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Fruit Morphology, Antioxidant Activity, Total Phenolic and Flavonoid Contents of *Salacca zalacca* (Gaertner) Voss by Applications of Goat Manures and *Bacillus velezensis* B-27

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Abstract

Snake fruit (*Salacca zalacca* (Gaertner) Voss) is one of indigenous fruits from Southeast Asia that has been consumed for its antioxidant contents. Improving the fruit quality might increase its benefits for human health. This study aims to analyze fruit morphology, antioxidant activity, total phenolic and flavonoid contents of the fruit applied with goat manures and *Bacillus velezensis* B-27. The research used two-factor Randomized Complete Block Design (RCBD) with three blocks as replications. The first factor is the level of goat manure application i.e. 0 kg plant^{-1}, 5 kg plant^{-1} and 10 kg plant^{-1}, while the second factor is the bacteria application i.e. applied with *Bacillus velezensis* B-27 and without *Bacillus velezensis* B-27. The research was conducted in June until December 2019 at Turi, Sleman, Yogyakarta, Indonesia. Fruit length, diameter and shape were measured as morphology parameters. Antioxidant activities were measured using 2,2-Diphenyl-1-picrylhydrazyl, whereas flavonoid and phenolic contents were determined by the aluminium-chloride colorimetric and Folin-Ciocalteu methods, respectively. Means of each parameter were analyzed using ANOVA, continued with the Tukey's HSD test at a 5% significance level. The result showed that the application of 10 kg goat manure per plant with bacteria increased the fruit length (76.78 mm) and the diameter (62.72 mm). Addition of 10 kg goat manure per-plant combined with *Bacillus velezensis* B-27 gave the highest antioxidant (IC50 of 37.83 µg mL^{-1}), flavonoid (5.35 mgGAE 100 g^{-1}) and total phenolic contents (44 mgQE 100 g^{-1}).

Keywords: antioxidant; flavonoid; goat manure; indigenous fruits; phenolic; snake fruit

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INTRODUCTION

Salacca zalacca (Gaertner) Voss, usually called snake fruit, is an indigenous exotic fruit from Southeast Asia such as Indonesia, Malaysia, Thailand and Brunei Darussalam that belongs to the palm group (Family: *Arecaceae*). It has reddish-brown scaly skin like snake and creamy-white fruit, which has honey-like taste (Saleh et al., 2018). In Indonesia, the snake fruit is an important fruit commodity, with the total production of snake fruit of 2.896 quintal year^{-1} (BPS - Statistics Sleman Regency, 2018). This underutilized fruit remains an issue about future sustainable utilization and commercial value enhancement, which the fruits are actually popular across international markets within South East Asia (Mazumdar et al., 2019).

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One of the problems is the lack of fruits quality because of inadequate cultivation practices, where the farmers rarely give attention to soil nutrition needed for plant growth and development. It also results in soil fertility and fruit productivity. The farmers have only used snake fruit leaves that have dried up as fertilizer (Adijaya and Yasa, 2015). Therefore, it is necessary to increase the fertility of land that utilizes familiar materials, such as the use of manure. There is goat manure, a kind of fertilizer that is important for increasing the growth of plant (Hariadi et al., 2016). The goat manure contains the highest macronutrients contents, such as 2.77% nitrogen, 1.78% P_2O_5 and 2.88% K_2O, among other animal manures (Chatzistathis et al., 2020). Mowa et al. (2017) have suggested that organic nutrients from goat manure can improve plant growth and yield performance of tomato. Nitrogen in fertilizer is one factor that affects the quality of the fruits (Lyu et al., 2019).

Moreover, the snake fruit is an excellent antioxidant source whose activities are higher compared to kiwifruit (Gorinstein et al., 2009; Saleh et al., 2018). Tilaar et al. (2017) have stated that several studies have proved that snake fruit has antioxidant activity that can be used for any purposes. The methanol extracts of the fruits showed a high antioxidant activity accompanied by its anti-hypertensive and anti-diabetic effects (Tan et al., 2020). Suica-Bunghez et al. (2016) revealed that the antioxidant activity of snake fruit is caused by the content of polyphenol, flavonoid, tannin and monoterpenoid compounds. Increasing the group of these chemical compounds will gain the benefits for the consumer of the fruit. However, the use of synthetic chemicals to improve fruit yield and phytochemical contents of snake fruits is known to have adverse effects both on health and environment. Thus, several studies to improve yield and quality of snake fruits by applying eco-friendly treatment are advisable.

