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Application of Soil Bacteria as Bioinoculants to Promote Growth of Cowpea (*Vigna unguiculata*)

Kedsukon Maneewan, Sirirat Deeseenthum, Worachot Saengha, Thipphiya Karirat, Vijitra Luang-In*

Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand.

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ABSTRACT

This work aimed to evaluate the capacity of soil bacteria as bioinoculants (biofertilizers) to promote cowpea (*Vigna unguiculata*) growth. Three pure bacterial cultures namely *Acinetobacter pittii* PT1.3.4 (AP), *Achromobacter* sp.C2.23 (AS), and *Achromobacter xylosoxidans* N3.4 (AX) were used as bioinoculants to enhance germination and development of cowpea seeds. Pre-decide formulations of single or mixed cultures were prepared, soaked with cowpea seeds, and cultivated on agar in a growth chamber for 7 days at 25°C. Shoot and root length were measured and percentage germination was determined. Similarly, bacterial formulations were prepared in talcum powder and were used as bioinoculants to adhere to cowpea seeds. The inoculated seeds were cultivated in pots for 28 days for the shoot and root length, fresh and dry weight, and percentage germination. Among the tested various formulations, treatment has *A. pittii* (AP) displayed the highest shoot length (14.67 cm) and fresh weight (0.58 g/plant) of cowpea under laboratory conditions after seven days of inoculation. Similarly, cowpea plants treated with *A. pittii* (AP) also have the tallest shoots (14.25 cm) under natural conditions after 7 days of inoculation, while the highest root length (10.5 cm) and fresh weight (1.57 g/plant) were recorded from the treatment of *Achromobacter* sp. (AS). Further, the results of the study also revealed that soil bacteria can survive for one month in talcum powder at 4°C and room temperature storage. These bioinoculants can be used for agricultural application by local farmers to mitigate the cost of chemicals that cause environmental concerns to promote sustainable agriculture in Thailand.

* Corresponding author

E-mail: vijitra.l@msu.ac.th (Vijitra Luang-In)

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1 Introduction

Bioinoculation of plants with nitrogen-fixing bacteria is considered more ecofriendly, reduced the cost of fertilization, helps in sustainable agriculture, and can be used as an alternative to agrochemicals. Bacterial bioinoculants have been widely used to improve plant growth and yields and minimize the threat of plant diseases (Kalia et al. 2020). Among the most commonly available bioinoculants, *Azotobacter*, *Rhizobium*, *Azospirillum*, and *Burkholderia* strains are now marketed as agricultural biofertilizers and bioinoculants (Maitra et al. 2021). Reena Josephine and Thomas (2022) isolated *Acinetobacter pittii* F25 (Accession no. KM677194) from the maize rhizosphere soil and found plant growth stimulating effects including ammonia production, inorganic and organic phosphate solubilization, and nitrogen fixation. The capacity of this strain to promote plant development in sustainable agriculture was demonstrated under laboratory and greenhouse conditions by improved shoot and root length as well as increased biomass in treated maize seedlings compared to uninoculated groups. Intriguingly, Liu et al. (2022) constructed a synthetic bacterial community (SynCom) from the bacteria isolated from the wheat rhizosphere; this synthetic bacterial community included three species of *Bacillus*, two species of *Acinetobacter*, one species of each *Enterobacter*, *Xanthomonas*, and *Burkholderia*. These researchers reported a significant effect of this SynCom on the growth, root development, and biomass output of wheat plants.

Achromobacter is a gram-negative straight rod-shaped motile bacterial genus that belongs to the family Alcaligenaceae order Burkholderiales. Members of this genus are found in soil and both fresh and saltwater (Isler et al. 2020) and have various agricultural benefits. In addition to nitrogen fixation, *Achromobacter xylosoxidans* BOA4 exhibits some other significant plant growth promoting biochemical features, including siderophore and IAA synthesis and phosphate solubilization (Rai et al. 2018). Recently three important bioinoculant bacteria namely *A. pittii* PT1.3.4 as phytase producer (AP), *Achromobacter* sp. C2.23 as chitinase producer (AS) and *A. xylosoxidans* N3.4 as nitrogen fixer (AX) were isolated from the untapped resource of Nasinuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand (Luang-In et al. 2021) were used as bioinoculants to study the impact on germination and growth of the local economic plant cowpea both in the laboratory environment and in soil.

