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Effect of phosphate solubilizing and nitrogen-fixing bacteria on Pontianak siam citrus (*Citrus ×nobilis var. microcarpa*) seed germination

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Abstract. *Adhyaningtyas RFI, Rahmawati, Mukarlina. 2023. Effect of phosphate solubilizing and nitrogen-fixing bacteria on Pontianak siam citrus (*Citrus ×nobilis var. microcarpa*) seed germination. Nusantara Bioscience 15: 245-250.* West Kalimantan is one of Indonesia's largest producing areas for Siam citrus (*Citrus ×nobilis var. microcarpa* Hassk.). It is necessary to carry out the proper treatments within the early stages of planting, which increases the speed of germination of citrus seeds to quickly increase the number of seedlings. The biological agents that play a role in promoting plant growth are a group of Plant Growth-Promoting Rhizobacteria (PGPR), consisting of Phosphate-Solubilizing Bacteria (PSB) and Nitrogen-Fixing Bacteria (NFB). This study aims to determine the effect of a combination treatment or a single treatment of phosphate-solubilizing and nitrogen-fixing bacteria, which is known to obtain the best results in the germination and growth of Siam citrus seeds. The study was conducted in a nonfactorial, Completely Randomized Design (CRD). The given treatments were control (without bacterial inoculum), nitrogen-fixing bacteria (NFB P1), phosphate solubilizing bacteria (PSB P2), a combination of NFB and PSB bacteria with a ratio of 1:1 (P3), a combination of NFB and PSB bacteria with a ratio of 2:1 (P4), and the combination of NFB and PSB bacteria with a ratio of 1:2 (P5). Observations were made for 4 weeks after planting. A single treatment with phosphate-solubilizing bacteria (P2) gave the best result on almost all parameters, such as germination percentage, emergence time of germination, root length, number of leaves and fresh weight of Siam citrus seedlings.

Keywords: Germination, nitrogen-fixing bacteria, phosphate-solubilizing bacteria, Pontianak Siam citrus

INTRODUCTION

Pontianak Siam citrus (*Citrus ×nobilis var. microcarpa* Hassk.) is a horticultural crop with a high production rate in Indonesia, among 160 other citrus cultivars (Dharmawan et al. 2009). West Kalimantan is one of the largest citrus-producing areas in Indonesia. It is known by its trade name, Pontianak citrus, which refers to the type of Siam citrus. Siam citrus production in West Kalimantan continues to increase yearly, reaching 1,190,770 quintals produced in 2018. The Pontianak Siam citrus is a type of citrus that is consumed widely and has a distinctive sweet taste (Sa'adah et al. 2022). Siam citrus also shows a sweet aroma and taste, thinned skin, and high water content (Wahibah et al. 2011). Siam citrus is a vitamin C source and contains various compounds such as glycemic and non-glycemic carbohydrates, potassium, folate, calcium, thiamin, niacin, vitamin B6, phosphorus, magnesium, riboflavin and other nutritional content (Ahmed and Saeid 2021).

Propagation of citrus plants is done in generative and vegetative methods. The generative method uses seeds as a propagation agent, while the vegetative method uses the vegetative parts of plants for plant propagation. Citrus growers commonly use the vegetative method by grafting two parts of the plant, scion and rootstock. Rootstocks are produced through certified source seeds or from planting seeds from trees to be propagated (Singh et al. 2018; Albrecht et al. 2019).

The rootstock production through seeds sown begins with the germination process on the growth media. Generally, the seeds begin to germinate perfectly on day 15, 18 to 45 days, depending on the type of citrus seeds sown (Neto et al. 2016; Prajapati et al. 2017). Several experiments were conducted to accelerate the germination of citrus seeds. Qessaoui et al. (2020) conducted research using *Pseudomonas* Migula 1894 to accelerate germination time within 14 days of germination. Bacteria that can increase plant growth are known as the Plant Growth Promoting Bacteria (PGPB), commonly found in the rhizosphere area, also known As Plant Growth-Promoting Rhizobacteria (PGPR). PGPR generally consists of bacteria that can provide plant nutrients, such as nitrogen-fixing, phosphate-solubilizing, and other nutrient-providing bacteria (Noumavo et al. 2013).