In terms of the antioxidant properties, the application plant growth-promoting rhizobacteria (PGPR), like *Bacillus* sp., can increase the antioxidant activity of fruit by increasing some metabolites level, such as phenolics and flavonoids (Rahman et al., 2018). Song et al. (2015) declared that PGPR can improve the plant root structure that gives positive effect on the fruit quality. In addition, *Bacillus velezensis*, an important PGPR, can promote the plant growth by producing some organic acids, ACC deaminase, the phytohormone indole-3-acetic acid (IAA) and siderophores, as well as and help nitrogen fixation and phosphate solubilization. Furthermore, the plants applied with the bacteria show growth and flowering improvement. Besides, PGPR exhibits antifungal activities and thus, has immense potency to support sustainable agriculture by minimizing fungicide use (Tiwari et al., 2019). According to Santos-sanchez et al. (2018), biosynthesis of antioxidant compounds, like polyphenol, flavonoid and tannin, occurs by shikimic acid pathway that begins with condensation of phosphoenolpyruvic acid, which is the result of glycolysis pathway. The pyruvate metabolism is one of important pathways in arillus of snake fruit (Fendiyanto et al., 2020). The nitrogen in the soil also gives some effects on glycolysis pathway that forms the pyruvate. Nevertheless, a few studies evaluate the metabolite levels as the results of fertilizer treatment (Iqbal et al., 2020).

Organic farming has become very interesting to change the old farming technique that uses non-natural chemicals in agricultural cultivation into an eco-friendly one. The organic farming also helps the consumers to obtain health organic products that can only be produced by organic farming method. However, in order to increase soil fertility, the use of organic matter still faces some challenges, such as a high ratio of organic fertilizer C/N, where the contents of complex compounds are higher than elements that can be absorbed by the roots of plants (Mayrowani, 2016). Therefore, combining fertilization with biological agent activities such as PGPR becomes one of the alternative solutions. Complex nutrients in manure will be broken down by PGPR as materials for metabolic activities so that there are elements that can be absorbed by the roots of plants. This combination is expected to increase the productivity and phytochemical properties of the snake fruit, without disrupting the environmental sustainability (Reddy et al., 2019; Sharma et al., 2019). However, studies investigating the effects of PGPR combined and goat manure to snake fruit are scarce, although it is highly important to increase fruit quality. Hence, in this present study, the combination of goat manure and PGPR that can improve the quality of snake fruits, both morphologically and for increasing its phytochemical contents is determined.

MATERIALS AND METHOD

Experimental site and materials

The experimental field of this study was located at “Si Cantik” organic snake fruit orchad, Ledoknongko Village, Turi, Sleman, Special Region of Yogyakarta, from June until December 2019 (7.650S and 110.360E). Meanwhile, the laboratory analysis was conducted in Horticulture Sub-laboratory, Faculty of Agriculture, Universitas Gadjah Mada.

The main materials used were the plants of snake fruit (*Salacca zalacca* var. pondoh) aged 20 years old, goat manures and *Bacillus velezensis*. The goat manures contained 0.32% of nitrogen, 24 ppm of phosphate, 0.59 Cmol kg^{-1} of potassium, with the pH value was 8.0 (Gichaba et al., 2020). The bacteria used in this study were *Bacillus velezensis* B-27 using gryB primers identification (Rahma et al., 2020).

Experimental design and procedures

Experimental design

The field experiment was laid out in a factorial randomized complete block design with two factors and three blocks as replications. The first factor was the level of goat manure application i.e. 0 kg $plant^{-1}$, 5 kg $plant^{-1}$ and 10 kg $plant^{-1}$. The second factor was *Bacillus velezensis* B-27 treatment i.e. *Bacillus velezensis* B-27 treatment and without bacteria treatment. There were six combinations of treatments for each block i.e. 0 kg goat manure per-plant without *Bacillus velezensis* B-27 (P0B0), 0 kg goat manure per-plant with bacteria (P0B1), 5 kg goat manure per-plant without bacteria (P1B0), 5 kg goat manure per-plant with bacteria (P1B1), 10 kg goat manure per-plant without bacteria (P2B0), 10 kg goat manure per-plant with bacteria (P2B1).

Procedures

Goat manure application

The goat manures were applied to the plants based on the experimental design. The manure application adopted the ring method developed by Adijaya and Yasa (2015) with several modifications, by making a circle hole around the tree approximately 50 cm from the base of the trunk. The goat manure was applied once after pollination using ring method at the day of pollination because nitrogen is one of factors affecting the viable of ovules including the fertilization (Hill-Cottingham and Williams, 2015).

