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THE EFFECT OF BIO-STIMULANT APPLICATION METHODS ON THE GROWTH AND PHYTOCHEMICAL CONTENT OF CHILLI SEEDLINGS

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Abstract: Bio-stimulants have gained vast recognition in recent years as vigorous research has been conducted to improve crop growth, yield, and quality. However, bio-stimulant utilization in soilless systems has been limited due to their rapid degradation and short lifespan. New formulations are essential to extend the longevity of bioactive compounds in the target site. This paper highlights the effect of different application methods, such as drenching soilless medium and foliar application, on the chlorophyll, total phenolic, flavonoid, and carotenoid contents in chilli leaves. A completely randomized block design (RCBD) consisting of five treatments and three replications with ten plants per replication was adopted in this study. Moringa leaf extract (MLE) was prepared in a nano-emulsion form while bacteria were incorporated in nano-gel. Data were analyzed using the SPSS software (IBM Statistics, version 28, USA). Foliar application of MLE nano-emulsion significantly increased the chlorophyll content (35.958±7.19 mg g-1) in chilli leaves than the drenching method (26.545±0.5 mg g-1) and non-encapsulated MLE foliar treatment (12.233±2.33 mg g-1). In addition, carotenoid (2.138±1.68 mg g-1) and phenolics contents (1.090±0.17 mg gallic acid equivalent g-1) in chilli leaves were higher when nano-emulsion of MLE used as foliar application than non-encapsulated MLE (1.725±1.08 mg g-1 of carotenoid and 0.993±0.16 mg gallic acid equivalent g-1 of phenolic content). Compared to other treatments, the seedlings treated with encapsulated bacteria and nano-emulsion of MLE showed a significant increase in plant height. It was found that MLE nano-emulsion improved the photosynthetic activities of chilli plants significantly by increasing the chlorophyll and carotenoid contents when used as a foliar application. Nano-encapsulation technique protects the active ingredients in MLE and increases the absorption due to their nano-size and large surface area.

Keywords: bio-stimulant, nano-encapsulation, bacteria, moringa, drenching, foliar

Introduction

Bio-stimulants are substances of natural origin that can improve the physiological functions of plants, resulting in increased uptake of nutrients, higher crop yield, and improved crop quality (Agliassa et al., 2021). Plant bio-stimulant studies have gained the recent attention of the scientific community and farmers due to their multi-functions, especially during this period of drastic climate change. It has

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greater scope to be developed into a more refined product to assist the plant in combating climate change while boosting growth and productivity.

Beneficial microorganisms could act as bio-stimulants, enhancing plant growth and crop production in response to abiotic stress in extreme environments (Romano et al., 2020). Trichoderma, Pseudomonas, Bacillus, Arbuscular Mycorrhizal Fungi (AMF), Phosphobacteria, Azosprillum, Azotobacter, Rhizobium, etc., were among the beneficial microorganisms that were introduced as bio-fertilizer or bio-stimulant for crop growth and yield (Kumari, Bhatnagar, Deepali, Mehla, & Vashistha, 2022). The inadequate development of microbial inoculants due to issues with formulating them has resulted in unsuccessful inoculants in performing their intended functions; hence, immobilization has been recommended to create effective formulations for PGPR inoculants (Vassilev et al., 2017).

Likewise, the application of bio-stimulants made from plant extracts is also deemed to boost the growth and yield of crops. Plant extracts that have been added as bio-stimulants were reported to bring benefits to plant growth and productivity. Moringa leaf extract is one of the plant-based bio-stimulant which is regarded as a cheap source of natural plant growth enhancers (Afzal, Akram, Rehman, Rashid, & Basra, 2020; Arora & Kaur, 2019; Refai et al., 2023). It contains various beneficial vitamins, nutrients, flavonoids, phenols, and phytohormones, which can positively impact crop growth and development (Mashamaite, Ngcobo, Manyevere, Bertling, & Fawole, 2022). A study has suggested that moringa leaf extract can serve as a natural bio-stimulant due to cytokinin (zeatin), gibberellic acid, ascorbic acid, phenolic compounds, and other minerals in significant quantities. These compounds can enhance plants' antioxidant and nutritional profile, thereby, increasing their growth and yield (Rana, Hoque, & Abedin, 2019). Additionally, it is proven that moringa leaves are rich in various active compounds that could enhance crop growth (Ahmad, Tanveer, Liaqat, & Dole, 2019; Alkuwayti, El-Sherif, Yap, & Khattab, 2020; Hussain et al., 2021; Rady, Kuşvuran, Alharby, Alzahrani, & Kuşvuran, 2019). However, to date, there has been limited research into developing an optimal formulation for using moringa leaf extract as a bio-stimulant.