In Thailand, cowpea (*Vigna unguiculata*) is an economic crop widely grown in all regions, especially in the north and northeast regions. It can be consumed as fresh pods or dry seeds, and are a rich source of carbohydrates and proteins (Haisirikul et al. 2020). Cowpea cultivation has various advantages including short harvest, drought tolerance, and easy cultivation on farmlands and paddy fields after harvest. In this study, the capacity of three bacterial strains i.e. AP, AS and AX isolated from the soil of Nasinuan Community Forest (both single strains and multi-strain formula) were evaluated to promote the growth of cowpea under laboratory and field conditions.

2 Materials & Methods

2.1 Isolation and cultivation of bacterial isolates

Three *Achromobacter* bacterial strains i.e. *A. pittii* PT1.3.4 (AP), *Achromobacter* sp. C2.23 (AS) and *A. xylosoxidans* N3.4 (AX) were isolated from the soil of Nasinuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand (Luang-In et al. 2021). Phytase-specific agar, chitin agar, and nitrogen-free agar were used as selective media to isolate AP, AS and AX, respectively as described by Luang-In et al. (2021). Phytase producers and chitinase producers' bacteria displayed clear zones around the colonies on selective media while nitrogen fixers turned green to blue media. The positive colonies were point inoculated on selective agars. The diameter of the clear zone or the blue area over the diameter of the colony was measured as the halo:colony ratio for identifying the most potential isolates as a phytase producer, chitinase producer, and nitrogen fixer. The bacteria AP, AS and AX with greater potential for use as bioinoculants were cultured in nutrient broth for 20 h and then spun down for 15 min at 10,000g. The cell pellets were resuspended in sterile water until OD_{600nm} reached 1.0 (1×10^8 CFU/mL).

2.2 Effect of bioinoculants on *in-vitro* cowpea seed germination and sprout growth

Cowpea seeds were purchased from Chia Tai Co., Ltd. Seeds with uniform size were selected and surface-sterilized by soaking in 10% Chlorox with 2 drops of Tween 20 for 15 min, this was followed by the washing of these seeds with sterile water. After this, seeds were soaked in a bacterial suspension of 1×10^8 CFU/mL concentration for 1 hour and following treatments (10 seeds per treatment in triplicate) were imposed: (T_1) AP (15 mL), (T_2) AS (15 mL), (T_3) AX (15 mL), (T_4) AP + AS (7.5 + 7.5 mL), (T_5) AS + AX (7.5 + 7.5 mL), (T_6) AP + AX (7.5 + 7.5 mL), (T_7) AP + AS + AX (5 + 5 + 5 mL) and (T_8) 15 mL water (control). Then seeds were placed on a Petri dish filled with ½ strength of Murashige and Skoog (MS) medium and allowed to germinate and grow for 7 days at 25°C in a 16 h light/8 h dark period in a growth chamber. Seed germination percentage, length of cowpea sprouts, fresh weight, and dry weight were recorded after 7 days.

2.3 Effect of bioinoculants on *in-vivo* cowpea growth in a pot experiment

For the pot experiment, 10 g of autoclaved talcum powder was mixed with predefined bacterial suspension concentration i.e. 1

$\times 10^8$ CFU/mL, and various treatments from T_1 - T_8 were formulated as per laboratory experiments while for field study an additional treatment T_9 (Talc powder) was formulated. Ten healthy, equal-sized cowpea seeds were adhered with each bioinoculant formulation uniformly per treatment in triplicate. Cowpea seeds were allowed to grow for 7, 14, 21, and 28 days in a pot containing autoclaved soil (1.5 kg per pot) under field conditions. Within one week, three or four plants per pot were reduced to one plant per pot. The plants were watered every day until harvested. At harvest, the pots were poured into a 2 mm sieve and the soil was carefully cleansed to separate the shoots and roots. The proportion of seeds that germinated, length of cowpea plants and roots, fresh weight, and dry weight were all reported.

2.4 Soil analysis

The pH and levels of phosphorus, nitrogen, and potassium in the top layer of the soil (0-20 cm) in the pot experiment were determined before and after the experiment using a Rapitest 1835 Digital 3-way soil analyzer (Luster Leaf, USA).

2.5 Storage condition of bioinoculants

The bioinoculants were stored in talcum powder at room temperature (35°C) and in a refrigerator at 4°C for 28 days. Microbial count (CFU/mL) was measured by the viable plate count method at intervals of 7 days until 28 days.

2.6 Statistical analysis

Completely randomized design (CRD) was implemented to conduct the study. Mean differences ± standard deviations of triplicates were compared using analysis of variance (ANOVA) and Tukey's multiple comparisons with GraphPad Prism 8.0 (GraphPad Software, Inc.). Statistically significant differences were considered at $p< 0.05$.

