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Organic Plant Bio-Stimulant for Early, Enhanced and Healthy Growth of Chili Seedlings †

Stella Matthews [1,2,*], Asgar Ali [2], Yasmeen Siddiqui [3] and Christina Vimala Supramaniam [2]

[1] Fertilizer Technology Programme, Soil Science, Water & Fertilizer Research Centre, Malaysia Agriculture Research & Development Institute (MARDI), Selangor, Malaysia
[2] School of Biosciences, Faculty of Science, The University of Nottingham Malaysia Campus, Semenyih 43500, Selangor, D.E., Malaysia; Asgar.Ali@nottingham.edu.my (A.A.); Chiristina.Supramaniam@nottingham.edu.my (C.V.S.)
[3] Laboratory of Plantation Science and Technology, Institute of Plantation Studies, University Putra Malaysia, Serdang 43400, Selangor, Malaysia; yasmeen@upm.edu.my
* Correspondence: stella@mardi.gov.my or stellamatthews1002@gmail.com
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Abstract: This research is aimed to explore natural and organic sources of plant bio-stimulant such as moringa leaves extract (MLE) and endophytic bacteria isolated from moringa roots to promote seed germination and seedling growth of chili. Various extraction methods using methanol, ethanol and distilled water were compared to select the best extraction method based on phytochemical properties that could help to alleviate abiotic stresses and enhance root growth. Pre-screened endophytic bacteria with plant growth promoting traits were also tested for its ability to increase the growth of chili seedlings. The effects of moringa leaves extract and endophytic bacteria as bio-stimulant on chili seedlings were determined using plant physiological analyses.

Keywords: sustainable; agriculture; environmentally friendly; plant growth promotion; nutrient use efficiency; green technology; safe for environment

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1. Introduction

The growth and productivity of plants mainly depend on the available nutrients and its uptake by plant roots. Bio-stimulant could amend plant's physiological processes to improve the nutrient absorption and optimize its consumption [1]. Research on the potential new sources of plant bio-stimulant to increase crop yield and quality despite of environmental stresses has gained a wide attention recently. Plant bio-stimulant that boosts nutrient absorption and nutrient use efficiency could also help to reduce the excess application of mineral fertilizers [2,3].

Moringa is a nutrient-rich plant with antioxidants [4] and naturally occurring cytokinin and zeatin [5]. It has been the research priority in the recent years as it could show promising results on seed germination, nutrient use efficiency, plant growth and yield under abiotic stress and improved the quality of post-harvest [6–8]. Endophytes are considered superior to rhizobacteria and could exhibit their beneficial activity more directly in the host as compared to rhizobacteria [9,10].

In this study, the effects of moringa leaves extract and moringa root's endophytic bacteria were tested on chili seedlings. Different extraction methods were compared to select the most efficient moringa leaves extract that could promote the growth of chili seedlings. This study is aimed to find potential organic plant bio-stimulant for chili cultivation. Organic plant bio-stimulant plays a vital role in the advancement of safe and sustainable agriculture practice.

2. Materials and Methods

2.1. Isolation and of Endophytes

Endophytic bacterium was isolated from Moringa oleifera roots. The roots were collected and stored at 10 °C until use. The soil attached to the roots was removed by washing with tap water. The roots were cut into small pieces and 1 g of roots were weighed in a conical flask filled up with sterile distilled water. The conical flasks were shaken in high speed for 30 min to remove the remaining soil attached to the root tips and rinsed a few times until sterile water remains clear. Then, the roots were macerated using a sterile pestle and mortar and put into 9 mL of sterile distilled water and mixed for 30 min at 100 rpm. To check the sterilization efficiency, sterile water from the final rinsing before maceration was inoculated on nutrient agar to ensure no bacterial growth. After 30 min, 1 mL from the root maceration solution was subjected to a serial dilution until 10^3 and spread onto nutrient agar and potato dextrose agar. The agar medium were incubated at room temperature for 48 h. The bacteria that grow on the medium was sub-cultured until a pure colony was obtained. The bacterial culture was tested for qualitative test for beneficial plant growth promoting activities such as indole acetic acid, phosphorus solubilisation, chitinase production and siderophore production using agar medium. The most potential endophytic bacteria, MR13 was selected for further experiment.. A pure colony of MR13 was cultured in Potato dextrose broth for 48 h at 80 rpm. Then, bacterial culture was transferred to a falcon tube and centrifuged at 10,000× rpm for 15 min. The pellet was washed with sterile distilled water and diluted with 30 mL of sterile distilled water. The bacterial culture was used as bacterial treatment for chili seedlings.