Preparation and application of bacterial suspension

Bacillus velezensis B-27 isolates were cultured in 500 mL liquid media in the form of nutrient broth (NB) in conical tubes. Each tube was placed in a shaking incubator with a speed of 150 rpm and a temperature of 27°C for 48 hours for bacterial growth. The bacterial solution was then centrifuged at a speed of 12,000 g and the pellets were washed with distilled water for three times to remove the nutrients from the media. Bacterial pellets were suspended in water and diluted to a concentration of 1×109 $CFUmL^{-1}$. Bacterial application was performed by hoeing the soil near the roots and then the bacterial solution was splashed to the roots and leaked at the base of the stem near the roots. The *Bacillus velezensis* B-27 was treated once a week for seven times, began at the day the goat manure applied.

Fruit harvesting and extraction

Fruits were harvested 5.5 months after pollination when the smooth thorns of the fruit skin were starting rarely. Then, the fruits were extracted to ensure that polyphenol and flavonoid compounds could be separated from fruit tissue. The extraction also minimized the degradation of polyphenols due to phenolase enzyme activity. The solvent used was 96% ethanol with a volume of 2 mL for 2 g of fruit samples. As much as 2 g of snake fruit was crushed with pestle and mortar until smooth then dissolved in 2 mL of 96% ethanol. The fruit solution in ethanol was then heated with a temperature of 70°C for 1 hour and filtered using filter paper to produce fruit extracts. Extract storage was carried out at -20°C (Belwal et al., 2019).

Measurement of fruit morphology

Diameter, length and shape of fruit were measured as the morphology parameters. The diameter was determined from the horizontal axis of fruit, whereas length was measured by the vertical axis (Figure 1). The shape of the fruit was quantitatively measured by the scoring method. The score of the fruit shape was determined by giving a score of 1 for the fruit with flattened shape, while the score of 2 for oval shape and a score of 3 for round shape.

Good quality fruit has a shape that tends to be rounded so that it received the highest score (Nurrochman et al., 2013).

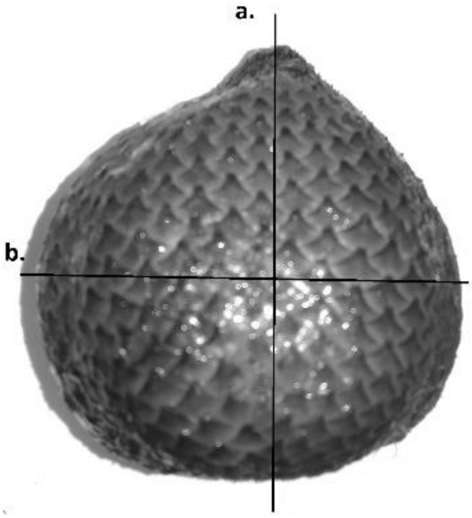


Figure 1. Morphology diagram of snake fruit: a. the fruit length, b. the fruit diameter

Antioxidant Activity Assay using 2,2-Diphenyl-1-picrylhydrazyl

The antioxidant activities were measured by modifying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Jimoh et al., 2019). Snake fruit extracts were made in seven concentrations, namely 120, 100, 80, 60, 40, 20 and 10 µg mL^{-1}. As much as 2 ml of snake fruit extract at each concentration was added with 2 mL of a 0.1 mM DPPH solution and then incubated for 30 minutes in a dark place. The absorbance of the solution was measured by a UV-Vis spectrophotometer with a wavelength of 517 nm. The value of % inhibition was then measured using absorbance values that have been measured by the formula:

$$\% \text{ inhibition} = [(A0 - As) / A0] \times 100\%$$

Note:

A0 = DPPH solution without sample

As = DPPH solution with sample

A regression equation between % inhibition and concentration was sought to measure the antioxidant activity. The antioxidant activity of the sample was expressed by the inhibition of free radical DPPH by 50% (IC50). IC50 value indicates a sample concentration that can inhibit DPPH free radicals by as much as 50%, so it can be used to show the antioxidant activity of a solution (X) from the regression equation. DPPH test with quercetin was also performed as a comparison.