3 Results

3.1 Bioinoculants promoted the growth of cowpea

Seed germination percentages in all treatment and control were reached 100% after 7 days of inoculation on ½ strength MS agar medium. Among the tested formulations highest fresh weight (0.58 g/plant) and longest shoots (14.67 cm/plant) were reported in the seeds treated by AP after 7 days of treatment under laboratory conditions (Figure 1A; Table 1). The longest roots (7.17 cm/plant) were reported from the combined application of AP + AX. Further, plant fresh weight was not showing any significant difference among various formulations, while dry weight was significantly different among the various treatments (Table 1). This showed that bioinoculants significantly promoted the growth of cowpea on ½ MS agar under laboratory conditions compared with the control.

The effect of bioinoculants on cowpea growth in a pot experiment under field conditions for 7, 14, 21, and 28 days was also studied (Figure 1; Table 2). Like laboratory conditions, the longest shoot on days 7^{th}, $14^{th,}$ and 21^{st} (14.25, 19.50, 21.75 cm/plant respectively), was reported in the plants treated with bioinoculants AP (T_1) (Figure 1B, 1C, 1D; Table 2), while, on the day 28^{th}, longest shoots i.e. 32.00 cm/plant were recorded in the pot treated with AS (T_2) (Figure 1E and 1F; Table 2). Similarly, on day 7^{th}, the longest roots (10.5 cm/plant) was reported from the pots treated with bioinoculants AS (T_2), while on day 14 and 21, the highest root length (10.13 cm/plant and 11.75 cm/plant, respectively) was reported from the pot treated with AX (T_3) and on day 28, it was reported 12.67 cm/plant from the plants treated with AP (T_1), in this manner a mixed response was reported in case of root length and it varies with the time spent. In the case of a combination of various bioinoculants, no significant differences were reported during the study. Further, in the case of fresh weight, on day 7^{th} and 14^{th}, the highest fresh weight (1.57 g/plant and 1.96 g/plant, respectively) was reported in the plants treated with bioinoculants

Table 1 Effect of bioinoculants on cowpea growth in ½ MS media under laboratory conditions for 7 days

Treatment	Germination (%) on day7	Length (cm/plant)		Fresh weight (g/plant)	Dry weight (g/plant)
		shoot	root		
AP	100	14.67± 0.75^a	3.07±0.98^c	0.58 ± 0.09	0.06 ± 0.01^a
AS	100	11.83± 0.50^b	4.73 ± 0.85^b	0.35 ± 0.09	0.03 ± 0.00^c
AX	100	7.67± 0.35^c	2.67 ± 0.51^d	0.28 ± 0.16	0.02 ± 0.00^c
AP+ AS	100	13.87 ± 0.64^a	6.33 ± 0.85^a	0.58 ± 0.12	0.06 ± 0.01^a
AS + AX	100	12.93 ± 0.65^b	7.17 ± 0.35^a	0.51 ± 0.16	0.05 ± 0.00^a
AP + AX	100	11.67 ± 0.55^b	2.23 ± 0.68^d	0.41 ± 0.18	0.04 ± 0.00^b
AP+ AS+ AX	100	10.83 ± 0.31^b	3.87 ± 0.76^b	0.41 ± 0.19	0.04 ± 0.01^b
Water (control)	100	2.47 ± 0.81^d	2.87 ± 0.71^d	0.24 ± 0.10	0.03 ± 0.00^c

* Disinct superscripts in columns represent statistically significant differences ($p< 0.05$)

Table 2 Effect of bioinoculants on cowpea growth in the pot experiment under field conditions