PGPR treatment with single kind action of bacteria has been shown to increase several growth parameters such as root length, seedling height, dry and fresh weight, germination percentage, number of leaves and number of flowers that tested on sorghum plants (Widawati and Suliasih 2018), *Vicia faba* L. beans (Demissie et al. 2013), and rice (Bandeppa 2022). Wahyuni et al. (2020) conducted similar research using Banjar siam citrus, the same genus of orange, in different locations, yet resulting insignificantly in effect on plant growth. Therefore, nitrogen-fixing bacteria and phosphate-solubilizing bacteria were chosen in the Pontianak Siam seed germination test to obtain good quality rootstock with shorter germination time.

MATERIALS AND METHODS

Preparation of bacterial inoculum

The isolates of Phosphate-Solubilizing Bacteria (PSB) and Nitrogen-Fixing Bacteria (NFB) used in this experiment were obtained from the Microbiology Laboratory, Department of Biology, Universitas Tanjungpura, Pontianak District, Indonesia. This isolate was isolated from the rhizosphere of citrus plants in Siam citrus plantations in the Singkawang area, West Kalimantan, Indonesia. The isolates were first propagated in the Nutrient Broth (NB) media and grown until the cell density reached 10^8-10^9 cells/mL or equivalent to 0.5 Optical Density (OD).

Seed extraction

Seeds were obtained from Siam citrus selected directly from Siam citrus plantations. Seeds were taken aseptically from the pulp using sterile tweezers. The removed seeds are then washed in water until there is no mucus covering the seeds. During this immersion, the seed viability was also selected with the criteria that the seeds were not flat, hollow, or floating. Then, the seeds were soaked in water at 52˚C to prevent Phytophthora sp. growth for 10 min.

Preparation of planting media

The planting medium used in the germination and growth of Siam citrus seeds is peat soil, which has been neutralized for its pH to 6.5 with dolomite. The peat soil was then sterilized in an autoclave at 121˚C for 15 min. Then, the soil was divided into 500 grams for each pot.

Making PSB and NFB cultures and treatment applications

The treatments given to the germination of Siam citrus seeds were P0 (control); P1 (NFB 100%); P2 (PSB 100%); P3 (NFB: PSB = 1:1); P4 (NFB: PSB = 2:1); and P5 (NFB: PSB = 1:2). One treatment was carried out in 10 replications, each replication contains 4 seeds. The inoculum made for one treatment was 5 mL. Therefore, each seed was inoculated with 0.125 mL culture with a cell density of 10^8 cells/mL or equivalent to 0.5 OD (Rahman et al. 2015) on nutrient broth media. Applying treatment to seeds is carried out every three days (Widyaningsih et al. 2020). Plant nurture was carried out daily by watering and weeding the weeds on the soil. Planting and observations were carried out 4 weeks after planting (WAP).

Seedling germination, growth, and parameters observation

Parameters observed in this experiment were germination percentage, sprout time emergence, the height of Siam citrus seeds, number of leaves, root length, and fresh weight. Parameters of seedling height, the number of leaves, root length, and wet weight were carried out at the end of the observation, which was four weeks after planting. Germination percentage and time of emergence were observed every day until the end of planting. The data of these two parameters are calculated by the equation below (Guragain et al. 2021).

$$\textit{Sprouts time emergence} = \frac{N1T1+N2T2+.....+NxTx}{\textit{Total sprouting seeds}}$$

Information:
N : Number of sprouts that appear in a certain time unit
T : Day of observation

$$\textit{Germination Percentage (\%)} = \frac{\textit{Total sprouts}}{\textit{Total potted seeds}}$$

Data analysis

The data obtained from observations during 4 WAP on the Siam citrus plants were then analyzed using Analysis of Variance (ANOVA) to determine the effect of the treatment of suspension of phosphate-solubilizing and nitrogen-fixing bacteria on the germination of Siam citrus seeds. Results that have an effect will be followed by Duncan's test with a level of 5% in differentiating several treatments.