2.2. Moringa Leaves Extraction

Healthy and matured moringa leaves were collected and washed. The extraction process was done using both dry (DL)and fresh leaves (FL). Leaves were dried in hot oven at 40 °C for 3 days. After drying, the leaves were crushed and ground using a blender. The ground powder was sieved with 0.5 mm mesh size sieve. Fresh leaves that were collected one day earlier were kept at 10 °C and used the next day. Three extraction methods were used in this study namely maceration, infusion and doction. The solvents used were distilled water, 50% ethanol *v/v* and 50% methanol v/v. For maceration, 10 g of dry leaf sample and 35 g of fresh leaf was mixed with 450 mL of solvent and left in a shaker for 72 h at100 rpm. Fresh leaves were homogenized using mortar and pestle before extraction. The extract was filtered using muslin cloth and the marc was re-extracted by the same process and solvent until the extraction was exhausted. The solvents such as ethanol and methanol were evaporated using rotary evaporator. The remaining balance after evaporation process was collected and stored at −20 °C until further use. For infusion method, distilled water (50°), cold methanol (50%) and cold ethanol (50%). About 10 g of dry leaf sample and 35 g fresh leaf sample was mixed with 450 mL of solvent and left for 72 h at room temperature. The extract was filtered and the marc was re-extracted by the same process and solvent until the extraction was exhausted. The solvents such as ethanol and methanol were evaporated using rotary evaporator. The remaining balance after evaporation process was collected and stored at −20 °C until further use. For doction method, 10 g of dry leaf sample and 35 g fresh leaf sample was boiled with 450 mL of distilled water for 30 min and left overnight. The extract was filtered using muslin cloth and stored at −20 °C until further use.The treatments used in this experiment were described in Table 1.

Table 1. Moringa leaves extraction methods.

Treatment Code	Leaf Type	Solvent & Condition	Method
T1	DL	50% methanol	Maceration
T2	DL	50% ethanol	Maceration
T3	DL	Distilled water	Maceration
T4	FL	50% methanol	Maceration
T5	FL	50% ethanol	Maceration
T6	FL	Distilled water	Maceration
T7	DL	Cold 50% methanol	Infusion
T8	DL	Cold 50% ethanol	Infusion
T9	DL	Distilled water 50 °C	Infusion
T10	FL	Cold 50% methanol	Infusion
T11	FL	Cold 50% ethanol	Infusion
T12	FL	Distilled water 50 °C	Infusion
T13	DL	Distilled water 100 °C	Doction
T14	FL	Distilled water 100 °C	Doction

[1.] DL: Dry leaf; FL: Fresh leaf.

2.3. Phyto-Chemistry Analyses

Water based treatments were centrifuged at 12,000× *g* for 20 min and the supernatant was collected and stored at −20 °C. For treatments with solvent, centrifuged at 12,000× *g* for 20 min and the supernatant was collected and was kept in water bath set at 40° C for three days before drying using a rotary evaporator. The balance solution after evaporation was stored at −20 °C until further analysis.