Application methods of bio-stimulant such as foliar spray, seed treatment and soil amendment may have different effect on crop performance. Direct bio-stimulant application in the soil and application via fertigation is also considered as soil treatments. It was found that bio-stimulant applied via soil resulted in 10% higher yield than foliar and seed application (Li, Van Gerrewey, & Geelen, 2022). On the other hand, foliar application of bio-stimulant were reported to increase photosynthetic efficiency (Quintero-Calderón, Sánchez-Reinoso, CháVez-Arias, Garces-Varon, & Restrepo-Díaz, 2021). A few other reports have indicated that there were no significant difference between foliar and soil application on plant growth enhancement (Nassar, El-Magharby, Ibrahim, Kandil, & Abdelsalam, 2023; Sujata, Goyal, Baliyan, Avtar, & Mehrotra, 2023). Therefore, this study is aimed to determine the effect of soil and foliar application of nano-encapsulated bio-stimulant on the growth of chilli seedlings.

Materials and Methods

Nano-encapsulated Bio-stimulant Preparation

Bacillus and Streptomyces (MR3 and MR13) were freshly grown in potato dextrose broth (PDB, Merck, Germany. The cells were collected via centrifugation at 5, 000 rpm for 5 minutes. Bacterial cells were diluted with sterile distilled water to ensure that the density of endophytic bacteria was within the range of 107-109 CFU/ml (bacterial density was checked using the total plate count method).

Sodium alginate (Sisco Research Laboratories, Pvt. Ltd., India) 0.5% (w/v), modified corn starch (0.1% w/v), zeolite (0.1% w/v), and skimmed milk (0.1% w/v) were mixed with bacterial cells using a magnetic stirrer for 10 min. A plastic syringe was used to extrude the bacterial-alginate mixture into a calcium chloride solution, 0.5 M under stirring conditions. The encapsulated bacteria in alginate gel were left overnight at room temperature for a stable gel formation. The nano-gel was washed thrice with distilled water. Then, excess water was removed by straining the gel, and the nano-gel was collected in a clean container before storing it at 10°C.

For moringa leaf extract nano-emulsion, 10% (w/v) of dry moringa leaf was macerated with sterile distilled water in a blender and left at room temperature overnight. The extract was collected by filtering the extract using a muslin cloth. The extract was diluted 10-1 and mixed with vegetable oil at a ratio of 1:5 (oil: moringa extract). The oil phase was combined in a magnetic stirrer at 50°C for 15 min. Then, 1% of the surfactant with an HLB value of 10 was added dropwise while stirring for 30 min. The oil phase-surfactant mixture was homogenized at 10 000 rpm for 2 min and diluted with 90% of the aqueous phase of sterile distilled water. The same process was used for dry moringa leaf extract obtained via decoction to prepare oil-in-water nanoemulsion.

Experimental design for the application method of bio-stimulant

SJ2-461, F1 hybrid hot chilli seeds were purchased from SAKATA Corp. Chilli seeds were soaked in water overnight before sowing into wet peat moss. The polybags were covered with black plastic sheets and kept at room temperature in a dark room until it germinates. Once, the radicle is visible, the black sheets were removed and the polybags were kept in glass-house under shade until true leaves emerged. Peat moss was sprayed with water every two days once or when it was dry. After the 14th day of sowing, fertilizer AB with EC reading of 0.9 was poured in a tray and the seedling tray was placed on it for maximum absorption of nutrients. Transplanting of seedlings was done 35 days after sowing. The planting medium, cocoa peat was prepared at in a polybag of 16” x 16” cm. A completely randomized block design (RCBD) (Fig.7.10) with three replications and 10 plants per replication were adopted to test the bio-stimulant application method in a shade-house. Five application methods (treatments) were tested as described below. Briefly, the bio-stimulants were encapsulated in nano-emulsion form for moringa leaves extract and in alginate gel form for endophytic bacteria. For nono-encapsulated bio-stimulant, liquid endophytic culture and moringa leaves extracts were used directly on soil and plants. Endophytic bacteria were applied on soil while moringa leaves extract was tested by foliar application and soil application methods.