RESULTS AND DISCUSSION

Germination percentage

Germination percentage with the treatment of phosphate-solubilizing and nitrogen-fixing bacteria in single or combined forms was not significantly different from the control ($P \geq 0.05$). Between treatments, P2 (PSB 100%) and P0 (Control) had a similar and highest percentage value among the other treatments. The combination treatment (P3, P4, and P5) and the single treatment of 100% NFB (P1) were not able to increase the germination percentage on the last day of observation (Table 1).

Time of emergence of sprouts

The time of emergence of sprouts with the treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria in treatment P1 (100% NFB) gave significantly different results at the 5% confidence level ($P \leq 0.05$) for all treatments with an average germination day of 10, 45 days. Single treatment of phosphate solubilizing bacteria (P2) and combination treatment (P3 and P4) had a higher average germination day than P0 (Control), which was 14 days. The P5 treatment (NFB 1: PSB 2) took longer for sprouts to emerge than all treatments (Table 2).

Seedling height

Parameters of seedling height with the administration of phosphate-solubilizing bacteria and nitrogen-fixing bacteria either in single or combined treatments could not give a significant difference at the 5% confidence level ($P \geq 0.05$) to the Control treatment (P0). Based on the values resulting from each treatment, it was shown that treatment P1 (NFB 100%) was the seed with the longest height among the other treatments, which was 6.28 cm, followed by treatment P4 (NFB 2 : PSB 1), which had a seed length of 6.16cm. Treatments P5 (NFB 1 : PSB 2) and P0 (Control) were included in the lowest average length of seedlings (Table 3).

Number of leaves

The average number of leaves in treatment P2 (PSB 100%) was significantly different from treatments P1 (NFB 100%) and P3 (NFB 1 : PSB 1) but not significantly different from treatment P0 (Control), P4 (NFB 2 : PSB 1), and P5 (NFB 1 : PSB 2). Among the P0 treatment (Control) with the treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria, both in a single form and in combination, the average value of the highest number of leaves sequentially was P2 (PSB 100%) as many as 15.1 strands, then followed by the treatment P0 (control) was 14.0 strands, P4 (NFB 2 : PSB 1) was 13.4 strands, and in P5 (NFB 1 : PSB 2) was 13.0 strands. The lowest average number of leaves was shown in treatment P1 (100% NFB), with 12.0 leaves and in treatment P3 (NFB 1 : PSB 1), with 11.4 leaves (Table 4).

Root length

The root length parameter from the results of the analysis of variance with a confidence level of 5% in treatment P1 (NFB 100%) was significantly different from treatment P3 (NFB 1 : PSB 1) and P5 (NFB 1 : PSB 2), but not significantly different from treatment P0 (Control), P2 (PSB 100%), and P4 (NFB 2 : PSB 1) ($P \leq 0.05$). When compared based on the root length value of each treatment, the highest root length value was found in treatment P1 (100% NFB), which was 6.34 cm, and P2 (100% PSB) was 6.04 cm (Table 5).

Fresh weight

All treatments showed that either single or combined treatment of phosphate-solubilizing bacteria showed no significant difference ($P \geq 0.05$). Based on a comparison of the average wet weight of Siam citrus seedlings, P2 (PSB 100%), P4 (NFB 2 : PSB 1), and P5 (NFB 1 : PSB 2) treatments were the highest wet weight. The lowest average wet weight was shown by treatment P0 (Control) and P3 (NFB 1 : PSB 1) (Table 6).

Table 1. Germination percentage (%) Siam citrus seeds

Treatment	Germination Percentage (%)
P0 (Control)	100.00 ± 0.00^{b}
P1 (NFB)	90.00 ± 12.90^{a}
P2 (PSB)	100.00 ± 0.00^{b}
P3 (NFB 1 : PSB 1)	97.50 ± 7.90^{ab}
P4 (NFB 2 : PSB 1)	92.50 ± 12.07^{ab}
P5 (NFB 1 : PSB 2)	97.50 ± 7.90^{ab}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level through Duncan's test

Table 2. The average time of emergence (days) of Siam citrus seeds

Treatment	Average Time of Sprouts Emergence (Days)
P0 (Control)	15.72 ± 5.06^{a}
P1 (NFB)	10.45 ± 1.62^{b}
P2 (PSB)	14.90 ± 3.58^{a}
P3 (NFB 1 : PSB 1)	14.50 ± 3.97^{a}
P4 (NFB 2 : PSB 1)	14.48 ± 3.69^{a}
P5 (NFB 1 : PSB 2)	16.18 ± 3.27^{a}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level through Duncan's test

