain AGKT4; T9, NKP + Formulation of CBS and *Bacillus amyloliquefaciens*; T10, KP + Formulation of peat moss and *Pseudomonas aeruginosa* strain AGKT1; T11, NKP + Formulation of peat moss and *Serratia marcescens* strain AGKT4; T12, NKP + Formulation of peat moss and *Bacillus amyloliquefaciens*; T13, NKP + Formulation of liquid *Pseudomonas aeruginosa* strain AGKT1 (M1); T14, NKP + Formulation of liquid *Serratia marcescens* strain AGKT4 (M2); T15, NKP + Formulation of liquid *Bacillus amyloliquefaciens* (M3). The treatments were the combination of the bacterial formulations and fertilizers (Urea as Nitrogen source and KCl as potassium source and $Ca_3(PO_4)_2$) with exception of treatments T1, T2 and T3. Application of N was carried out using Urea, P using $Ca_3(PO_4)_2$ and K using KCl at the rate of 1.38 g N/plant, 0.56 g P/plant and 0.45 g K/plant, respectively. Fertilizers of N and K were applied at fortnightly intervals starting from 30 DAS up to the fifth month, while P was applied after 30 DAS as the only one-application. Treatment T1 was served as the control without any application of fertilizer. Treatment T2 was the standard nursery fertilizer application using NPK blue 12:12:12+ TE which was applied at the rate

of 10 g/plant split to fortnightly intervals as recommended by Teoh (1980). Treatment T3 consisted of fertilizer from N and K source at the same rate as in treatments, T4 to T15. Treatments of T4 to T12, dry bioinoculant formulations were applied at 37.5 g/plant once (≈10^{14} cfu/g), near the root zones of cocoa seedlings in a circular furrow of 2 cm in depth and 5 cm apart from the basal stem of cocoa seedling.

Treatments of T13 to T15, which were liquid bioinoculant formulations, applied at 25 mL/plant (≈10^{14} cfu/mL) at monthly intervals and applied near the root zones of cocoa seedlings, 5 cm apart from the basal stem. All pots were also amended with lime using CaCO at the rate of 6 g/potto raise the pH of soil. Filtered deionized water was used as watering the seedlins in the pots at the field capacity (22%) using ELGA deionized cartridge connected to the water supply. Regular maintenance of pest and disease was carried out. The plants were harvested at twenty weeks (5 months) of planting. The root, stem and leaves were separated and and put into a handmade brown translucent paper (brown onionskin) envelop. Then the root, stem and leaves were weighed. The weight of fresh root was divided by the weight of fresh shoot (the total weight of fresh leaves and stem) to get fresh root:shoot ratio. Leaf analyses for total nitrogen (N), total phosphorus, potassium, calcium, magnesium were determined by the method as described by SIRIM (1980). All data in this study were subjected by analysis of variance (ANOVA) and separation of means by Fisher's least significant difference test (LSD) by using SAS Enterprise Guide 4.1 (4.1.0.1009) and SAS Version 9.1.3.

RESULTS AND DISCUSSION

Effect of treatments on the fresh weight of cocoa seedlings

Table 1 shows that the treatments had the significant effect on the fresh weight of plant components. Treatments of T4, T5, T6, T7 and T8 resulted of significant increase of fresh weight of leaf, stem, root, total fresh of plant as well as shoot as compared to the other treatments. Most of these treatments had low ratio of root:shoot indicating that the effect of treatments was greater on the growth of the upper part of plant than the root growth. This case might explain that most nutrient uptake was on the upper part of vegetative growth, stem and leaves. These treatments were better than the standard nursery fertilizer application. Dry formulation using PSB from *Pseudomonas aeruginosa* strain AGKT1, *Serratia marcescens* strain AGKT4 and *Bacillus amyloliquefaciens* in all carrier materials from cocoa pod husk, cocoa bean shell and peat moss (T4,T5,T6, T7,T8, T9,T10,T11and T12) also resulted in greater effectiveness on fresh weight of plant components as compared to liquid formulations (T13,T14 and T15). Nevertheless, in liquid formulations, all PSB also showed significant different as compared to the control (T1), standard fertilizer application (T2), and straight fertilizer NKP (T3-without PSB). As shown in Figure 1, most of the formulations increased the fresh weight of plant components with at least one parameter of the measurement of plant components as indicated by higher increase on the relative percentage as compared to the control. Mostafa and Abo-Baker (2010) obtained similar results in which biofertilizer consisted of phosphate solubilizing bacteria enhanced fresh dry of sunflower. Most of the formulations increased the fresh weight of plant components with at least one plant component as indicated by higher relative percentage increase of biomass than the control. Similar observation of the plant promotion of maize using *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35 increased 99% and 94% of the plant biomass under glasshouse conditions. Another study also showed that *Gluconacetobacter* sp. and *Burkhoderia* sp. significantly increased shoot biomass of cowpea with the inoculation of these PSB (Linu *et al*., 2009). From this study, it was clearly indicated that the use of the PSB in a few formulations enhanced the fresh biomass of plant components and of cocoa seedlings. The enhancement of the crop