2.3.1. Phenolic Content

Test tube containing 100 µL of plant extract, 500 µL of water and 100 µL of Folin-Ciocalteu reagent were prepared in triplicates. The mixture was allowed to stand for 6 min before adding 1 mL of sodium carbonate solution (7.5% *w*/*v*) and 500 µL of water. Then the tubes were kept in dark room at RT for 30 min. Absorbance was recorded against a reagent blank at 760 nm using a UV-Vis spectrometer after 30 min of incubation in a dark room. Total phenolic content was calculated using the standard calibration curve of gallic acid in methanol and expressed as mg gallic acid equivalent/g.

2.3.2. Total Flavanoids

Plant extract (100 µL) was mixed with 500 µL of distilled water and then with 100 µL of 5% of sodium nitate in test tubes and allowed to stand for 6 min. Then 150 µL of 10% aluminium chloride solution was added and allowed to stand for 5 min and finally 200 µL of 1M sodium hydroxide was added. The absorbance of the samples was measured at 510 nm using a UV-Vis spectrometer. Blank contained all reagents except $AlCl_3$ that was replaced with distilled water. The results were calculated using the standard calibration curve of quercetin in methanol and expressed as quercetin equivalents (mg/g).

2.3.3. Free Radical Scavenging Activity of 2,2-diphenyl-1-picryl-1-phenyl-hydrazyl-hydrate (DPPH)

A fresh 0.02% DPPH solution was prepared in methanol. A 50 µL of moringa leaves extract and 3 mL of DPPH solution was added to test tube and mixed vigorously. It was allowed to stand in dark at 37 °C for 30 min. The decrease in the absorbance was measured in each solution at 517 nm using a UV-Vis spectrometer. A standard graph was prepared using gallic acid of different concentrations. Blank was prepared without Gallic acid. The concentration of sample required to scavenge 50% of DPPH was determined from the

curve of percentage inhibition. Free radical scavenging activity was calculated as per formula below.

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.4. Seedlings Growth

Chili seeds were pre-germinated in sterile water and transferred into seedling tray filled up with peat moss. It was let to grow for about one week or until 2–4 leaves emerged from the seedlings. Then 2 mL of the moringa leaves extract (T1–T14), MR13 (Endophytic bacteria) and Distilled water (control) were applied to the peat moss. Every treatment was applied two weeks once with five replications. Thirty days after seed germination, the seedlings were subjected to plant physiology analyses such as seedlings height, total root length, total root surface area, relative chlorophyll content and root shoot ratio.

2.4.1. Root Length and Root Surface Area

WinRhizo root scanner (Epson) was used to measure the total root length and total root surface area. Chili roots were washed thoroughly in tap water to remove the soil debris prior to imaging the roots in root in WinRhizo root scanner.

2.4.2. Relative Chlorophyll Content

Relative Chlorophyll Content was measured using SPAD-502 chlorophyll meter (SPAD 502, Minolta-Camera Co., Osaka, Japan). The data points were recorded at five positions along the leaf blade and then the data points were averaged as a single value.

2.4.3. Plant Height and Root: Shoot Ratio

Seedlings height were measured from the collar of the stem to the highest leave. The roots and shoots were separated, washed and dried in oven at 70 °C for 3 days before measuring the dry weight of root biomass and dry weight of shoot biomass.

3. Results and Discussion

Medicinal plants are rich with antioxidants which could alleviate environmental stresses and lead to plant growth. Among all the extraction methods, the antioxidant properties such as phenols, flavonoids and DPPH radical scavenging activities of moringa leaves extract of various extraction method revealed that apart from polar solvents that are commonly used for the extraction of antioxidant compounds, water extraction has also used considerable antioxidant activities (Table 2). IC_{50} value is used to indicate the antioxidant capacity where the concentration of the sample that causes 50% reduction of the initial DPPH concentration was calculated based on a linear regression graph (not shown) of the mean percentage of antioxidant activity against concentration of gallic acid (μg/mL). The range of DPPH radical scavenging activity is 26.41–73.96 μg/mL. The least free radical scavenging activity was recorded by maceration of fresh moringa leaves. All the other extraction methods exhibited more than 50% free radical scavenging activity which corresponds to more than 8.39 μL/mL of DPPH concentration. Polar solvents extraction using methanol and ethanol have shown better antioxidant activities as compared with water extraction. However, water extraction of fresh moringa leaves extract via infusion method (T12) at 50 °C was comparable to polar solvent extraction method. The total phenolic content in all the extraction method ranged between 0.16–0.72 gallic acid equivalent (GAE) mg/L while total flavonoid content ranged from between 0.25–75.44 quercetin equivalent mg/mL. Methanol extraction using maceration method (T1) showed the highest phenolic content and water extraction using infusion method (T9) showed the lowest phenolic content. Extraction methods using ethanol (T11) and water (T3) exhibited high flavonoid content followed by cold methanol infusion (T10) and water boiling of fresh