T1: AB fertilizer (control)
T2: AB fertilizer + Soil application of non-encapsulated endophytic bacteria + Soil application of non-encapsulated moringa leaves extract
T3: AB fertilizer + Soil application of encapsulated endophytic bacteria + Soil application of encapsulated moringa leaves extract
T4: AB fertilizer + Soil application of encapsulated endophytic bacteria + Foliar application of encapsulated moringa leaves extract
T5: AB fertilizer + Soil application of non-encapsulated endophytic bacteria + Foliar application of non-encapsulated moringa leaves extract

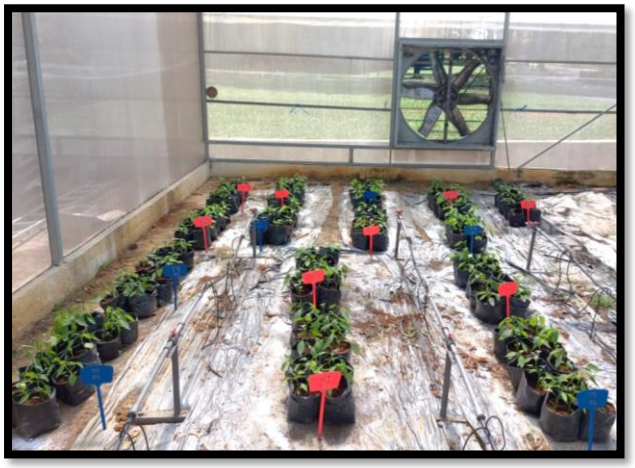

Fig. 7.11 Shade-house trial on application methods of bio-stimulant

Vegetative growth promotion of chilli seedlings

The chilli seedlings were grown for 30 days. Vegetative growth analysis such as seedlings height, root length, root biomass, shoot biomass and chlorophyll content were measured.

Chlorophyll measurement

For chlorophyll content analysis, 1.0 g of fresh chilli leaves were ground with 4 mL of 80% acetone using a pestle and mortar. The mixture was transferred into a falcon tube and left for 1-2 hours. Then the sample was centrifuged for 5 min at 10,000 g and the supernatant was used to measure the absorbance at 470 nm (chlorophyll a), 652 nm (chlorophyll b) and 665 nm (carotenoid content).

Phenolic and flavonoid content in chilli leaves

Fresh chilli leaves, 1.0g was extracted with 4 ml of 80% ethanol in water. It was left in ultrasonic bath at 60°C for 1 h. The samples were let to cool down and centrifuged at 8000 rpm for 5 min. The supernatant was filter sterilized with 0.25 µm filter membrane. Total phenolics were estimated with the modified Folin–Ciocalteu colorimetric method (Kopta et al 2020).

For phenol content, the supernatant (0.2 ml) was mixed with 0.2 ml of Folin–Ciocalteu's reagent (1:1 v/v) and 0.6 ml of deionized water. After 5 min, 1 ml of sodium carbonate was added to the test tubes and kept in dark for 1 h. The absorbance of the resulting blue colour was measured at 765 nm using a UV-VIS (Ultraviolet–visible spectroscopy) Helios Beta spectrophotometer (Waltham, MA, USA) against a reference solution. The results were expressed as gallic acid equivalents (GAE), mg GAE per 1 g fresh weight. Gallic acid calibration curve was prepared in between 20-100 mg/L. Standard gallic acid was prepared by mixing 1g of gallic acid with 10 ml methanol and kept at dark vial at 4°C.

For flavonoid content, 500 µl supernatant was mixed with 150 µl of 5% sodium nitrate and 2 ml of distilled water. After 5 min, 150µl of Al2Cl3 was added and left for 1 min. Then 2ml of NaOH and 1.2ml of distilled water. The mixture was incubated for 30 min at RT and the absorbance was read at 510 nm. The results were calculated using the standard calibration curve of quercetin in methanol and expressed as quercetin equivalents (mg/g).

