Tropical Natural History 24 (2024): 107-114
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Development of a Biofertilizer from Plant Growth Promoting Rhizobacteria Isolated from Millipede Fecal Pellets

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Received: 2 October 2023; Accepted: 29 April 2024

ABSTRACT. – Plant Growth Promoting Rhizobacteria (PGPR) are commonly used to produce biofertilizers. As such, the purpose of the present research was to develop a PGPR biofertilizer formulation from four *Streptomyces* strains isolated from millipede fecal pellets and to select suitable carriers for them. The four *Streptomyces* isolates were *Streptomyces* sp. KLS-AC04, *S. zaomyceticus* KLD-AC02-1, *S. zaomyceticus* KLD-AC16 and *S. zaomyceticus* KLD-AC30. Their compatibility was tested on Actinomycetes isolation agar by the cross-streak method and the results showed that the four isolates can coexist. A viability test was done with three different types of carriers: coconut coir, cow dung and peat moss. The best carrier for all isolates was peat moss (viability: $5.3 \times 10^4 - 8.5 \times 10^5$ cfu/g). The isolate that exhibited the highest survival rate across the three carriers was *Streptomyces* sp. KLS-AC04. Seven PGPR biofertilizer formulations were developed, of which formulation 6 (cow dung: peat moss: KLS-AC04: KLD-AC02-1: KLD-AC16: KLD-AC30) showed the highest viability (6×10^5 cfu/g). The effects of the PGPR biofertilizer formulations on plant growth were explored on Chinese kale. This showed that the use of the four *Streptomyces* strains had a significant positive effect on germination percentage (range 47.5% − 80.0%), fresh weight (range 0.060 − 0.086 g), shoot length (range 36.1 − 60.4 mm), and root length (range 15.3 − 30.8 mm).

KEYWORDS: Chinese kale, *Streptomyces*, *Thyropygus resimus*

INTRODUCTION

In terrestrial ecosystems, millipedes (Diplopoda) play a crucial role by enhancing soil quality through their involvement in decomposing organic matter and facilitating nutrient cycling (Wang et al., 2018). Their fecal pellets constitute a significant source of vital soil nutrients, including nitrogen, phosphorus, and potassium (Sutthisa et al., 2023). Additionally, these fecal pellets serve as habitats for a diverse array of microorganisms, such as Plant Growth Promoting Rhizobacteria (PGPR). PGPR are beneficial bacteria that enhance plant growth and health by various mechanisms. The connection between millipedes and PGPR is rooted in the distinctive symbiotic relationship between these soil-dwelling arthropods and the microorganisms present in their fecal pellets. The millipede digestive system provides an environment that enhances the growth and colonization of various microorganisms, especially PGPR. As millipedes consume plant materials and organic debris, they also ingest PGPR, which travel through their digestive system to establish themselves in the millipede fecal pellets. One intriguing aspect of this relationship is the potential of millipede fecal pellets as a reservoir for diverse PGPR strains that can settle and establish themselves in the pellets, rather than in the millipede gut tractus (Hopkin and Read, 1992; Smit and Van Aarde, 2001).

PGPR play a pivotal role in maintaining soil fertility and fostering sustainable crop growth by improving plant health, producing phytohormones, enhancing phosphate solubility, facilitating mineralization, fixing nitrogen, and mediating enzymatic degradation (Chandran et al., 2021). In this way, PGPR contribute to sustainable soil fertility management and promoting ecologically responsible practices in crop production, while safeguarding the environment and long-term soil health. It is hence no surprise that a lot of research is focusing on the development and evaluation of PGPR biofertilizers as potential soil enhancers and biocontrol agents. In this context, dry formulations of PGPR biofertilizers are often preferred over liquid ones, because they are more convenient for field application and commercialization (Aloo et al., 2022) As such, (dry) PGPR biofertilizers offer environmentally friendly solutions to enhance soil fertility, improve soil structure, and manage soil-borne plant diseases within the domain of organic farming (Ahamad et al., 2023), a progressively favored approach towards sustainable agriculture that augments human health. For example, PGPR biofertilizers using *Streptomyces aureoverticillatus* HN6 effectively prevent plant diseases and promote plant growth by improving soil fertility and regulating the microbial community of the rhizosphere (Olanrewaju and Babalola, 2019; Wang et al., 2022). Other PGPR biofertilizers contain bacteria such as *Bacillus* spp., *Trichoderma* spp., and *Streptomyces* spp. that may suppress soil-borne pathogens, promote plant

growth, and induce plant resistance to (a)biotic stresses (Berg, 2009; Li et al., 2022).