Measurement of flavonoid content

Flavonoid content was measured by aluminium-chloride colorimetric method proposed by Chang et al. (2002), with modification. First, a standard quercetin curve was created by preparing six series of aqueous quercetin solution with the concentration of 20, 40, 60, 80, 100 and 120 µg mL^{-1}. Then, 0.5 mL of each solution was mixed with 1.5 mL ethanol 95%, 0.1 mL KCH_3COOH 1 M and 2.8 distilled water and then incubated for 30 minutes at 25°C. Absorbance of the solutions was measured by a UV-Vis spectrophotometer at a wavelength of 415 nm. The standard equation was determined by regression between the absorbance and concentration of quercetin. Second, flavonoid contents were measured by the same step with flavonoid content of quercetin. The value of flavonoid contents was determined using the standard equation and expressed as milligram flavonoid per-100 gram of sample (mgQE 100 g^{-1}).

Measurement of total phenolic content

Total phenol contents were determined using the Folin-Ciocalteu method with some modifications based on Zargoosh et al. (2019) with gallic acid as standard. A series of gallic acid with a concentration of 1.1; 2.2; 3.3; 4.4; 5.5; 6.6; 7.7; 8.8; 9.9; 10.10 and 11 mg mL^{-1} were prepared to make a standard curve. Briefly, each of standard (1 mL) and extract (1 mL) were placed in different test tubes and three mL of distilled water, 100 µl of Folin-ciocalteu reagent were added and mixed and then incubated for three minutes. Next, 300 µl Na_2CO_3 2% was mixed and then added with ethanol until reaching a total volume of 5 mL. After that, each solution was incubated for two hours at 25°C and absorbance was measured by a UV-Vis spectrophotometer at a wavelength of 760 nm. Total phenol content was expressed as milligram phenol per-100 gram of sample (mgGAE 100 g^{-1}).

Data analysis

Mean of each parameter was analyzed using Analysis of Variance (ANOVA) continued with Tukey's HSD test at 5% of significance level. Then, the antioxidant activity, flavonoid and total phenolic contents were correlated using correlation test with SPSS 16.0.

RESULTS AND DISCUSSION

Fruit morphology

Measurement of fruit length, diameter and shape score was conducted as morphological parameter of fruit quality. The data of length of the fruits are shown in the following Table 1. Based on Table 1 presents that the levels of goat manure applications and bacteria treatments showed a positive interaction to increase the fruit length. The best treatment of all was the application of 10 kg goat manure per-plant combined with *Bacillus velezensis* B-27. Then, by comparing the *Bacillus velezensis* B-27 treatment and control, we got that bacteria could significantly increase the fruit length.

Table 1. Fruit length of snake fruit by goat manure and *Bacillus velezensis* B-27 applications

Bacillus velezensis B-27 applications	Fruit length (mm) Goat manure level (kg per-plant)			The average of fruit length by *Bacillus velezensis* B-27 treatments (mm)
	0	5	10	
Without *Bacillus velezensis* B-27	53.73 f	57.17 e	67.18 c	59.36
With *Bacillus velezensis* B-27	64.74 d	69.33 b	76.78 a	70.28
The average of fruit length by goat manure treatments (mm)	59.24	63.25	71.98	(+)
Coefficient of variance (%)		12.14		

Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to Tukey's HSD test. A positive sign (+) shows positive interaction between two factors

The following Table 2 figure out that goat manure and *Bacillus velezensis* B-27 gave a positive interaction to increase the fruit diameter. The application of 10 kg goat manure per-plant with *Bacillus velezensis* B-27 could significantly improve the diameter of the fruit.

Table 2. Fruit diameter of snake fruit by goat manure and *Bacillus velezensis* B-27 applications

Bacillus velezensis B-27 applications	Fruit diameter (mm) Goat manure level (kg per-plant)			The average of fruit diameter by *Bacillus velezensis* B-27 treatments (mm)
	0	5	10	
Without *Bacillus velezensis* B-27	36.92 e	43.44 d	48.13 c	59.36
With *Bacillus velezensis* B-27	47.55 c	58.85 b	62.72 a	70.28
The average of fruit diameter by goat manure treatments (mm)	42.24	51.15	55.43	(+)
Coefficient of variance (%)		18.55		

Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to Tukey's HSD test. A positive sign (+) shows positive interaction between two factors

Table 2 demonstrates that the application of *Bacillus velezensis* B-27 without manure still gave longer diameter than the application of 5 kg goat manure per-plant. The *Bacillus velezensis* B-27 addition also increased the fruit diameter compared with the application without bacteria. This indicates that the bacteria supplement could be an alternative to make the nutrients absorption more efficient.