biomass was due to the increase in nutrient uptakes and the availability of nutrients in the soil. Most of the formulations increased the fresh weight of plant components with at least one plant component as indicated by higher relative percentage increase of biomass than the control. Similar observation of the plant promotion of maize using *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35 increased 99% and 94% of the plant biomass under glasshouse conditions (Mishra *et al*., 2011). Another study also showed that *Gluconacetobacter* sp. and *Burkhoderia* sp. significantly increased shoot biomass of cowpea with the inoculation of these PSB (Linu *et al*., 2009). From this study, it was clearly indicated that the use of the PSB in a few formulations enhanced the fresh biomass of plant components of cocoa seedlings. The enhancement of the crop biomass was due to the increase in nutrient uptakes and the availability of nutrients in the soil.

Table 1: The effects of treatments on the fresh weight of leaf, stem, root, total weight, shoot and the ratio of root:shoot of 5 months-old cocoa seedlings.

Treatment	Fresh Weight (g)					
	Leaf	Stem	Root	Total	Shoot	Root:shoot
T1	9.75d	5.71f	7.71e	23.16a	15.45d	0.54a
T2	15.33cd	9.31bcdef	12.54ab	37.195ab	24.64c	0.52a
T3	16.63c	7.66def	7.51e	31.80bcd	24.29cd	0.31c
T4	26.20a	11.64abcd	11.23abcd	49.06cdef	37.84ab	0.30c
T5	24.59ab	12.56abc	9.17cde	46.31cdef	37.15ab	0.25c
T6	19.58bc	11.72abcd	9.59bcde	40.89cde	31.29bc	0.31c
T7	27.76a	12.91ab	12.15abc	52.81ef	40.66a	0.30c
T8	25.73a	15.63a	13.13a	54.488cdef	41.36a	0.32c
T9	28.08a	11.86abc	12.88ab	52.815f	39.94ab	0.33c
T10	18.35c	8.88bcdef	13.74a	40.968cde	27.23c	0.51a
T11	18.75c	7.34ef	9.20cde	35.283f	26.09c	0.36bc
T12	16.96c	9.76bcdef	8.41de	35.128cde	26.72c	0.33c
T13	18.55c	8.97bcdef	9.80bcde	37.318abcd	27.52c	0.35bc
T14	14.95cd	10.51bcde	11.43abcd	36.883ab	25.46c	0.50ab
T15	14.97cd	8.51cdef	11.40abcd	34.875abcd	23.48cd	0.53a
LSD(p≤0.05)	6.57	4.06	3.36	11.26	9.16	0.15

Means followed by the same letter in the same column are not significantly differences according to the Fisher's least significant difference (LSD) test (p≤0.05). The p-value is for the analysis of variance (ANOVA). Each value is the mean of four replicates.