leaves (T14). The lowest flavanoid content was observed in treatment T4 (maceration using methanol) and T12 (Water infusion at 50 °C). In general, fresh leaves extraction has recorded high flavonoid content except for sample T3 and high DPPH free radical scavenging activity except for samples T7 and T8. Dry leaves extraction showed high phenolic content.

Table 2. Phytochemical analyses of moringa leaves juice extracted via various methods.

Treatment	DPPH Scavenging Activity %	Phenolic Content (mg/mL)	Flavanoid Content (mg/mL)
T1	63.70 ± 0.23	0.72 ± 0.01	18.61 ± 11.98
T2	61.25 ± 0.35	0.50 ± 0.00	69.60 ± 1.60
T3	47.39 ± 0.94	0.16 ± 0.00	74.89 ± 0.40
T4	66.13 ± 0.32	0.34 ± 0.00	0.35 ± 0.02
T5	70.29 ± 0.18	0.27 ± 0.00	13.1 ± 11.13
T6	26.41 ± 1.02	0.32 ± 0.00	69.82 ± 1.51
T7	70.58 ± 0.04	0.67 ± 0.00	74.42 ± 0.60
T8	71.73 ± 0.05	0.60 ± 0.00	0.29 ± 0.03
T9	54.91 ± 0.85	0.12 ± 0.00	12.65 ± 12.02
T10	73.96 ± 0.01	0.30 ± 0.00	70.14 ± 2.11
T11	72.38 ± 0.03	0.28 ± 0.00	75.44 ± 1.83
T12	69.32 ± 0.00	0.24 ± 0.00	0.25 ± 0.01
T13	43.22 ± 0.78	0.52 ± 0.00	12.83 ± 12.37
T14	44.50 ± 0.00	0.31 ± 0.00	70.78 ± 2.26

[1] Standard deviation of five independent measured were calculated for each analysis.

The plant growth parameters such as relative chlorophyll content, seedling height, total root length, total root surface area and the root to shoot ratio were measured for moringa leaves of various extraction methods, endophytic bacteria and control. Relative chlorophyll content is a non-destructive and indirect method that indicates the health and nutritional status of the plant. The relative chlorophyll content was not significant among treatments except for T7 (cold methanol infusion of dry leaves) and T9 (water infusion of dry leaves at 50 °C) (Figure 1). Plant height was significantly higher in all treatments as compared to control particularly in sample T1 (methanol maceration of dry leaves) and T6 (water maceration of fresh leaves). The seedlings height ranged between 12.1 cm–16.5 cm (Figure 2). Total root length and total root surface area are important indicators that could show the direct effect of bio-stimulant on chili seedlings. Total root surface measurements were comparable among all the treatments (Figure 3). Root length and proliferation root hairs could enhance nutrient absorption and nutrient use efficiency. The highest root length was observed in sample T2 (dry leaves of ethanol extraction), T10 (fresh leaves of methanol extraction), T8 (dry leaves of ethanol extraction), T9 (dry leaves of water extraction) and MR13 (endophytic bacteria) (Figure 4). Root/shoot ratio is a vital criterion for assessing plant health. Samples T13 (water extraction of dry leaves by boiling method) and T4 (methanol extraction of fresh leaves by maceration method) showed the highest root/shoot ratio (Figure 5).

