



## Research Article

# The Role of Anthocyanin Content of Garden Balsam's (*Impatiens balsamina* L.) Flower Extract on the Growth of *Ralstonia solanacearum*

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## ABSTRACT

The study was aimed to determine the response of bacterial growth of *Ralstonia solanacearum* Race I of biovar III phylotype I treated with flower extract of Garden Balsam (*Impatiens balsamina* L.) on different color of flower, that was able to give the strongest antibacterial compound and to determine the physical characteristics of *I. balsamina* in order to facilitate the purpose of the garden balsam cultivation as antibacterial. In addition, this study also aimed to determine the highest total anthocyanin content (TAC) and to determine the molecular characteristics of anthocyanin-coding genes and DNA base sequences of *I. balsamina* that indicated antibacterial properties. The research used Completely Randomized Design (CRD) 2×4 each with 3 replications. The first factor was the method of plating bacteria culture (pour plate and streak plate method), the second factor was the color of the garden balsam flower extracts consisted of white, red, purple and magenta. Therefore, the experiment consisted of 8 treatments of combination. Data were analyzed using CRD ( $\alpha = 0.05$ ). The results showed that the average combination of pour plate and red flower extract gave the value of resistance velocity on the 3rd day with the diameter of the inhibition zone was 33.46 mm, with the inhibitory zone diameter of the garden balsam extract of 22.90 mm. Red flower plants showed a bright red stem on the braches and the pointed leaf edge. Although this red does not show the total content of anthocyanin compounds higher than purple, however from RAPD analysis for red color indicated the anthocyanin of pelargonidin as an anthocyanin compound responsible for its antibacterial properties.

Keywords: antibacterial, anthocyanin, flower color, *Impatiens balsamina*, *Ralstonia solanacearum*

## INTRODUCTION

Garden balsam (*Impatiens balsamina* L.) is one of the plants that its extract contains flavonoid compounds as antibacterial. Garden balsam flower has various colors, i.e. white, red, purple, magenta, pink, and light purple (Dalimartha, 2003). The various colors of garden balsam flower indicated certain metabolite compounds. Anthocyanin is a class of flavonoids that are responsible for flower coloring. Darker flower color revealed a higher anthocyanin accumulation. However, high anthocyanin accumulation does not indicate its ability as a stronger antibacterial. Generally, anthocyanin is divided into three basic pigments, i.e. pelargonidin, cyanidin, and delphinidin (To & Wang, 2006).

The extracts of leaf, stem, and flower of garden balsam as antibacterial have been previously

reported on *Aeromonas hydrophila* by the size of the clear zone around the paper disc, where the leaf and stem extracts are classified in the strong group, with inhibitory zones of 11.2 mm and 13.7 mm, respectively. While the ability of flower extracts to inhibit the growth of *A. hydrophila* with inhibition zones of 21.4 mm showed that the extract is included in a very strong group (Kusuma, 2014). The application of 35% ethanol from garden balsam extract can reduce the mortality rate of 45% in control (without dosing) (Ishiguro, 1992 *cit.* Ishiguro 2015). Adfa (2007) stated that methanol extract of garden balsam leaves with white flowers contain coumarin, quinone, flavonoids, steroids, triterpenoids, phenolics, and saponins while ethanol extracts of garden balsam leaves with purple flowers contain alkaloid, antron, anthraquinone, coumarin, flavonoids, and phenolics (Amrullah & Yuliani, 2015).

The color of red and blue flowers is caused by anthocyanin. The red-magenta anthocyanin has the highest TAC (Total Anthocyanin Content) value on *Mirabilis jalapa* followed by *Impatiens balsamina*, 338.61 mg/kg and 336.56 mg/kg, respectively (Vankar & Srivastava, 2010). Because the number of efforts for finding the local plants that able to suppress the growth of bacteria in important plants in Indonesia has increased, the extract of garden balsam is considered able to increase natural germplasm as a plant-based pesticide (antibacterial). In addition, garden balsam also acts as an antibacterial in humans and animals, hence it is also may able to act as an antibacterial against pathogenic plant bacteria.