Table 3. Average seed height (cm) Siam citrus

Treatment	Average Height of Siam Citrus Seeds (cm)
P0 (Control)	5.84 ± 0.87^{a}
P1 (NFB)	6.28 ± 0.69^{a}
P2 (PSB)	5.91 ± 1.03^{a}
P3 (NFB 1 : PSB 1)	5.93 ± 0.85^{a}
P4 (NFB 2 : PSB 1)	6.16 ± 0.82^{a}
P5 (NFB 1 : PSB 2)	5.82 ± 0.61^{a}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Table 4. Average number of leaves (strands)

Treatment	Average Number of Leaves (Strands)
P0 (Control)	14.0 ± 2.53^{ab}
P1 (NFB)	12.0 ± 2.70^{a}
P2 (PSB)	15.1 ± 2.60^{b}
P3 (NFB 1 : PSB 1)	11.4 ± 1.83^{a}
P4 (NFB 2 : PSB 1)	13.4 ± 4.22^{ab}
P5 (NFB 1 : PSB 2)	13.0 ± 2.78^{ab}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Table 5. Average root length (cm)

Treatment	Average Root Length (cm)
P0 (Control)	5.92 ± 0.97^{ab}
P1 (NFB)	6.34 ± 0.51^{b}
P2 (PSB)	6.04 ± 1.22^{ab}
P3 (NFB 1 : PSB 1)	5.15 ± 0.76^{a}
P4 (NFB 2 : PSB 1)	5.53 ± 0.92^{ab}
P5 (NFB 1 : PSB 2)	5.30 ± 1.07^{a}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Table 6. Average height, number of leaves, root length, and wet weight of Siam citrus seeds

Treatment	Average Wet Weight of Siam Citrus Seeds (g)
P0 (Control)	0.22 ± 0.06^{a}
P1 (NFB)	0.23 ± 0.36^{a}
P2 (PSB)	0.26 ± 0.56^{a}
P3 (NFB 1 : PSB 1)	0.22 ± 0.56^{a}
P4 (NFB 2 : PSB 1)	0.25 ± 0.34^{a}
P5 (NFB 1 : PSB 2)	0.24 ± 0.47^{a}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Discussion

Germination of seeds generally depends on several environmental factors: seed quality, growing media, temperature, light and moisture of the surroundings. The moisture content of the planted seeds must not be lower than 6% to make it not inhibitory to germinate. The seed must be kept moist by using the seeds as soon as possible after extracting it from the host fruit. Seed requires a humid environment to germinate; hence, the first thing to concern is the storage of the seeds. The germination will be delayed if the seeds are left dry (Orbović et al. 2013). The other factor, growing media, determines the germination's moisture level. The germination process needs water to end the dormancy and start imbibition (Haj Sghaier et al. 2022). The proper growing media that keeps the moisture content could increase the seed's and seedlings' germination percentage and plant height (Handayani and Yuzammi 2019). Temperature, light intensity and other limiting factors carried the germination process then impacted the quantity of seedlings' height, root length, number of leaves, and fresh weight.

Treatment of 100% phosphate-solubilizing bacteria (P2) gave the best results in the parameter of germination percentage, number of leaves, and fresh weight of seedlings. The results obtained in the P2 treatment indicated the presence of P content in the organic and inorganic soil, which had been dissolved by phosphate-dissolving bacterial agents from the rhizosphere of Siam citrus. Phosphate content in the growing media becomes available and can be used by plants as a supply of macronutrients needed, thus increasing plant biomass distributed in plant parts such as crowns, roots, and leaves. Phosphate solubilizing bacteria cause the availability of P to be absorbed by plants by producing several compounds that play a role in dissolving P that is not dissolved in the soil. Several compounds produced by phosphate-solubilizing bacteria, such as organic acids, exopolysaccharides, and other enzymes, will make P available to plants (Sharma et al. 2013). Soil contains phosphate, which binds to cations such as Ca^{2+}, Al^{3+} and Fe^{3+}. This cation forms a chelate with the carboxyl and hydroxyl groups of organic acids produced by phosphate-dissolving bacteria and releases phosphate bonds into a dissolved form (Kalayu 2019). The roots will absorb the available P element. Element P is channeled through the stem to the leaves to help the photosynthesis process in the leaves. Hormones produced by bacteria also influence increased growth by phosphate-solubilizing bacteria. Plant roots produce exudates in the form of tryptophan, which acts as a signaling molecule to produce IAA (Tariq and Ahmed 2022). Phosphate-solubilizing bacteria *Bacillus* Cohn 1872 and *Pseudomonas* produced the auxin, increasing plant mass and plant root growth (Kudoyarova et al. 2017). In another study, *Bacillus* sp. produces different types of growth hormones: IAA, jasmonic acid, and gibberellic acid (Mažylytė et al. 2022). In addition, ACC deaminase, the key enzyme synthesized by phosphate-solubilizing bacteria, hydrolyzed the precursor of ethylene (ACC), plant growth inhibitory agents (Zaidi et al. 2009).