Treatment	Length (cm/plant)								Fresh weight (g/plant)			
	Day 7		Day 14		Day 21		Day 28		Day 7	Day 14	Day 21	Day 28
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root				
AP (T_1)	14.25 ± 0.25^{a}	5.50 ± 0.36^{c}	19.50 ± 0.50^{a}	3.70 ± 0.61^{b}	21.75 ± 0.75^{a}	8.25 ± 0.75^{b}	29.50 ± 1.80^{a}	12.67 ± 0.58^{a}	1.14 ± 0.07^{a}	1.61 ± 0.00^{a}	2.60 ± 0.36^{a}	3.95 ± 0.31^{a}
AS (T_2)	13.75 ± 0.75^{a}	10.50± 0.00^{a}	14.07 ± 0.12^{c}	5.23 ± 0.40^{b}	20.75 ± 0.75^{a}	10.50 ± 0.50^{ab}	32.00 ± 2.65^{a}	9.30 ± 0.58^{b}	1.57 ± 0.14^{a}	1.96 ± 0.15^{a}	2.07 ± 0.21^{a}	3.72 ± 0.45^{a}
AX (T_3)	13.25 ± 0.75^{a}	8.75 ± 0.25^{b}	14.07 ± 0.12^{c}	10.13 ± 2.23^{a}	20.25 ± 0.75^{a}	11.75 ± 1.25^{a}	23.00 ± 0.87^{b}	9.17 ± 1.04^{b}	1.33 ± 0.04^{a}	1.53 ± 0.15^{a}	1.37 ± 0.40^{c}	3.14 ± 0.45^{a}
AP+ AS (T_4)	12.5 ± 1.00^{a}	6.25 ± 0.25^{c}	14.83 ± 0.29^{c}	7.07 ± 0.12^{b}	16.50 ± 1.00^{d}	7.00 ± 1.00^{bc}	24.33 ± 0.58^{b}	9.67 ± 1.53^{b}	1.25 ± 0.18^{a}	1.58 ± 0.24^{a}	1.83 ± 0.25^{a}	3.55 ± 0.29^{a}
AP+ AX (T_5)	11.75 ± 0.25^{a}	9.00 ± 0.50^{b}	13.00 ± 0.00^{d}	4.33 ± 0.58^{b}	18.00 ± 0.50^{b}	4.50 ± 1.50^{c}	25.00 ± 0.00^{b}	10.67 ± 0.58^{a}	1.12 ± 0.17^{a}	1.67 ± 0.36^{a}	1.33 ± 0.06^{c}	3.30 ± 0.35^{a}
AS+ AX (T_6)	11.25 ± 0.25^{b}	8.00 ± 0.00^{b}	14.47 ± 0.57^{c}	7.53 ± 0.84^{b}	17.50 ± 0.50^{c}	10.25 ± 0.75^{ab}	21.17 ± 1.26^{b}	8.50 ± 1.04^{c}	1.29 ± 0.23^{a}	1.40 ± 0.20^{a}	1.67 ± 0.21^{b}	3.01 ± 0.11^{a}
AP+ AS+ AX (T_7)	10.75 ± 0.75^{b}	7.25 ± 1.25^{b}	13.83 ± 0.76^{d}	9.03 ± 0.06^{a}	20.00 ± 0.00^{a}	9.00 ± 0.00^{b}	22.50 ± 1.80^{b}	9.00 ± 0.87^{c}	0.95 ± 0.09^{b}	1.48 ± 0.16^{a}	1.73 ± 0.15^{b}	3.16 ± 0.88^{a}
Water (T_8)	7.75 ± 0.75^{c}	2.70 ± 0.75^{d}	16.33 ± 1.53^{b}	4.33 ± 1.04^{b}	18.75 ± 0.25^{b}	8.00 ± 0.00^{bc}	20.83 ± 1.89^{b}	8.00 ± 1.00^{c}	0.69 ± 0.24^{b}	1.10 ± 0.21^{b}	2.17 ± 0.06^{a}	1.69 ± 0.13^{b}
Talc (T_9)	9.50 ± 2.00^{c}	4.50 ± 1.00^{c}	12.50 ± 0.50^{d}	9.50 ± 3.04^{a}	15.25 ± 2.75^{d}	6.00 ± 2.00^{bc}	20.83 ± 0.29^{b}	10.50 ± 1.32^{a}	0.73 ± 0.33^{b}	1.21 ± 0.32^{b}	1.60 ± 0.46^{b}	2.07 ± 0.39^{b}

* Disinct superscripts in columns represent statistically significant differences (p< 0.05).