Actinomycetes (order: Actinomycetales) comprise numerous PGPR, with the genus *Streptomyces* standing out as the most abundant and significant among them. This genus is a good source of bioactive compounds that can be used in biofertilizers, including antibiotics, extracellular enzymes, and plant growth enhancers (Olanrewaju and Babalola, 2019). As such, *Streptomyces* spp. may promote plant growth by (1) producing phytohormones (auxins, cytokinins, and gibberellins) and siderophores (which scavenge ferric iron from the environment), (2) fixing nitrogen, (3) controlling plant pathogens, and (4) buffering stress (via 1-aminocyclopropane-1-carboxylate deaminase activity). However, the utility and efficiency of PGPR biofertilizers depend on several factors, including the local environment and the plant species for which they are applied (Alabouvette et al., 2006). This research aims to develop a dry PGPR biofertilizer formulation using *Streptomyces* isolates from millipede fecal pellets and select suitable carriers. Therefore, this study investigates the compatibility of four *Streptomyces* isolates and identifies the most viable carrier. Multiple formulations of the biofertilizer are developed and compared for their efficacy based on viability assessments. The research also evaluates the beneficial impact of the *Streptomyces* based PGPR biofertilizer on the growth of Chinese kale (*Brassica oleracea* var. *alboglabra*).

MATERIALS AND METHODS

Streptomyces isolates

The four PGPR isolates utilized in this research were obtained from the fecal pellets of the widely distributed Thai millipede species *Thyropygus resimus* Attems, 1938. The specific *Streptomyces* isolates included in this study are: (1) *Streptomyces* sp. KLS-AC04, (2) *S. zaomyceticus* KLD-AC02-1, (3) *S. zaomyceticus* KLD-AC16, and (4) *S. zaomyceticus* KLD-AC30 (Sutthisa et al., 2023). These isolates were carefully selected for their potential as PGPR and their significance in sustainable agricultural practices. All experiments were conducted at the Fungi and Bioproduct Laboratory, Department of Biology, Faculty of Science, Mahasarakham University, Thailand, ensuring the reliability and accuracy of the results presented in this study.

Compatibility among *Streptomyces* isolates

Streptomyces isolates were grown on Actinomycetes isolation agar (AIA) at 30 °C for 24−72 h. The cross-streak method of Purivirojkul and Areechon (2007) was employed to assess their compatibility. In this method, one isolate was streaked over approximately 4 cm at the center of the AIA plate, while the other isolates were streaked perpendicularly to the first one, maintaining a separation of 1−2 cm. Three different experimental treatments were applied: (1) KLD-AC02-1: KLD-AC30: KLD-AC16: KLS-AC04, (2) KLD-AC30: KLD-AC16: KLS-AC04, and (3) KLD-AC16: KLS-AC04. Each experimental treatment was replicated three times and incubated at 30 °C. After 48 h, the AIA plates were examined to determine positive growth of the isolates and any growth inhibition in the zones where the isolates intersected.

Viability of the *Streptomyces* isolates in various carrier materials

Three carrier materials, including coconut coir, cow dung, and peat moss, were tested under uniform conditions. These materials were prepared with 10% moisture content, sifted through a 2 mm sieve, and packed in polyethylene bags, each containing 10 g of the respective carrier. All material was sterilized at 121 °C for 30 min by autoclaving twice with a 24 h interval. Each *Streptomyces* isolate was cultured in International *Streptomyces* Project-2 (ISP-2) Medium, shaken at 125 rpm and 25 °C for 24−72 h, and the cell suspension was adjusted to 10^8 cells/ml. Then 1 ml of *Streptomyces* suspension was mixed with the prepared carrier material in a 1:10 ratio for three replicates per treatment and stored at 25 °C. The viability of *Streptomyces* was tested on AIA medium and recorded by the total plate count technique after storage for 0 and 7 days.