The score of fruit shape signified the quality of fruit, in which the highest value represented round shape fruit. The application of goat manure and *Bacillus velezensis* B-27 did not show positive interaction to make the shape score higher than control. From Table 3, we could see that the level of manure that gave a significantly higher value than control was 10 kg goat manure per-plant. Based on (Handajaningsih et al., 2019), incorporating organic matter like goat manure can improve soil fertility, then increase the fruit weight and diameter in *Cucumis melo* L.

For the *Bacillus velezensis* B-27 application to the plant, the score for the shape did not show the significant improvement from the application with bacteria and without bacteria. However, compared with control treatment of manure, added 10 kg goat manure per-plant could enhance the score of the shape, meaning that the fruit tended to be rounded than flattened.

Commercially, farmer sorted the rounded fruit as the best grade of snake fruits (Nurrochman et al., 2013).

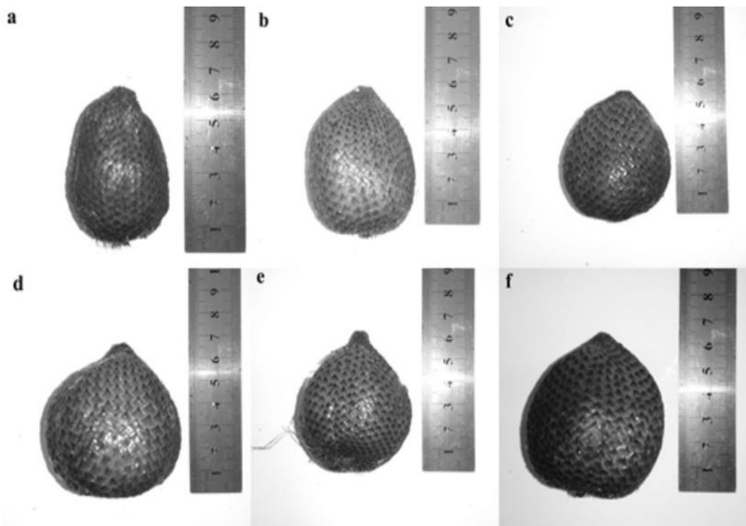
Table 3. Fruit shape scoring of snake fruit by goat manure and *Bacillus velezensis* B-27 applications

Treatments	Shape score
Goat manure level	
0 kg per-plant	1.83 b
5 kg per-plant	2.33 ab
10 kg per-plant	2.83 a
Bacillus velezensis B-27 applications	
Without *Bacillus velezensis* B-27	1.89 p
With *Bacillus velezensis* B-27	2.78 p
Interaction	(-)
CV (%)	34.08

Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to HSD-Tukey Test. A negative sign (-) showed negative interaction between 2 factors

The application of goat manure combined with *Bacillus velezensis* B-27 gave variation to the size and shape of the fruits. The illustration of the fruit morphology can be seen in Figure 2. Improvement of size and shape are affected by nutrition supply of manure and microbial activity of *Bacillus velezensis* B-27 through inclination of phytohormones level (Rahman et al., 2018).

The fruit morphology of the snake fruit shows an improvement for the size and shape of goat manure and *Bacillus velezensis* B-27 treatments, compared with control treatment (Figure 2). This study gave the same result as the study by Wang et al. (2020), which figured out that *Bacillus* sp. could improve the morphology of banana fruit, including the length and diameter of the fruit. The banana root exudate might enhance the colonization of the bacteria, whereas they could produce auxin as a plant growth regulator. Another study from Gül et al. (2008), also reported a consistent result, revealing that *Bacillus* sp. treatment for tomatoes could increase the fruit diameter and thus increased marketable fruits. Rahma et al. (2020) confirmed that *Bacillus velezensis* strain B-27 could significantly reduce disease infections and improved vegetative growth and yield of shallot. However, goat manure and *Bacillus velezensis* B-27 did not give significant effect on the fruit shape (Table 3). The shape might be encoded by complex genes that bring up shape character. Hence, to improve the shape quality of fruits needs a combination with another technique like phytohormone application to induce fruit growth to reach maximum shape.