*Ralstonia solanacearum* is a bacterial species complex (Trianom *et al.*, 2018) which is an important pathogenic bacterium of many types of plants in Indonesia. *R. solanacearum* first appeared in Indonesia in 1864 in the Deli area of North Sumatra on tobacco plants. In addition, this disease also attacks other plants such as peanuts, bananas, potatoes, tomatoes, ginger, patchouli, sesame, and various other host plants (Arwiyanto, 2014).

This study aimed to determine the response of bacterial growth of *R. solanacearum* race I of biovar III phylotype I treated with flower extract with different colors, which provide the ability as the strongest antibacterial compound. In addition, this study also aimed to facilitate the selection of local plants as an antibacterial by observing the physical characteristics of stem, leaf, and flower; chemical characteristics by calculating the total anthocyanin compounds in the flowers; and molecular analysis to determine the characteristics of anthocyanin-coding genes and DNA base sequences of garden balsam that indicate characteristics as an antibacterial.

## MATERIALS AND METHODS

### *Place and Time of Research*

The study was conducted from April 2016 to April 2018 in the Laboratory of Plant Genetics and Breeding, and Laboratory of Plant Disease Science, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

### *Selection of Garden Balsam Flower as Antibacterial*

The selection of flower colors is according to “RHS Color Chart” with standardized colors for

white (N155B), red (41 A), purple (N78B), and magenta (N66A) (The Royal Horticultural Society, 2001). Garden balsam flowers were obtained from Jatimulyo, Jambon, Sleman, Yogyakarta.

### *Extraction of Garden Balsam Flower*

Simplisia in the form of 50 g of fresh garden balsam flower (converted with every 25 flowers), washed and drained. The flowers were then soaked immediately after the draining process by sterile water solvents. Simplisia that has been soaked was then macerated, incubated over 24 hours in a cool place and protected from sunlight at room temperature. The material was squeezed using gauze until water or starch comes out then mashed by mortar, and then filtered with filter paper. The extract solution was separated using a centrifuge speed of 3000 rpm for 5 minutes. The extract obtained was stored in a freezer (-20°C) used for further tests.

### *Test of the Inhibitory Growth of Ralstonia solanacearum*

0.1 ml of *R. solanacearum* Race I of biovar III phylotype I with a density of  $10^8$  CFU/ml in a test tube was poured into YPGA (Yeast Pepton Glucose Agar, with a ratio of 3 g of yeast, 10 g of peptone, 10 g of glucose, and 15 g/liter of difco agar) and inoculated in petridish. Bacteria were grown using two methods: pour plate (with pouring) and streak plate (with scratches/zig-zag using L glass). 10  $\mu$ l of garden balsam flower extract was poured on a paper Whatman No. 42 with (5 mm in diameter) was then placed on an agar medium contained bacterial culture. Petri dishes were incubated at 37°C for 24 hours.

### *Observation of Plant Morphology*

Observation of plant morphology was done by observing the stem, leaf, and flower of garden balsam that indicate the accumulation of anthocyanin which was shown in red-purple in a certain part of the plant. Plant parts that indicated anthocyanin accumulation was then compared between its colors to distinguished its characteristics as the early identification stage. The purpose of this observation is as a quick step in determining the material to be used as an antibacterial compound. Morphological observations are expected to facilitate the collection of antibacterial base ingredients. The physical/morphological variables of the plants observed were to compare: (a) the color of the stems on the branches that accumulated

anthocyanin in each garden balsam color, (b) the shape of the leaves and the number of leaves that formed on the apical buds of each flower color garden balsam, (c) flower color and intensity of flower color brightness using the RHS Color Chart.

#### **Determination of Total Anthocyanin Compounds**

The total content of anthocyanin compounds was performed according to the procedure by Glusti and Wrolstad (2001) using the extracts of stems, leaves, and flowers of garden balsam. The observation aimed to find out the highest anthocyanin accumulation, then compared it with the ability of its extract as an antibacterial, as a follow up in knowing the role of anthocyanin as an antibacterial of garden balsam.