Table 3 Effect of bioinoculants on soil N and K levels in the pot experiment

Treatment	Soil N and K levels (ppm)					Soil P level (ppm)				
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 0	Day 7	Day 14	Day 21	Day 28
AP (T_1)	74 ± 12	134 ± 12	100 ± 18^{ab}	80 ± 0^{cd}	80 ± 0^{cd}	5.6 ± 0.8	9.6 ± 0.8	7.3 ± 1.2^{ab}	6.0 ± 0.0^{cd}	6.0 ± 0.0^{cd}
AS (T_2)	80 ± 15	158 ± 15	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	11.2 ± 1.0	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AX (T_3)	80 ± 0	155 ± 15	110 ± 0^{a}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	11.0 ± 1.0	8.0 ± 0.0^{a}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AP+ AS (T_4)	83 ± 12	155 ± 15	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.2 ± 0.8	11.0 ± 1.0	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AP+ AX (T_5)	83 ± 12	143 ± 12	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.2 ± 0.8	10.2 ± 0.8	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AS+ AX (T_6)	80 ± 0	143 ± 12	100 ± 18^{ab}	140 ± 30^{ab}	140 ± 30^{ab}	6.0 ± 0.0	10.2 ± 0.8	7.3 ± 1.2^{ab}	10.0 ± 2.0^{ab}	10.0 ± 2.0^{ab}
AP+ AS+ AX (T_7)	80 ± 0	143 ± 12	100 ± 18^{ab}	125 ± 15^{b}	125 ± 15^{b}	6.0 ± 0.0	10.2 ± 0.8	7.3 ± 1.2^{ab}	9.0 ± 1.0^{b}	9.0 ± 1.0^{b}
Water (T_8)	80 ± 0	143 ± 12	80 ± 0^{b}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	10.2 ± 0.8	6.0 ± 0.0^{b}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
Talc (T_9)	80 ± 0	140 ± 12	110 ± 0^{a}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	10.0 ± 0.8	8.0 ± 0.0^{a}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}

* Disinct superscripts in columns represent statistically significant differences (p< 0.05).

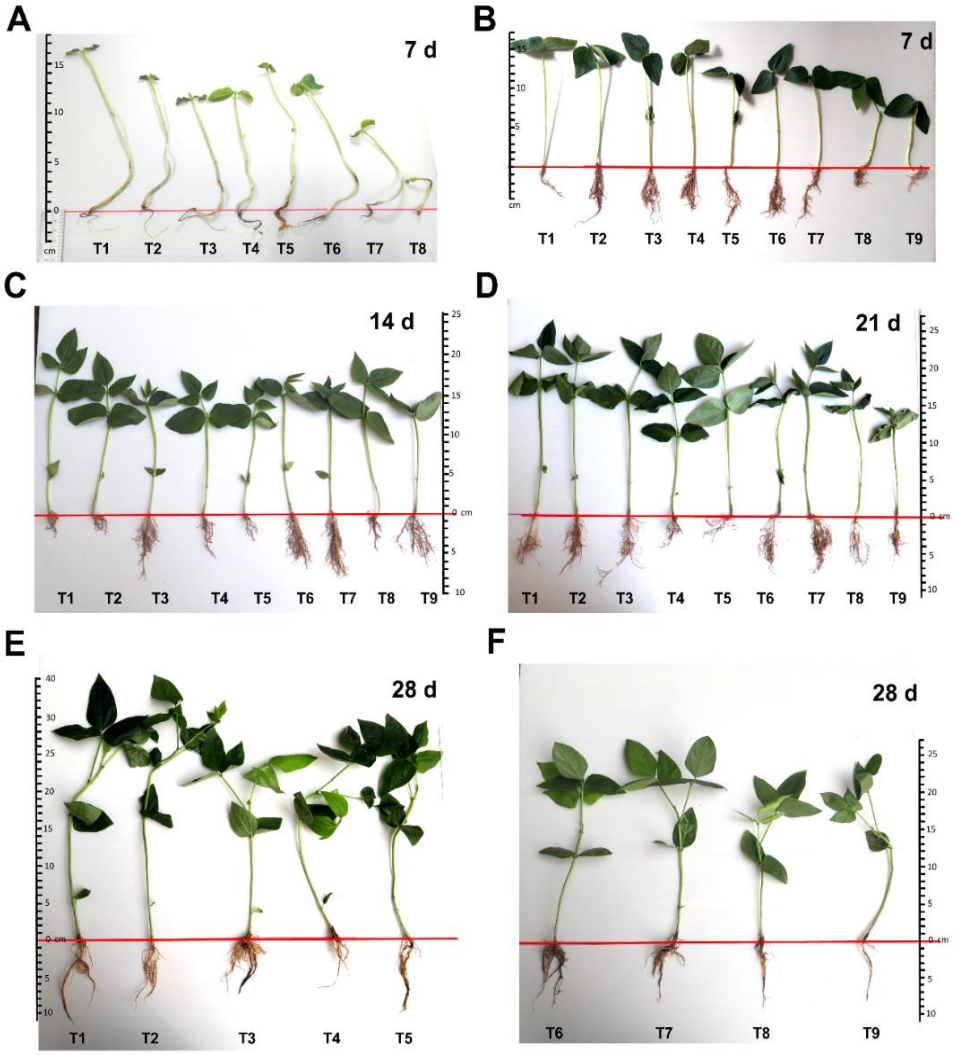

Figure 1 Growth of cowpea (A) on ½ strength MS liquid media under laboratory conditions after 7 days of bioinoculants treatment; Growth of cowpea in pot experiment under field conditions (B) at 7 days; (C) at 14 days; (D) at 21 days; (E) at 28 days (T1-T5); (F) at 28 days (T6-T9)

AS (T_2), while on day 21st and 28th, highest fresh weight (2.60 g/plant and 3.95 g/plant, respectively) was recorded from the plants treated with bioinoculant AP (T_1). Dry weight was not significantly different (data not shown) for all treatments. These findings underscored that bioinoculants significantly promoted the growth of cowpea in the pot experiment under field conditions at 7, 14, 21, and 28 days compared with the controls (water or talcum only).