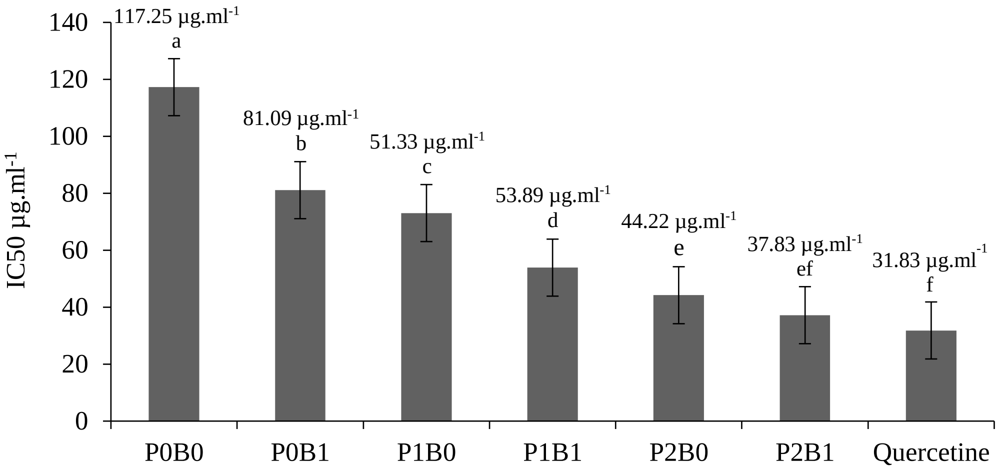
Note: a. P0B0 = 0 kg per-plant of goat manure without *Bacillus velezensis* B-27; b. P0B1 = 0 kg per-plant of goat manure + *Bacillus velezensis* B-27; c. P1B0 = 5 kg per-plant of goat manure without *Bacillus velezensis* B-27; d. P1B1 = 5 kg per-plant of goat manure + *Bacillus velezensis* B-27; e. P2B0 = 10 kg per-plant of goat manure without *Bacillus velezensis* B-27; f. P2B1 = 10 kg per-plant of goat manure + *Bacillus velezensis* B-27

Figure 2. Fruit morphology by the application of goat manure combined with *Bacillus velezensis* B-27

Using microbial treatment in agriculture remains a challenge because we should choose suitable species of bacteria to increase the yield. However, the study to determine the effects of PGPR to enhance the quality and quantity of plant production is still required, especially to realize the sustainable agriculture. Cultivation technique using organic substances and biological agents is an alternative to improve plant growth and combat some abiotic stresses or pathogens. Some studies have noted that *Bacillus* sp. can increase fruit quality (Rahman et al., 2018). The growth and development improvement induced by *Bacillus* sp. are mostly affected by indole-acetic-acid produced by the bacteria and through their ability to overcome several stresses caused by environment or pathogen like *Pseudomonas* sp. (Albanchez et al., 2018). Subsequently, the *Bacillus* sp. treatment was also reported to decrease ring rot disease in apple fruit, as a postharvest handling method of the fruits (Li et al., 2013). For the postharvest treatment of snake fruit, the effect of *Bacillus* sp. to inhibit the activity of pathogenic microbes is necessary to be analyzed in snake fruit.

Antioxidant activity

The DPPH test of snake fruit represent how fruit extracts can scavenge 50% of DPPH radicals. The measured value was defined as IC50. The IC50 value is opposite with antioxidant activity. The high IC50 value means low antioxidant activity (Oubihi et al., 2020). The results reveal that the snake fruits with 10 kg goat manure per-plant and *Bacillus velezensis* B-27 applications had the highest antioxidant value of all treatments although it was insignificantly different with antioxidant activity of quercetin as positive control and snake fruit with 10 kg goat manure and without *Bacillus* application (Figure 3).



Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to Tukey's HSD test. P0B0 = 0 kg per-plant goat manure without *Bacillus velezensis* B-27; P0B1 = 0 kg per-plant goat manure + *Bacillus velezensis* B-27; P1B0 = 5 kg per-plant goat manure without *Bacillus velezensis* B-27; P1B1 = 5 kg per-plant goat manure + *Bacillus velezensis* B-27; P2B0 = 10 kg per-plant goat manure without *Bacillus velezensis* B-27; P2B1 = 10 kg per-plant goat manure + *Bacillus velezensis* B-27

Figure 3. Antioxidant activity of snake fruit treated with three levels of goat manure and *Bacillus velezensis* B-27 compared with quercetin as standard

As a result, application of 10 kg goat manure per-plant could increase the antioxidant value of snake fruit and then its combination with *Bacillus velezensis* B-27 also produced higher the IC50 value. Salehi et al. (2019) found that the increase of the antioxidant activity by the applications of goat manure may be caused by the existence of organic manure in providing macronutrients, such as nitrogen, phosphorus and potassium that have a role for antioxidant activity. According to Rahman et al. (2018), giving *Bacillus velezensis* B-27 treatment in the roots of strawberry plants produced higher antioxidant activity than the treatment without bacteria. Therefore, the combination of goat manures and *Bacillus velezensis* B-27 treatments possibly increase the substances engaged in the synthesis process of some secondary metabolite pathways. As reported before, using microbes can enhance some genes in the phenylpropanoid pathway that result in increased flavonoid compounds (Ali and McNear, 2014). In line with the report of Leontowicz et al. (2006), the IC50 value of snake fruit without any treatment was 72.9 µg mL^{-1}, representing lower antioxidant activity than the fruit with goat manure and *Bacillus velezensis* B-27 applications.