Statistical analysis

Statistical analysis was performed using the Univariate analysis with Tukey's test ($p < 0.05$) by the SPSS software (IBM Statistics, version 28, USA) to determine the significance of the data.

Results and Discussion

Effect of drenching and foliar application methods on the phytochemical contents of chilli leaf and vegetative growth of seedlings

Nano-encapsulated bio-stimulant was applied using the drenching method in all the previous experiments. In general, the drenching of the soilless medium could promote the vegetative growth, yield, and quality of chilli except for several parameters including phenolics content. In this experiment, moringa leaves extract nanoemulsion was tested using drenching and foliar spraying methods while nano-gel containing endophytic bacteria was drenched into the soilless medium. Most of the literature reviews have reported foliar application as an ideal application method to be used as a bio-stimulant and very few have been applied via soil drenching but none have tested the efficiency on soilless medium. The experiment aimed to compare the effectiveness of two different application methods, drenching, and foliar application, for a nanoemulsion containing moringa leaf extract.

In addition to this, the effect of both encapsulated and non-encapsulated methods was studied by looking into their capability to promote the chlorophyll content that has a direct relationship with photosynthesis activity in plant growth promotion. Carotenoid content in the leaf refers to the amount of carotenoid pigments in the leaf tissue. Carotenoids are important organic pigments that give leaves their colors, and they play essential roles in photosynthesis and other biological processes. Measuring carotenoid content in leaves can provide insight into the health and nutrition of the plant, as well as its ability to carry out photosynthesis and other metabolic processes.

Foliar application of moringa leaves extract nanoemulsion increased the chlorophyll content in chilli leaves significantly compared to the drenching method (Table 1.1). Interestingly, encapsulated nano-emulsion has promoted significantly higher chlorophyll production than non-encapsulated nano-emulsions when applied as a foliar treatment. The drenching method was unsuitable to increase the chlorophyll content using encapsulated and non-encapsulated bio-stimulants as the value was lower than the control plants. No significant difference was observed for other parameters between treatments. As this analysis was conducted during the initial phase of seedling growth, approximately one month after transplanting, it is possible that significant variations may arise as the plant continues to mature and develop. However, an increasing trend could be observed in carotenoid and phenolics contents of chilli leaves treated with encapsulated nano-emulsion applied as foliar application as compared with other treatments. When applied through foliar application, plant extracts can be absorbed more efficiently by the leaves, which have a large surface area and are capable of rapid uptake of nutrients and other substances. It also allows precise application of the bio-stimulant to the

plant's leaves, where it can directly stimulate the chlorophyll production and photosynthesis activity. Foliar application can also minimize the loss of the active ingredients.

The vegetative growth of seedlings with different application methods was investigated (Table 1.2). Non-encapsulated endophytes showed the highest average root biomass and root length as compared to nano-encapsulated endophytes. As the evaluation was carried out during the early stage of seedling growth, the impact of encapsulated endophytes could not be observed since the slow release of microbes from the nano-gel would require a longer period. The similar observation was observed for the average aboveground biomass. However, all the above mentioned vegetative growth parameters were not significantly different among treatments. Compared to other treatments, the seedlings that received the T3 treatment, consisting of encapsulated endophytic bacteria and a nano-emulsion of moringa leaf extract, showed a significant increase in height. This could be due to the effect of nano-emulsion of moringa leaf extract that exhibited highest chlorophyll content as discussed earlier. The objective of using nano-gel is to prolong the survival of the endophytic bacteria, which could potentially enhance the product's shelf-life. Consequently, the release of bacteria into the root zone would occur slower than that of non-encapsulated bacteria, resulting in a gradual response in root growth.

Weight loss was measured by weighing the chilli fruit on balance at day 0 and the end of each storage interval. The total weight loss during that storage interval was measured by the difference between initial and final chilli weight, and it was computed as percentages on a fresh weight basis by the standard AOAC (1984) method.

Phenolic and flavonoid content in chilli leaves

Fresh chilli leaves, 1.0g extracted with 4 ml of 80% ethanol in water. It was left in an ultrasonic bath at 60°C for 1 h. The samples were let to cool down and centrifuged at 8000 rpm for 5 min. The supernatant was filter sterilized with 0.25 µm filter membrane. Total phenolics were estimated with the modified Folin–Ciocalteu colorimetric method (Kopta et al 2020).