Development of the PGPR biofertilizer

The development of the PGPR biofertilizer started by evaluating the compatibility and growth performance of the four *Streptomyces* isolates. These isolates underwent cultivation in ISP-2 Medium with continuous shaking at 125 rpm and a temperature of 25 °C for a duration of 24−72 h. Following the incubation period, the resulting cell suspension was adjusted to achieve a concentration of 10^8 cells/ml. Subsequently, the cell suspension of each *Streptomyces* isolate was blended with specific carrier materials, leading to the creation of seven distinct formulations. About 50 g of each formulation was then meticulously packed into plastic bags and subjected to two rounds of autoclaving at 121 °C for 30 min each, ensuring thorough sterilization. Next, 5 ml of *Streptomyces* suspension were carefully introduced to each carrier material and mixed thoroughly. The seven carrier formulations tested were: (1) cow dung: peat moss, (2) cow dung: peat moss: KLD-AC30, (3) cow dung: peat moss:

KLD-AC02-1, (4) cow dung: peat moss: KLD-AC16, (5) cow dung: peat moss: KLS-AC04, (6) cow dung: peat moss: KLS-AC04: KLD-AC02-1: KLD-AC16: KLD-AC30, and (7) cow dung: peat moss: coconut coir: KLS-AC04.

During the development of the seven formulations, the mixtures were initially incubated at 25 °C for 3 days. Following this incubation period, the formulations were subjected to a drying process at 50 °C for 72 h. A pH meter (Mettler Toledo) was used to measure the pH of the dry formulations after reconstituting them with deionized water, with a ratio of 1g of dry formulation in 99 ml of deionized water. The moisture content of the dried formulations was assessed as follows: 1 g of each formulation was placed in a separate aluminum foil container and dried at 105 °C for 5 h in a hot air oven. Subsequently, the samples were immediately transferred to a desiccator and allowed to cool down for weighting (Sawatphanit et al., 2022). Moisture content (%) was then calculated using the formula:

$$\frac{(W_2 - W_1) - (W_3 - W_1)}{(W_2 - W_1)} \times 100$$

With W_1 the weight of the aluminum foil container, W_2 the weight of the formulation and the aluminum foil container before drying, and W_3 the weight of the formulation and the aluminum foil container after drying.

Viability of *Streptomyces* in PGPR biofertilizer formulations

The viability of the *Streptomyces* isolates in each formula was examined by measuring the number of microorganisms that survive after the formulation was serially diluted between 10^{-3} and 10^{-6}. The total number of *Streptomyces* cells was determined using the spread plate technique on AIA medium, incubated at 25 °C for 24−72 h. The results were reported in cfu/g.

Effect of the new PGPR biofertilizer on the growth of Chinese kale

Potting soil to grow Chinese kale was prepared by autoclaving 120 g of peat moss at 121 °C for 30 min, twice. Chinese kale seeds were soaked in 10% clorox for 5 min, washed twice with sterile distilled water for 2 min each time, and dried. The effect of the *Streptomyces* based PGPR biofertilizer on the growth of Chinese kale was tested by the soil mixing method. To this end, 10 g of PGPR biofertilizer was mixed with 30 g of potting soil. The Chinese kale seeds were planted in this PGPR biofertilizer enriched potting soil according to the 7 aforementioned carrier formulations. The experiment was conducted in a randomized complete block design, with 10 seeds planted per pot, 3 replicates per formulation, and 3 pots per replicate. The plants were watered every day for 7 days and the growth of the plants was followed by measuring plant height, root length, fresh weight, and germination percentage.