The success of a single treatment of phosphate-solubilizing bacteria was shown by the research of Viruel et al. (2014), which can increase the germination parameters and plant height of corn. This was also shown in the increase in the number of leaves by the 100% PSB treatment, which was caused by the available P absorption activity. The increase in wet weight by adding phosphate-solubilizing bacteria has also been proven in the research by Ehsan et al. (2014), who tested on wheat plants. Demissie et al. (2013) tested seed germination of *V. faba* bean with the addition of phosphate-solubilizing bacteria, resulting in increased germination. Gholami et al. (2009) suggested that these bacteria can synthesize a hormone that stimulates seed germination, the gibberellin hormone. Gibberellin hormone triggers the activity of specific enzymes that support germination, α-amylase, which increases starch assimilation.

Treatment of 100% nitrogen-fixing bacteria (P1) can produce the best parameter values on the time of sprout emergence, seedling height, and root length. The average germination time by this treatment gave the best value among the other treatments, which was 10.45 DAP. It is reported that citrus seed's germination time is 18 and 22 days on paper substrate (Ragagnin et al. 2022). Similar results were also found in the study of Naing and Zin (2013), which utilized nitrogen-fixing bacteria from the genus *Azotobacter* Beijerinck 1901 and *Azospirillum* Tarrand et al. 1979 in rice plants, resulting in an increase in plant height growth on the 30th day after planting, root length on the 40th day after planting, and wet weight. Plants on the 20th day after planting compared to the control treatment.

The combined treatment consisting of P3 (NFB and PSB 1:1), P4 (NFB and PSB 2:1) and P5 (NFB and PSB 1:2) could not give the best results for all parameters. This is thought to be caused by several conditions, namely competition between bacteria in different growth media, differences in elemental composition found between peat soil media and adapted bacterial growth media, and differences in the growth phases of two different bacteria. The results in this study are contrary to the findings of Li et al. (2020), which resulted in increased yields for all growth parameters in a consortium treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria in wheat plants. This difference is thought to cause competition between the bacterial consortia in the new substrate, although the synergism test results show that the two isolates can grow together in Nutrient agar media. This statement is supported by Deng and Wang (2016) that differences in substrate composition will affect bacterial interactions in their research using bacteria grown on two different types of media, but in one of the media, it causes competition between bacteria. The competitive result between bacteria and host plants for the source of needed elements, such as nitrogen, has been recorded by Kuzyakov and Xu (2013). It stated that one of the factors of competition between microorganisms and plants is the different stages of nutrient demand of each organism. When the plant is on a high level of N demand, it will decrease the microorganism uptake of N nutrients.

In conclusion, some growth parameters were affected by the single treatment, P1 (nitrogen-fixing bacteria) and P2 (phosphate-solubilizing bacteria). P2, which consists of singular phosphate-solubilizing bacteria, gives the best results on citrus seed germination and growth, especially on germination percentage, sprout time emergence, number of leaves, and fresh weight. P1, consisting of singular nitrogen-fixing bacteria, only gives the best seedling height and root length results. This means that singular treatment was better than three variations of combined treatment. Chemical and molecular tests can identify those potential bacteria (PSB and NFB) and could be used to make biofertilizers to utilize the bacterial function.

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