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For flavonoid content, 500 µl supernatant was mixed with 150 µl of 5% sodium nitrate and 2 ml of distilled water. After 5 min, 150µl of Al2Cl3 was added and left for 1 min. Then 2ml of NaOH and 1.2ml of distilled water. The mixture was incubated for 30 min at RT and the absorbance was read at 510 nm. The results were calculated using the standard calibration curve of quercetin in methanol and expressed as quercetin equivalents (mg/g).

Table. 1.1 Chlorophyll measurement, phenolic, carotenoid, and flavonoid content in chilli leaves

Treatments	Method of application	Total chlorophyll (mg/g)	Carotenoid (mg/g)	Phenol content (mg gallic acid equivalent/g)	Flavonoid content (mg of quercetin equivalent /g)
AB fertilizer (control)	Drenching of planting material	30.480±2.26^{ab}	1.686±0.4^{a}	1.057±0.14^{a}	0.0105±0.00047^{a}
AB fertilizer + non-encapsulated endophytic bacteria and moringa leaves extract	Drenching of planting material (both endophytic bacteria and moringa leaves extract)	26.178±1.47^{b}	2.032±0.25^{a}	1.076±0.19^{a}	0.0029±0.00928^{a}
AB fertilizer + encapsulated endophytic bacteria and moringa leaves extract	Drenching of planting material (both nano-gel of endophytic bacteria and nano-emulsion of moringa leaves extract)	26.545±0.57^{ab}	2.000±0.11^{a}	1.065±1.07^{a}	0.0001±0.0019^{a}
AB fertilizer + encapsulated endophytic bacteria and moringa leaves extract	Drenching of planting material (endophytic bacteria nano-gel) and foliar application (nano-emulsion of moringa leaves extract)	35.958±7.19^{a}	2.138±1.68^{a}	1.090±0.17^{a}	0.0174±0.00188^{a}
AB fertilizer + non-encapsulated endophytic bacteria and moringa leaves extract	Drenching of planting material (endophytic bacteria) and foliar application (moringa leaves extract)	12.233±2.33^{c}	1.725±1.08^{a}	0.993±0.16^{a}	0.0173±0.01521^{a}

Table 1.2 Vegetative growth of seedlings treated with various application methods.

Code	Treatment	Average root biomass (g)	Average aboveground biomass (g)	Seedling height (cm)	Root length (cm)
T1	AB fertilizer (control)	0.198±0.05[a]	0.771±0.20[a]	16.19±3.73[c]	11.26±3.33[a]
T2	AB fertilizer + Soil application of non-encapsulated endophytic bacteria + Soil application of non-encapsulated moringa leaves extract	0.250±0.05[a]	0.857±0.08[a]	17.92±3.43[bc]	12.24±4.12[a]
T3	AB fertilizer + Soil application of encapsulated endophytic bacteria + Soil application of encapsulated moringa leaves extract	0.224±0.05[a]	0.840±0.18[a]	19.99±3.99[a]	12.27±4.07[a]
T4	AB fertilizer + Soil application of encapsulated endophytic bacteria + Foliar application of encapsulated moringa leaves extract	0.199±0.03[a]	0.768±0.13[a]	18.19±3.66[ab]	12.39±3.8[a]
T5	AB fertilizer + Soil application of non-encapsulated endophytic bacteria + Foliar application of non-encapsulated moringa leaves extract	0.270±0.14[a]	0.923±0.19[a]	19.20±3.8[ab]	12.69±4.1[a]

Conclusion

Foliar application of nano-emulsion of moringa leaf extract exhibited positive effects in increasing chlorophyll and carotenoid content and increasing the seedling height compared to non-encapsulated foliar application of moringa leaves extract. Applying nano-gel-containing endophytic bacteria did not demonstrate any significant impact on the chlorophyll, carotenoid, phenolic, and flavonoid content in the leaves, or the vegetative growth of chilli seedlings. The primary objective of producing nano gel is to prolong the viability of bacteria during shelf-life and facilitate a gradual release of bacteria into the medium for long-term efficacy. Thus, in the short-term evaluation of its impact on seedlings, nanogel was found to be less efficient than non-encapsulated bacteria, which demonstrate an immediate effect on plant growth.

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