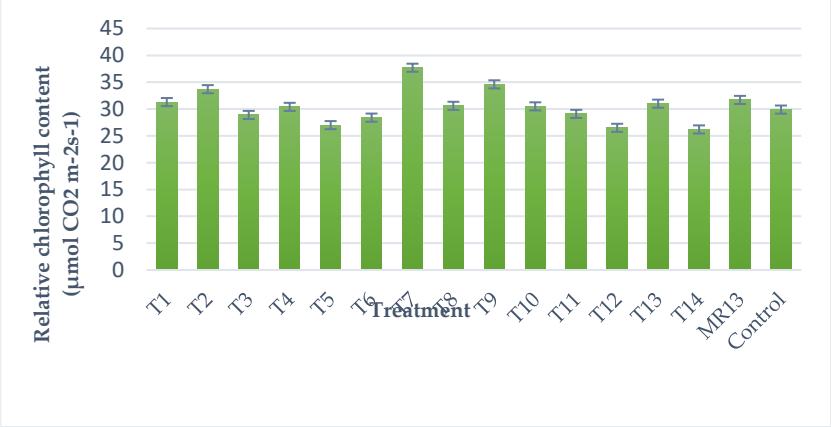

Figure 1. Relative chlorophyll content of chili seedlings treated with endophytic bacteria and moringa leaves extract of various extraction methods. The values are an average of five independent measurements.

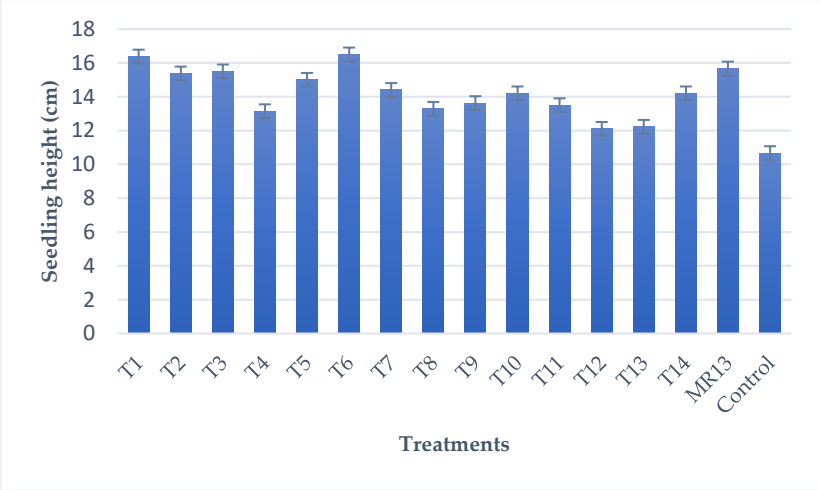

Figure 2. The height of chili seedlings treated with endophytic bacteria and moringa leaves extract of various extraction methods. The values are an average of five independent measurements.

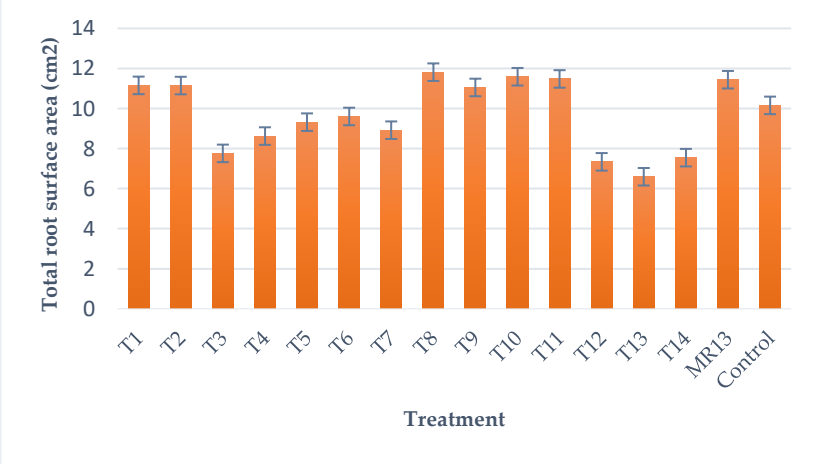

Figure 3. Total root surface area of chili seedlings treated with endophytic bacteria and moringa leaves extract of various extraction methods. The values are an average of five independent measurements.