Treating snake fruit with *Bacillus velezensis* B-27 showed higher antioxidant than control and then combined it with goat manure produced the best result (Figure 1). Kadaikunnan et al. (2015) revealed that active compound in the cell surface of *Bacillus* sp. contains some antioxidant enzymes like NADH-oxidase, SOD, NADH peroxide and non-heme catalases. Since antioxidant comprises chain-breaking antioxidants like phenols and flavonoids and also antioxidant enzymes (Pisoschi et al., 2021), increasing the secondary metabolites and enzymes by *Bacillus* sp. can enhance antioxidant activity to reduce oxidative stress. We know that society consume fruits not only to enjoy the delicious taste but also to take several substances that are beneficial for health, including antioxidant substances, that can improve antioxidant activity by bio-fertilizers and substitute synthetic inputs, which can decrease physical texture of the soil.

Flavonoid contents

The flavonoid content figured out the concentration of flavonoid compounds that equivalent to quercetin per-100 gram of samples. Positive interaction was showed by the goat manure level and *Bacillus velezensis* B-27 application to increase the flavonoid content of snake fruit (Table 4).

Table 4. Flavonoid content of snake fruit by applications of goat manure and *Bacillus velezensis* B-27

Bacillus velezensis B-27 applications	Flavonoid content (mgQE 100 g^{-1}) Goat manure level (kg per-plant)			The average of flavonoid content by *Bacillus velezensis* B-27 treatments (mgQE 100 g^{-1})
	0	5	10	
Without *Bacillus velezensis* B-27	5.04 cd	5.02 d	5.07 cd	5.04
With *Bacillus velezensis* B-27	5.12 c	5.24 b	5.35 a	5.24
The average of flavonoid content by goat manure treatments (mgQE 100 g^{-1})	5.08	5.13	5.21	(+)
Coefficient of variance (%)		2.33		

Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to Tukey's HSD test. A positive sign (+) shows positive interaction between two factors

Table 4 depicts that the best application was 10 kg goat manure per-plant combined with *Bacillus velezensis* B-27 that produced the highest content of flavonoid (5.35 mgEQ 100 g^{-1}), followed by 5 kg goat manure per-plant with bacteria application (5.24 mgEQ 100 g^{-1}) as the second-highest of flavonoid content. Application of *Bacillus velezensis* B-27 without manure produced the third highest value of flavonoid content (5.12 mgEQ 100 g^{-1}) that did not show a significant difference with an application of 10 kg goat manure per-plant without bacteria (5.07 mgEQ 100g^{-1}). Then, the application of 10 kg goat manure per-plant only, also gave an insignificant difference with 5 kg goat manure per-plant without bacteria (5.02 mgEQ 100 g^{-1}). At the last, the control treatment showed the lowest flavonoid content of the all (5.04 mgEQ 100 g^{-1}), which was not significantly different from an application of 5 kg goat manure per-plant without bacteria.

Several studies showed that *Bacillus* sp. could increase the flavonoid contents by some compounds in its pathway of metabolism. An *in vitro* assay showed that *Bacillus* sp. could significantly increase the flavonoid level through β-glucosidase and protease activities (Yang et al., 2019). In addition, the naringinase, an enzyme that contributes to the formation of some flavonoid compounds, is also purified from *Bacillus* sp. (Zhu et al., 2017). Since flavonoid compounds are not synthesized by the human body, consuming them from fruit becomes an alternative to supply antioxidant needs, because mainly flavonoids are the rich antioxidant resources. Naturally, the snake fruit contains flavonoid compounds that show inhibition effect to tyrosinase, the rate-limiting enzyme that provides melanin. Thus, this fruits extract is potential as a skin lightening agent (Tilaar et al., 2017). Besides, the snake fruit contains 0.31 mg g^{-1} of catechin, an important flavonoid compound, that can be used for several purposes in the field of health (Gorinstein et al., 2011).