#### **Molecular Identification to Determine Anthocyanin-Coding Genes**

This test was conducted to find a more specific picture of the antibacterial characteristics by molecular identification of anthocyanin-coding genes that play a role in flower coloring. The procedure was carried out to obtain a specific description of anthocyanin genes that encode the color of the flower according to Doyle and Doyle (1990) in the DNA Analysis Training book, Laboratory of Plant Breeding, Faculty of Agriculture, UGM (2016). By identifying the flower color anthocyanin genes, then could be determined the type of anthocyanin that acts as an antibacterial against *R. solanacearum*. The anthocyanin coding genes used in this study were F3'H, F3'5'H, DFR, ANS, CHI, CHS, UFGT, PR1 (To & Wang, 2006).

#### **Observation**

Observation of the bacterial inhibition zone diameter was carried out by measuring the clear zone around the bacterial colony in the petridish. Inhibition zone diameter measurement was done using the electric slide caliper to get the magnitude value for the inhibition zone easily and quickly. The diameter of the bacterial inhibition zone was calculated using the mean diameter vertically and horizontally of the clear zone produced on the *n* day, while the increase of the inhibition zone was done by subtracting the value of the *n* inhibition zone from the previous day. The unit for measurement of bacterial inhibition zones was in mm. Morphological observations were performed by visual observations using the RHS color chart. Chemical observations were conducted by calculating the total content of

anthocyanin compounds in ppm. The molecular observation was carried out by the PCR technique and the results were evaluated by comparing the presence or absence of DNA bands in each color sample.

#### **Data Analysis**

This study used a Completely Randomized Design 2×4 each with 3 replications with the following combination of treatments: PtP (white, pour plate), MeP (red, pour plate), UgP (purple, pour plate), MgP (magenta, pour plate), PtS (white, streak plate), MeS (red, streak plate), UgS (purple, streak plate), MgS (magenta, streak plate). Data were analyzed by ANOVA ( $\alpha = 5\%$ ) using SAS (Statistical Analysis System) software. Bacterial resistency velocity was calculated by measuring the diameter vertically and horizontally, then calculated its average.

## **RESULTS AND DISCUSSION**

#### **The Inhibition Test of *Ralstonia solanacearum***

The resistance velocity on *R. solanacearum* began on the third day with a significant increase in inhibition zone diameter after treatment. The highest diameter of the inhibitory zone of *R. solanacearum* growth is in the treatment combination using the pour plate method in the extract of red flower color of 33.46 mm with an average inhibition zone increase of 22.90 mm (Table 1 and Table 2). This result revealed that methods and flower extracts used have a significantly different reaction. On the fourth and fifth days, the red garden balsam extract had the highest inhibitory diameter compared to the other garden balsam extracts in inhibiting the growth of *R. solanacearum*. The pour plate method used on the fifth day affected the growth of *R. solanacearum* bacteria as well as the blocking area system (the zone between the agar that was overgrown with bacteria and the antibacterial clear zone) by antibacterial compounds of garden balsam flower extracts. Basically, the streak plate and pour plate methods have a similar technique. The application of the pour plate has an advantage in terms of growing bacteria. Besides the colony could grow evenly, the pour plate technique also provides an opportunity for bacteria to grow faster because the density of bacterial growth areas is relatively the same on the media surface. Bacterial growth after the second day showed almost the same density

Table 1. The inhibitory zone diameter of garden balsam flower extract (*Impatiens balsamina*) on different flower colors and techniques on the growth of *Ralstonia solanacearum* on the 3rd day

Treatment	Inhibitory zone (mm)
Sreak plate–white	7.22b
Sreak plate–red	13.32b
Sreak plate–purple	7.58b
Sreak plate–magenta	7.28b
Pour plate–white	7.42b
Pour plate–red	33.46a
Pour plate–purple	7.38b
Pour plate–magenta	7.49b

Remarks: Values in the same column followed by the same letter were not significantly different according to CRD (factorial test) ( $\alpha = 0.05$ )

Table 2. The increase of the inhibitory zone diameter of garden balsam flower extract (*Impatiens balsamina*) on different flower colors and techniques on the growth of *Ralstonia solanacearum* on the 3rd day

Treatment	Inhibitory zone (mm)
Sreak plate–white	0.11b
Sreak plate–red	4.33b
Sreak plate–purple	0.12b
Sreak plate–magenta	- 0.05b
Pour plate–white	0.27b
Pour plate–red	22.90a
Pour plate–purple	0.06b
Pour plate–magenta	0.06b