RESULTS

Compatibility among *Streptomyces* isolates

After 48 h of incubation, inspection of the AIA plates revealed no visible growth inhibition in the zones where the isolates intersected. This absence of growth inhibition indicates that the four *Streptomyces* isolates are compatible and can coexist without adversely affecting each other's growth (Fig. 1). This compatibility among the isolates positions them as promising candidates for PGPR biofertilizer development, enabling further experimentation and formulation.

Viability of the *Streptomyces* isolates in various carrier materials

Table 1 shows the viability of different *Streptomyces* isolates in various carrier materials. Three isolates, viz. KLD-AC02-1, KLD-AC30, and KLD-AC16 showed no detectable viability on coconut coir, suggesting that this carrier material is unsuitable for these isolates. In contrast, KLS-AC04 survived in the three carriers, with the highest viability in peat moss (8.5×10^5 cfu/g), followed by cow dung (3.3×10^5 cfu/g), and lastly, coconut coir (5.9×10^5 cfu/g). Hence, KLS-AC04 survives better in the tested carrier materials than the other isolates, making it a potentially more suitable candidate for further development in PGPR biofertilizer formulations.

Development of the PGPR biofertilizer

The seven PGPR biofertilizer formulations yielded biofertilizers with distinct texture characteristics depending on the type of carrier material used. The moisture content of these biofertilizers ranged from 0.32% to 5.37%. The data on texture characteristics, moisture content, and pH values of the biofertilizers are presented in Table 2. All formulations were weakly acidic, with pH values ranging from 5.91 to 6.83.

Viability of *Streptomyces* in PGPR biofertilizer formulations

All PGPR biofertilizer formulations, except formulation 1 without PGPR inoculation, supported the survival of *Streptomyces* isolates, with viabilities ranging from 6.0×10^2 up to no less than 6.4×10^5 cfu/g, for formulation 6 (Table 3).