Total phenol contents

The total phenol content represents whole phenol group compound contained in the snake fruit treated with goat manure and *Bacillus velezensis* B-27. The result shows that the interaction of goat manure level and *Bacillus velezensis* B-27 application raised total phenol (Table 5).

Table 5. Total phenol content of snake fruit by applications of goat manure and *Bacillus velezensis* B-27

Bacillus velezensis B-27 applications	Total phenol content (mgGAE 100 g^{-1}) Goat manure level (kg per-plant)			The average of total phenol content by *Bacillus velezensis* B-27 treatments (mgQE 100 g^{-1})
	0	5	10	
Without *Bacillus velezensis* B-27	4.13 c	4.14 bc	4.22 bc	4.16
With *Bacillus velezensis* B-27	4.26 b	4.30 b	4.44 a	4.33
The average of total phenol content by goat manure treatments (mgQE 100 g^{-1})	4.14	4.22	4.33	(+)
Coefficient of variance (%)		2.59		

Note: Mean values followed by the same letters are not significantly different at $p < 0.05$ according to Tukey's HSD test. A positive sign (+) shows positive interaction between two factors

The positive interaction between two factors brought 10 kg of goat manure per-plant with *Bacillus velezensis* B-27 became the best treatment to increase total phenol content (4.44 mgEGA 100 g^{-1}). The second highest of total phenol was treatment using 5 kg goat manure per-plant combined with *Bacillus velezensis* B-27 (4.30 mgEGA 100 g^{-1}). This value was not significantly different from the total phenolic of *Bacillus velezensis* B-27 without manure application (4.26 mgEGA 100 g^{-1}). Then, treatment with 10 kg goat manure per-plant without bacteria (4.22 mgEGA 100 g^{-1}) was also insignificant different with the application of *Bacillus velezensis* B-27 only and 5 kg goat manure per-plant without bacteria (4.14 mgEGA 100 g^{-1}). Treatment without manure nor bacteria produced the lowest total phenol (4.13 mgEGA 100 g^{-1}) that did not show significant differences with other treatments without bacteria. The lowest value of total phenol was control.

From the data, we can see that 10 kg of goat manure per-plant combined with *Bacillus velezensis* B-27 gave the highest total phenolic value of all treatments (Table 5). This result is also supported by another study revealing that *Bacillus* sp. inoculation can produce indole-3-acetic-acid (auxin) that induce plant growth and improve the synthesis of amino acids like phenylalanine and tyrosine, which enter shikimic acid pathway, which has the responsibility to product some phenolic compounds such as gallic acid, ferulic acid and p-coumaric acid (Cisternas-Jamet et al., 2020; Sutini et al., 2020). Gowtham et al. (2018) also showed that *Bacillus* sp. treatment was in line with higher activity of some enzymes like phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO), which play a role on phenolics biosynthesis and other enzymes like peroxidase (POX) and β-1,3-glucanase, which show antioxidant activity. Since phenolic compounds naturally act as plant protection compounds, the improvement of these compounds also facilitates the plant's ability to overcome some diseases or abiotic stresses.

The snake fruit is one of the indigenous fruits becoming popular in Indonesia as the good fiber and antioxidant sources. The high antioxidant value by *Bacillus velezensis* B-27 and goat manure treatment correlates with a phytochemical compound contained. The strong correlation between antioxidant, total phenolic and flavonoid content shows that the antioxidant activity of the snake fruit is mostly affected by several compounds from the phenolic and flavonoid groups. In line with the results, a study that was undertaken by Esmaeili et al. (2015) showed a strong correlation between phenol and flavonoid contents with DPPH radical scavenging activities in red clover. Total phenolic and flavonoid contents show a remarkable scavenging effect on DPPH free radicals, although more studies with in vivo model should be done to examine antioxidant activity in plants and the relationship between phenolic and flavonoid compounds and antioxidant activity by using other methods to clarify the use of compounds as sources of antioxidants (Phuyal et al., 2020).

CONCLUSIONS

In conclusion, the application of *Bacillus velezensis* B-27 combined with goat manure can increase the antioxidant level, flavonoid and total phenol contents. The best combination treatment was 10 kg goat manure per-plant with bacteria. Besides, the size of fruit is also improved by 10 kg goat manure per-plant enriched with *Bacillus velezensis* B-27. Advanced research about the effect of *Bacillus velezensis* B-27 on fruit development seems to be promising to propose genomic and proteomic changes caused by the bacteria activity.

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