3.2 Effect of bioinoculants on soil fertility levels and soil pH

The collected soil samples were analyzed as per the manual of a Rapitest 1835 Digital 3-way soil analyzer and reported optimal levels of nitrogen (N), phosphorus (P), and potassium (K) in the range of 50-200 ppm, 4-14 ppm and 50-200 ppm, respectively. Results of soil fertility properties showed that on day 0, soil Nitrogen and potassium levels were 74-83 ppm (Table 3) and these mineral levels significantly increased to 134-158 ppm on day 7; however, on day 21-28, the level of Nitrogen and potassium declined to 80-140 ppm. Likewise, the level of phosphorus followed the same trend (Table 3) and on day 0, the P level was 5.6-6.2 ppm which was significantly increased on day 7 (9.6-11.2 ppm). However, on day 28, the P level dropped to 6-10 ppm (Table 3).

The case of optimal soil pH for general plants ranged from 6.5-7.0. On day 0, soil pH ranged from 6.1-6.8 (normal), but after 7 days there was a significant decrease in soil pH ranging from 5.5-6.0, indicating increased acidity (Table 4). On days 14 and 21, soil pH increased to neutral at 7.0 with no significant difference in all treatments, while on day 28 the pH decreased to a weakly acidic level of 5.93-7.13 (Table 4).

Table 4 Effect of bioinoculants on soil pH in the pot experiment

Treatment	Soil pH				
	Day 0	Day 7	Day 14	Day 21	Day 28
AP (T_1)	6.8 ± 0.0^{a}	6.0 ± 0.3^{a}	7.00 ± 0.00	7.00 ± 0.00	5.93 ± 0.12^{c}
AS (T_2)	5.8 ± 0.3^{e}	5.7 ± 0.0^{a}	7.27 ± 0.46	6.60 ± 0.10	6.00 ± 0.00^{c}
AX (T_3)	6.1 ± 0.1^{de}	5.7 ± 0.1^{a}	7.00 ± 0.00	7.00 ± 0.00	6.00 ± 0.00^{c}
AP+ AS (T_4)	6.3 ± 0.1^{cd}	5.5 ± 0.1^{b}	7.00 ± 0.00	7.00 ± 0.00	5.90 ± 0.17^{c}
AP+ AX (T_5)	6.4 ± 0.1^{bcd}	5.7 ± 0.2^{a}	6.93 ± 0.12	6.60 ± 0.40	6.07 ± 0.12^{c}
AS+ AX (T_6)	6.3 ± 0.1^{cd}	5.5 ± 0.1^{b}	7.00 ± 0.00	7.00 ± 0.00	6.10 ± 0.17^{b}
AP+ AS+ AX (T_7)	6.5 ± 0.1^{abc}	5.7 ± 0.2^{a}	7.10 ± 0.17	6.90 ± 0.10	6.20 ± 0.00^{b}
Water (T_8)	6.2 ± 0.1^{cd}	5.7 ± 0.2^{a}	7.00 ± 0.00	7.00 ± 0.00	6.67 ± 0.58^{ab}
Talc (T_9)	6.3 ± 0.1^{cd}	5.8 ± 0.1^{a}	7.00 ± 0.00	7.00 ± 0.00	7.13 ± 0.23^{a}

* Disinct superscripts in columns represent statistically significant differences ($p< 0.05$).