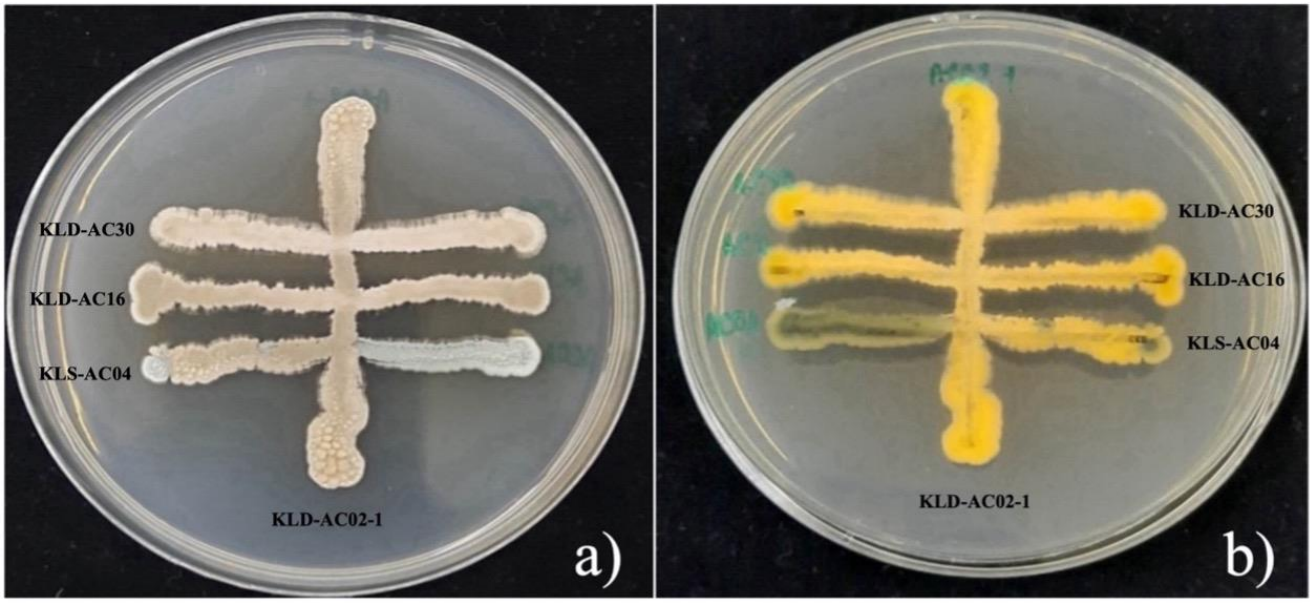

FIGURE 1. Compatibility test of four *Streptomyces* isolates (experimental treatment 1: KLD-AC02-1: KLD-AC30: KLD-AC16: KLS-AC04). a) Top view on the colonies. b) Colonies seen from the underside of the plate.

TABLE 1. Viability of the *Streptomyces* isolates in various carrier materials.

Isolate	Viability (cfu/g)*		
	coconut coir	peat moss	cow dung
KLD-AC02-1	0.00^{c}	2.7×10^{5bc}	2.5×10^{4c}
KLD-AC30	0.00^{c}	5.3×10^{4bc}	3.8×10^{3c}
KLD-AC16	0.00^{c}	8.5×10^{4bc}	1.8×10^{3c}
KLS-AC04	5.9×10^{5ab}	8.5×10^{5a}	3.3×10^{5ab}

* Means followed by the same letter were not significantly different according to Duncan's new Multiple-Range Test, $P < 0.05$.

Effect of the new PGPR biofertilizer on the growth of Chinese kale

Among the seven PGPR biofertilizer formulations tested, formulation 2 (cow dung: peat moss: KLD-AC30) showed the highest germination percentage (80 ± 10.80%), although this was not significantly different from the other formulations, whose germination percentage ranged from 47.5 ± 6.30 to 75 ± 9.57% (Table 4).

Formulation 5 (cow dung: peat moss: KLS-AC04) yielded Chinese kale seedlings with the longest roots (37.0 ± 1.9 mm), but this was not significantly different from the root lengths obtained with formulations 3, 4, 6, and 7 (Table 4). The tallest Chinese kale plants were obtained with biofertilizer formulation 7 (60.4 ± 1.6 mm), but again, this was not significantly different from formulations 2-6, whose shoot lengths ranged from 52.1 ± 1.3 to 57.3 ± 7.7 mm. In contrast, formulation 1 led to significantly smaller plants (Table 4).

Chinese kale seedlings grown on formulations 5 and 6 had the highest fresh weights (0.086 ± 0.010 g and 0.086 ± 0.006 g, respectively). However, once more, these values were not significantly different from those of formulations 2, 3, 4, and 7, with fresh weights ranging from 0.060 ± 0.018 to 0.081 ± 0.025 g (Table 4). Thus, different PGPR biofertilizer formulations have variable effects on Chinese kale growth parameters, with formulation 2 showing notable benefits in germination percentage, formulation 5 promoting root length, and formulation 7 resulting in taller plants. Yet, no single formulation consistently and significantly outperformed the others, even if formulation 1 (without *Streptomyces*) appeared less optimal than the formulations with *Streptomyces*.

TABLE 2. Characteristics of the PGPR biofertilizer formulations.

Formulation	Description	Moisture content (%)	pH
1. cow dung: peat moss	black to greyish black, not fine, loosely 	3.46	6.11
2. cow dung: peat moss: KLD-AC30	dark brown, not fine, loosely 	0.68	5.91
3. cow dung: peat moss: KLD-AC02-1	dark brown to black, slightly loosely 	2.17	6.06
4. cow dung: peat moss: KLD-AC16	dark brown to black, loosely 	5.37	6.12
5. cow dung: peat moss: KLS-AC04	brown, loosely 	1.71	6.07
6. cow dung: peat moss: KLD-AC30: KLD-AC02-1: KLD-AC16: KLS-AC04	dark brown, slightly loosely 	4.27	6.03
7. cow dung: peat moss: coconut coir: KLS-AC04	light brown, coarse texture, very loosely 	0.32	6.83

TABLE 3. Viability of *Streptomyces* isolates in PGPR biofertilizer formulations after drying at 50 °C for 3 days.