Remarks: Values in the same column followed by the same letter were not significantly different according to CRD (factorial test) ( $\alpha = 0.05$ )

(the media to be covered by bacterial growth), but the blocking area was shown by the red garden balsam flower extract in both methods still showed its ability as an antibacterial. This finding showed that red garden balsam extracts able to act as an antibacterial until the fifth day. Figure 1 showed the image of inhibition zone diameter of the red garden balsam flower extract

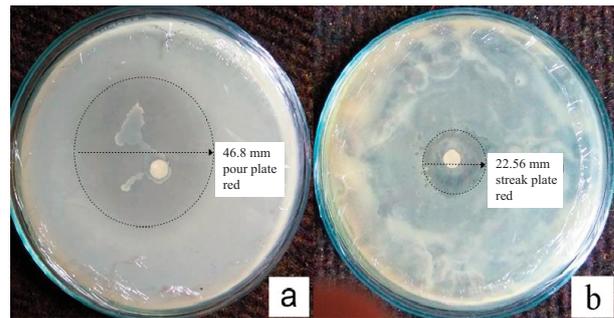


Figure 1. The average of the inhibitory zone diameter of the red garden balsam flower extract (*Impatiens balsamina*) on the 5th day using (a) pour plate technique (46.8 mm) and (b) streak plate technique (22.56 mm)

in the pour plate and streak plate that the red flower extract may contain a certain secondary metabolite compound that inhibits *R. solanacearum*.

#### **Anthocyanin Accumulation in the Stem, Leaf, and Flower of Garden Balsam**

Based on the visual observation, the red flower plant stems have a more intense red color than purple, white and magenta on the node that may have a higher accumulation of anthocyanin than other stem parts. Anthocyanin color is generally dominated by bright colors in certain parts of the plant, usually in the flowers (To & Wang, 2006). Therefore, we can observe from the early identification that the plant has a dark red color that may have anthocyanin compound as an antibacterial. The red garden balsam has wider and jagged leaves that may act as the highest antibacterial shown by the physical characteristics in Figure 2b. Physical characteristics based on the color of the flower are considered easier to be observed than the stem or leaf. However, accuracy is still needed because the physical factors of plants are influenced by the environment. Although visually white, red, purple and magenta can be distinguished, a color standard is still needed to ensure when the

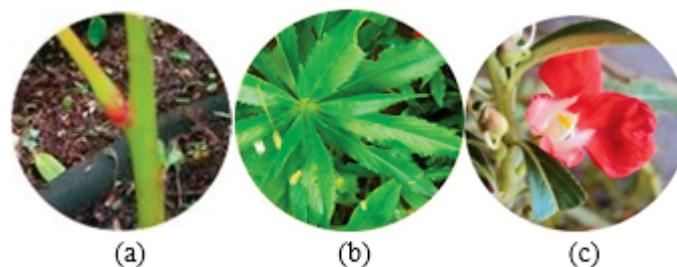


Figure 2. Morphological characteristics of the red garden balsam parts (*Impatiens balsamina*) [stem (a), leaves (b), flower (c)]

selection of the antibacterial material is conducted.

Garden balsam plants have the free-stamens with the propagation method using vegetative parts and seeds. Naturally, the segregation of garden balsam occurs on a large scale. As a result, plants with certain flower colors do not always reflect genetic traits as sought. Hence the color of the flowers that are visually similar to the color standard sometimes does not have the exact same antibacterial characteristics as the exact same color. Intermediate colors, such as pink and light purple on white flowers, generally have very thin color gradations that affect the ability of garden balsam flower extract as an antibacterial agent.