Table 5 Microbial counts of bioinoculants stored at 4°C and 35 °C (room temperature)

Treatment	Day 0	Microbial counts (CFU/mL) at 4°C				Microbial counts (CFU/mL) at 35°C			
		Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
AP (T_1)	8.68 ± 0.06^{b}	8.71 ± 0.04^{b}	8.78 ± 0.03^{b}	8.83 ± 0.01^{a}	8.85 ± 0.08^{a}	8.85 ± 0.19^{b}	8.98 ± 0.08^{b}	8.99 ± 0.09^{b}	9.19 ± 0.14^{a}
AS (T_2)	8.57 ± 0.04^{b}	8.60 ± 0.05^{b}	8.62 ± 0.02^{b}	8.71 ± 0.02^{a}	8.67 ± 0.01^{a}	8.68 ± 0.01^{b}	8.83 ± 0.06^{a}	8.91 ± 0.16^{a}	9.04 ± 0.08^{a}
AX (T_3)	8.71 ± 0.08	8.70 ± 0.07	8.70 ± 0.09	8.63 ± 0.03	8.59 ± 0.12	8.90 ± 0.09^{b}	9.08 ± 0.01^{a}	9.21 ± 0.06^{a}	9.22 ± 0.13^{a}
AP+ AS (T_4)	8.82 ± 0.10	8.78 ± 0.09	8.80 ± 0.08	8.81 ± 0.07	8.85 ± 0.06	8.86 ± 0.12^{b}	8.99 ± 0.10^{b}	9.19 ± 0.16^{a}	9.35 ± 0.09^{a}
AP+ AX (T_5)	8.58 ± 0.12^{b}	8.60 ± 0.08^{b}	8.62 ± 0.11^{b}	9.00 ± 0.05^{a}	9.05 ± 0.04^{a}	8.78 ± 0.21^{b}	8.84 ± 0.20^{b}	9.38 ± 0.04^{a}	9.43 ± 0.04^{a}
AS+ AX (T_6)	8.60 ± 0.02^{b}	8.63 ± 0.01^{b}	8.62 ± 0.03^{b}	9.01 ± 0.05^{a}	9.07 ± 0.08^{a}	8.78 ± 0.02^{b}	8.92 ± 0.02^{b}	9.34 ± 0.04^{a}	9.46 ± 0.02^{a}
AP+ AS+ AX (T_7)	8.68 ± 0.06^{b}	8.62 ± 0.04^{b}	8.67 ± 0.05^{b}	8.98 ± 0.03^{a}	8.99 ± 0.09^{a}	8.95 ± 0.10^{a}	8.98 ± 0.08^{a}	9.00 ± 0.05^{a}	9.28 ± 0.03^{a}

* Disinct superscripts in rows represent statistically significant differences ($p< 0.05$).

3.3 Microbial counts of bioinoculants under different storage conditions

Microbial counts of bioinoculants during the storage conditions were carried out at 4°C and 35°C (room temperature) for 28 days. For this, bioinoculants along with talc powder are kept in a small glass jar at the selected temperatures. At 4°C, microbial counts were relatively stable at every **7** days at log 8-9 CFU/mL. On day 21st and 28th, except for AX (T_3) and AP + AS (T_4), the microbial counts were increased in most bioinoculant formulations (Table 5). Further, in the case of room temperature (35°C), microbial counts increased after **7** days in all bioinoculant formulations (Tables 5). This indicated that bioinoculants can be stored in a refrigerator or at room temperature in Thailand. This is convenient for farmers who do not need to use expensive instruments to store bioinoculants for at least a month.

4 Discussion

This is the first report demonstrating the utilization of three potential soil bacteria isolated from Na Si Nuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand. Bioinoculants from soil bacteria enhanced the growth of cowpea under laboratory and field conditions. Results of the study showed that phytase (AP) and chitinase-producing (AS) bacteria were the most effective in promoting the growth of cowpea in the soil during the selected experimental periods. The *A. pittii* PT1.3.4 bacteria produced phytase which degrades phosphorus into more readily usable forms, thereby facilitating plant growth, photosynthesis, biochemical oxidation, nutritional absorption, and plant cell division.

Our results were consistent with recent findings of Yaghoubi Khanghahi et al. (2021), which found phosphate solubilizing

capacity in *A. pittii*, *Acinetobacter calcoaceticus*, *A. oleivorans*, *Acinetobacter* sp., and *Pseudomonas alcaligenes*. In this study, all soil pH values following bioinoculant treatment on day 28 exhibited a shift toward the mild acidic range, indicating that organic acid exudation is involved in phosphate solubilization. Expectedly, the control groups had the highest pH levels (talc and water). In a recent study, Yaghoubi Khanghahi et al. (2021) also determined *A. pittii* JD-14 as the most active strain in improving the biomass of alfalfa at Hada Al Sham in Saudi Arabia.

The bioinoculant strain *Achromobacter* sp. C2.23 produces chitinase which can be able to degrade fungal and insect cell wall chitin into amino acids and nitrogenous sources for plants. Phosphate-solubilizing bacteria release gluconic, acetic, and oxalic acids, as well as quinic and succinic acids while growing *in vitro*. Organic acids generated on the outside face of the cytoplasmic membrane were shown to be the product of direct oxidation in an acidic (lower pH) environment (De Amaral Leite et al. 2020). An association has been also found between the pH drop and phosphorus solubilization in the liquid culture medium, as shown by the results of the pot experiment in this study where soil pH was reduced due to added bioinoculants.