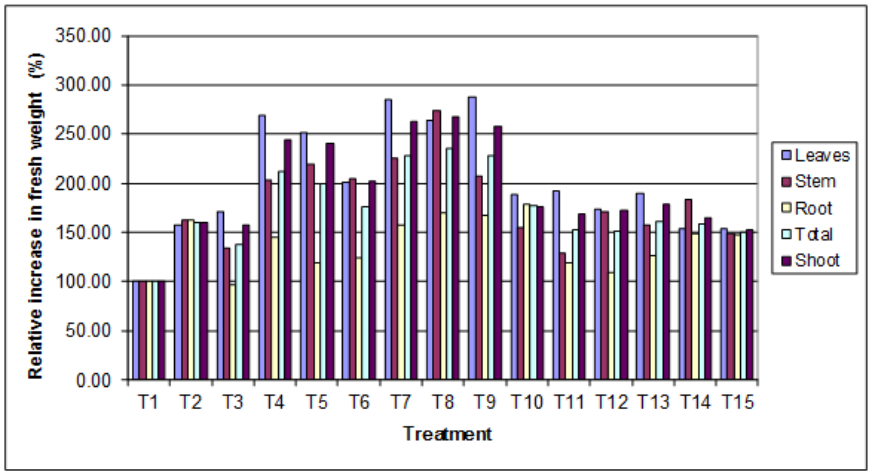

Figure 1. Percentage increase of fresh weight of plant components over the control (T1-no fertilizer application).

Effect of treatments on the leaf nutrients of cocoa seedlings

Effect of treatments on leaf nutrient status is shown in Table 2. The results indicated that the treatments had significant effect on the leaf nutrient status of 5-months old cocoa seedlings. The application of most formulations either dry and liquid formulation significantly increased the nutrient content such as N, P, K, Ca and Mg in the leaves as compared to the control (T1), standard nursery fertilizer application (T2) and straight fertilizer application (T3-uninoculated PSB). The pronounce effect of nutrient uptake in the leaves of 5-month old cocoa seedling can be seen in the treatments of T7, T8, T9 and T12. The results indicated that efficiency of nutrient uptake in N, P, K and Mg were due to the effect and efficacy of these formulations. The effectiveness of these formulations can be compared to the treatment of T3 (NKP-without inoculation of PSB). The present of PSB in the formulations from these treatments significantly increased the nutrients in the leaves. Similar results was observed by Mostafa and Abo-Baker (2010), which indicated that treatments of PSB on sunflower enhanced the nutrient content of N and P in the plant. Numerous research findings indicated that many PSB were capable of enhancing the nutrient uptake by the crops (Emine *et al*., 2006; Han and Lee, 2005; Patel *et al*., 2008; Dastager *et al*., 2010; Linu *et al*., 2009; Mishra *et al*., 2011; Hameeda *et al*., 2008; Castagno *et al*., 2011).

Table 2. The effects of treatments on the nutrient contents in the leaves of 5 months old cocoa seedlings.

Treatment		Nutrient content in leaves (%)				
		N	P	K	Ca	Mg
T1		1.91f	0.08d	0.97h	0.92a	0.29cd
T2		2.11cdef	0.09bcd	1.75efg	0.68bc	0.29cd
T3		2.12cdef	0.10bcd	2.27cde	0.69bc	0.21e
T4		2.23abcd	0.14ab	3.09ab	0.58cd	0.29cd
T5		2.11cdef	0.13abcd	3.25a	0.56d	0.28cd
T6		1.96ef	0.11abcd	3.08ab	0.56d	0.26de
T7		2.36abc	0.14a	3.16ab	0.63bcd	0.38a
T8		2.33abc	0.11abcd	2.73abc	0.67bcd	0.33abc

T9		2.40ab	0.13abc	3.36a	0.73b	0.36ab
T10		2.35abc	0.09bcd	2.23cdef	0.66bcd	0.25de
T11		2.19bcde	0.13abcd	2.53bcd	0.70b	0.31bcd
T12		2.46a	0.13abc	2.26cdef	0.69bc	0.26de
T13		2.12cdef	0.12abcd	1.89defg	0.65bcd	0.26de
T14		2.05def	0.11abcd	1.59fgh	0.72b	0.25de
T15		1.87f	0.09cd	1.48gh	0.68bc	0.28cd
LSD(p≤0.05)		0.27	0.04	0.69	0.12	0.06

Means followed by the same letter in the same column are not significantly differences according to the Fisher's least significant difference (LSD) test (p≤0.05). The p-value is for the analysis of variance (ANOVA). Each value is the mean of four replicates.

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