Formulation	PGPR Biofertilizer	Viability (cfu/g)*
1	cow dung: peat moss	0.00^{d}
2	cow dung: peat moss: KLD-AC30	3.4×10^{4cd}
3	cow dung: peat moss: KLD-AC02-1	6.0×10^{2d}
4	cow dung: peat moss: KLD-AC16	5.4×10^{4c}
5	cow dung: peat moss: KLS-AC04	1.6×10^{5b}
6	cow dung: peat moss: KLD-AC30: KLD-AC02-1: KLD-AC16: KLS-AC04	6.4×10^{5a}
7	cow dung: peat moss: coconut coir: KLS-AC04	2.6×10^{3d}

* Means followed by the same letter were not significantly different according to Duncan's new Multiple-Range Test, $P < 0.05$.

DISCUSSION

This study successfully developed PGPR biofertilizer formulations that enhance the growth of Chinese kale by utilizing a combination of four *Streptomyces* isolates derived from millipede fecal pellets. The observed compatibility among these four *Streptomyces* isolates, allowing them to coexist without inhibiting each other's growth, is a promising indicator for the development of additional PGPR biofertilizer formulations. This finding aligns with previous research highlighting the potential of certain *Streptomyces* species or strains in promoting plant growth (Olanrewaju and Babalola, 2019; Sutthisa et al., 2023).

Varying viabilities of *Streptomyces* isolates on different carriers were observed. While KLD-AC02-1, KLD-AC30, and KLD-AC16 did not thrive on coconut coir, KLS-AC04 exhibited robust survival on all three carriers. These findings underscore the significance of carrier materials in supporting the survival and activity of beneficial microorganisms in biofertilizers (Olanrewaju and Babalola, 2019; Sawatphanit et al., 2022). Specifically, peat moss and cow dung emerged as favorable environments for microbial growth and nutrient release, contributing to the overall success of biofertilizer formulations (Subramanian et al., 2018). These findings open new possibilities for using *Streptomyces* isolates from millipede fecal pellets in biofertilizer development, with carrier selection playing a crucial role in their effectiveness.

The formulations showed varying amounts of moisture (0.32% to 5.37%) and were weakly acidic (pH 5.91 to 6.83). These characteristics are essential for maintaining microbial viability and activity in the biofertilizers (Alabouvette et al., 2006; Ogbo et al., 2018). The weakly acidic pH values are favorable for the growth of *Streptomyces* species and their ability to produce bioactive compounds that promote plant growth (Sutthisa et al., 2023).

The viability density of *Streptomyces* isolates in this study ranged from 6.0×10^{2} to 6.4×10^{5} cfu/g, suggesting that the biofertilizer formulations provided favorable conditions for the growth and survival of these beneficial bacteria. Formulation 6 (cow dung: peat moss: KLS-AC04: KLD-AC02-1: KLD-AC16: KLD-AC30) showed the highest *Streptomyces* viability (6.4×10^{5} cfu/g), indicating that the combination of carrier materials and *Streptomyces* isolates in this formulation created an optimal environment for bacterial proliferation. Therefore, formulation 6 is a promising candidate for further agricultural applications.

The findings from this study are in line with similar research conducted by Sabaratnam and Traquair (2002), who developed *Streptomyces* formulations using vegetative propagules obtained from actively growing, non-sporulating liquid cultures. They utilized various carriers such as alginate beads, durum flour granules, and talcum powder to encapsulate *Streptomyces* Di-944. The formulations showed varying degrees of stability during storage at different temperatures. The powder and granular formulations stored at 4 °C were the most stable, retaining 100% viability for 14 and 10 weeks, respectively, over a 6-month storage period. However, the viability of the propagules in these formulations decreased over time, with the talcum powder formulation displaying the lowest stability. Similarly, Myo et al. (2019) developed a kaolin-based powder formulation as a carrier for *Streptomyces* NKZ-259. This carrier demonstrated good shelf-life stability, with the cell population showing no significant decrease over four months of storage at 4 °C. *In vivo* analysis revealed that tomato seedlings treated with kaolin powder containing NKZ-259 cells exhibited significant increases in root and

TABLE 4. Effects of PGPR biofertilizers on growth of Chinese kale.