In this study, N, P, and K contents in soil increased from day 1 to day 7 in every treatment, including the control. The used cultivation soil was of good quality at the start of the experiment. However, ketogluconic, oxalic, gluconic, and succinic acids, as well as other organic acids, were released into the soil over time, causing the pH of the soil to decrease with time.

Plant root exudates and decaying microbial matter are the two most important sources of organic acids in soil (Paul et al. 2021). Bacterial, fungal, and lichen species are significantly contributed to the release of organic acids into the soils. Decomposition of organic waste also adds to high levels of organic acids in the soil and improves the habitat for plants (Paul et al. 2021). Free phosphorus (P) can be released from complex mineral P such as hydroxyapatite or tricalcium phosphate by organic acid-aided chelation of divalent cations (e.g. Ca^{2+}). Thus, plants can take up free P more readily. Several gram-negative bacteria such as *Acinetobacter* sp. SK2 undergoes periplasmic glucose oxidation through pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) enzyme to produce gluconate that can be metabolized or enhanced the P solubilization effects (Bharwad and Rajkumar 2020).

The bioinoculant that contained nitrogen-fixing bacterial strain *A. xylosoxidans* N3.4 created soil acidic. Nitrogen-fixing bacteria are known to convert ambient nitrogen (N_2) into plant-utilizable ammonia (NH_3). This process consumes H^+ and, as a result, changes the pH. Certain NFB strains created neutral pH whereas others produced acidic pH (Oliveira et al. 2017). The pH discrepancies between NFB isolates might be attributed to differing N_2 fixing mechanisms across bacterial taxa (Das et al. 2022).

Strain AP gave the highest growth, shoot, and fresh weight of cowpea on ½ strength MS media in laboratory conditions; however, combined strains AP + AX gave the longest roots. Likewise, for cowpea growth in soil under the field condition, on day 28, strain (AP) recorded the highest growth, shoot, and fresh weight. These results are in agreement with the findings of Daur et al. (2018), who found that introduction of *Achromobacter* sp. 5B1 in *Arabidopsis thaliana* resulted in a fourfold increase in lateral root number and density, which is associated with a significant increase in the overall fresh shoot and root weight. Increased synthesis of photosynthetic pigments occurred as a result of microbial inoculation. Plants with more chlorophyll have a higher photosynthetic rate, which transforms more carbon dioxide and water into glucose, increases metabolic activity and subsequently improves plant development (Kumawat et al. 2022). *Achromobacter* sp. 5B1 has been reported to promote root development of *A. thaliana* via auxin signaling and redistribution (Jiménez-Vázquez et al. 2020). Further, the results of the study suggested that the combination of AS + AX bacterial strains and AP + AS + AX strains produced the highest mineral levels in the soil.

Jha and Kumar (2009) suggested that *A. xylosoxidans* produces indole acetic acid, nitrogenase activity, and phosphate solubilization which may explain the plant development potential of this genus. Similarly, Wang et al. (2020) established the indole-3-acetic acid excretion capabilities of *A. xylosoxidans* GD03 which help in the rice growth. Under drought stress, bioinoculants of the bacteria *E. cloacae* and *A. xylosoxidans* boosted growth, gas exchange characteristics, and nutrient concentrations in shoots and grains, as well as yield in maize (Danish et al. 2020). Additionally, inoculation of PGPR increased the amount of N, P, K, and Mg, these PGPR also improved a variety of processes including increased nutrient availability, greater nutrient absorption, and healthier root development. By releasing regular organic acids, PGPR boosts nutritional availability by solubilizing nutrients (Cataldi et al. 2020).

Conclusions

Results of the study can be concluded that the three bacterial strains namely *A. pittii* PT1.3.4 (AP), *Achromobacter* sp. C2.23 (AS) and *A. xylosoxidans* N3.4 (AX) have the highest potential for producing agriculture and can be used as bioinoculants to enhance cowpea growth. Talcum-based bioinoculants showed positive results on cowpea growth when they were applied to the seeds before planting in the soil. Inoculated plants thrived in cultivated soils significantly better than the controls. Local bacteria can be

used to promote the growth of cowpea, thereby mitigating the cost of chemicals to cultivate local crops in a safe environment for both farmers and consumers. Thai agriculture can develop toward sustainability using biological methods.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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