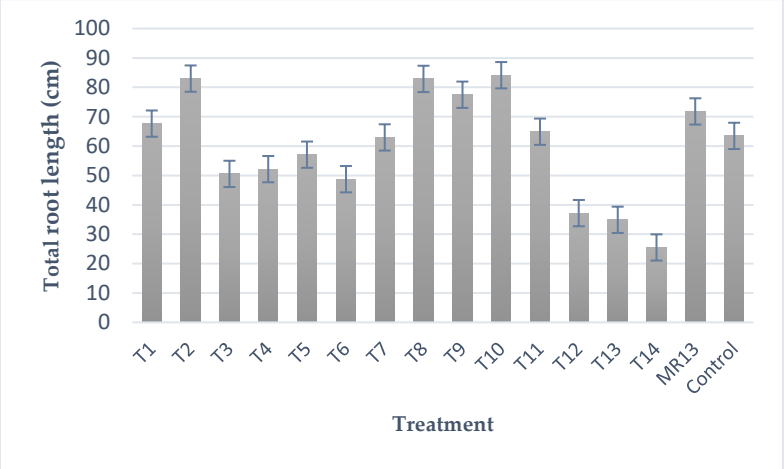

Figure 4. The root length of chili seedlings treated with endophytic bacteria and moringa leaves extract of various extraction methods. The values are an average of five independent measurements.

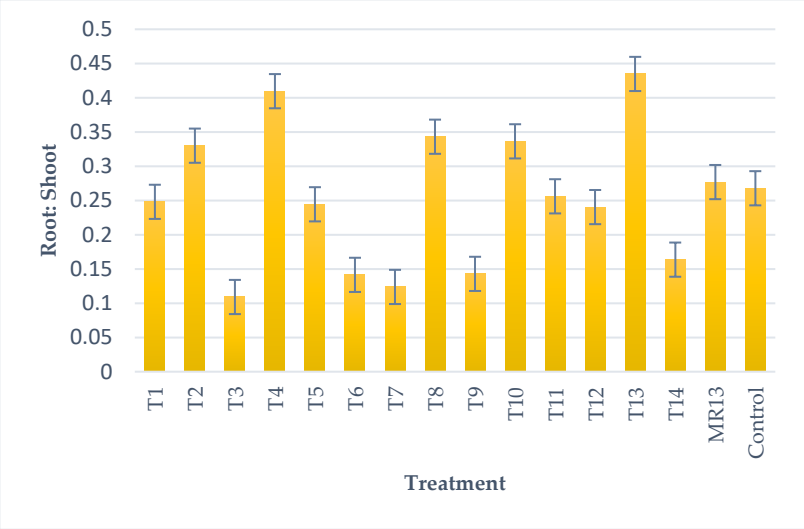

Figure 5. The root to shoot ratio of chili seedlings treated with endophytic bacteria and moringa leaves extract of various extraction methods. The values are an average of five independent measurements.

4. Conclusions

This study has revealed that apart from methanol and ethanol extraction, water extraction which is a cheap and feasible method of moringa leaves extraction could be used for the production of organic bio-stimulant of chili seedlings. Moringa leaves were not only rich with antioxidant contents but also could enhance the health and root growth of chili for better nutrient use efficiency. Endophytic bacteria too has the potential to be utilized as plant bio-stimulant as its efficiency is comparable to moringa leaves extract in improving chili seedling growth.

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