Formulation	Germination [ns] (%)	Root length[*] (mm)	Shoot length[*] (mm)	Fresh weight [ns] (g)
1	47.5 ± 6.30	15.3 ± 2.1[c]	36.1 ± 8.9[b]	0.060 ± 0.018
2	80.0 ± 10.80	26.7 ± 1.2[b]	52.1 ± 1.3[ab]	0.080 ± 0.005
3	65.0 ± 11.90	31.1 ± 1.6[ab]	56.7 ± 2.9[a]	0.081 ± 0.025
4	60.0 ± 14.72	34.2 ± 5.0[ab]	57.3 ± 6.1[a]	0.074 ± 0.013
5	75.0 ± 9.57	37.0 ± 1.9[a]	57.3 ± 4.3[a]	0.086 ± 0.010
6	60.0 ± 10.80	31.3 ± 3.7[ab]	57.3 ± 7.7[a]	0.086 ± 0.006
7	72.5 ± 16.83	30.8 ± 4.0[ab]	60.4 ± 1.6[a]	0.079 ± 0.003

[ns] not significant

[*] Mean ± Standard deviation; values followed by the same letter in the column were not significantly different according to Duncan's new Multiple-Range Test, $P < 0.05$.

shoot length, as well as fresh and dry weights. These results indicate that the kaolin-based powder was an effective carrier for NKZ-259 cells and held promise for commercial applications. Overall, the successful development of PGPR biofertilizer formulations in the present study illustrates the potential of using such formulations to enhance plant growth and crop productivity. However, further research and field trials are necessary to validate the performance of these formulations under different environmental conditions and with various crop species.

The PGPR biofertilizer formulations had varying impacts on different growth parameters of Chinese kale, even if most results were in statistical terms not significantly different. Still, the presence of *Streptomyces* in the formulations showed trends of positive effects on several plant growth characteristics of Chinese kale compared to formulations without *Streptomyces*. Specifically, formulation 2 (cow dung: peat moss: KLD-AC30) yielded the highest germination percentage, formulation 5 (cow dung: peat moss: KLS-AC04) increased root length and formulation 7 (cow dung: peat moss: coconut coir: KLS-AC04) produced taller plants. These consistent *Streptomyces*-linked trends toward improved growth parameters are in line with previous research that highlighted the beneficial effects of PGPR biofertilizers on plant growth and development (Wu et al., 2018; Chandran et al., 2021). Nevertheless, the lack of statistical significance of these positive *Streptomyces*-linked effects on Chines kale growth underscores the need for further studies with larger sample sizes, other formulations and a wider spectrum of target plants in order to assess the real impacts of *Streptomyces*-based biofertilizers on plant growth. Moreover, a better understanding of the underlying mechanisms by which PGPR biofertilizer formulations exert their effects on plant growth is necessary to optimize their performance and maximize their potential benefits in agriculture.

CONCLUSION

This study successfully developed PGPR biofertilizer formulations using *Streptomyces* isolates from millipede fecal pellets and diverse carrier materials to enhance Chinese kale growth. The compatibility of *Streptomyces* isolates without inhibitory interactions makes them promising candidates for biofertilizer formulations. Formulation 6 (cow dung: peat moss: KLS-AC04: KLD-AC02-1: KLD-AC16: KLD-AC30) demonstrated the highest *Streptomyces* viability and potential for practical agricultural applications. While the formulations showed promising trends in enhancing plant growth, it is important to note that these results were not statistically significant. Further research, including field trials under diverse environmental conditions, is necessary to validate the practical application and efficacy of these biofertilizers.

ACKNOWLEDGMENTS

This research project was financially supported by Mahasarakham University. Authors are grateful to Sathit Saratan (Sirindhorn Museum, Thailand) for assistance in collecting material.

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