#### **Total Anthocyanin Compounds in Garden Balsam**

The highest total content of anthocyanin was observed in the stem, leaf, and flower of garden balsam was the purple flower (88.83 ppm) followed by the red flower (51.785 ppm) (Table 3). This result was similar to Kusuma (2014) stated that the highest antibacterial ability is in the garden balsam flower extract. Vankar and Srivastava (2010) also stated that red-magenta flower has a high total anthocyanin content, a high anthocyanin content also found in darker color flowers. Based on the bacterial tests conducted showed that the total content of anthocyanin is not directly in line with its ability as an antibacterial. This is revealed by the highest average inhibition zone diameter is from red flower extract. Based on

Table 3. The total content of anthocyanins in the stem, leaf and flower of garden balsam (*Impatiens balsamina*) in each color

Part of plant	Color of flower	Total Anthocyanin content
Flower	White	8.115h
	Red	51.785b
	Purple	88.83 a
	Magenta	39.94 c
Leaf	White	15.46 g
	Red	18.18 e
	Purple	20.74 d
	Magenta	16.585f
Stem	White	2.905i
	Red	3.385i
	Purple	3.6 i
	Magenta	3.085i

Remarks: Values in the same column followed by the same letter were not significantly different according to CRD (factorial test) ( $\alpha = 0.05$ ). Data is the result of transformation.

the flavonoid biosynthesis pathway to produce anthocyanins and several flower-related flavonoid classes, the red color-coding gene for the highest antibacterial compound should be able to be detected by anthocyanin primers encoding cyanidin (magenta red) or pelargonidin (reddish-orange) (To & Wang, 2006). For this reason, early detection can be done to distinguish the type of anthocyanin red flowers as the highest antibacterial properties, namely by molecular analysis using RAPD techniques.

#### **Molecular Analysis of Anthocyanin-Coding Genes as An Indicator of the Type of Anthocyanins as Antibacterials**

Molecular identification was aimed to obtain a visual representation of anthocyanin genes associated with the red garden balsam flower gene that acts as the highest antibacterial against the growth of *R. solanacearum*. Eight anthocyanin primers used were chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3 $\beta$ -hydroxylase (F3'H), flavonoids 3'5' hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), flavonoids 2-O-glucosyltransferase (UGT) (To & Wang, 2006) and downy mildew resistance genes in maize encoding pelargonidin (Pr1) anthocyanin (Sharma *et al.*, 2011 *cit.* Fatmawati, 2016). The 8 primers selected were based on the biosynthesis pathway of flavonoids for anthocyanins in general because the specific primers of garden balsam plants as biosynthetic markers were unidentified yet. After obtaining the highest inhibitory zone of *R. solanacearum* in the antibacterial extract of the red flower, the molecular analysis conducted is considered able to provide genetic information regarding the encoding of anthocyanin for the garden balsam red flower. From the results of PCR and agarose gel electrophoresis by UV, it was found that from the 8 primers used, only Pr1 primer showed the brightest PCR amplification with 1 specific band.

Based on the agarose gel electrophoresis results by UV of PCR results on 8 primers and 8 annealing temperatures with 4 different colors revealed that the anthocyanin coding gene of Pr1 primer showed a clear band seen at 50°C–54°C. The selection of annealing temperature at 51°C is based on the bright band. The white color (PT) showed 2 bands at 500 bp and 1000 bp (unclear), while red (MR), purple (UG), and magenta (MG) indicated 1 band only on the DNA band length at 1000 bp. The anthocyanin

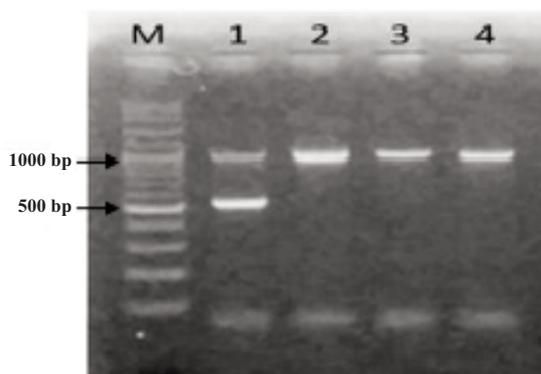


Figure 3. The result of agarose gel electrophoresis of 10  $\mu$ l volume in each color using PR1 primer at 51°C: (M = DNA marker 100 bp, 1 = white, 2 = red, 3 = purple, 4 = magenta)

encoding gene for Pr1 in maize shows its specific gene at 800 bp. The amplification of the Pr1 anthocyanin coding gene for white has detected the presence of these 2 bands, that might happen because the RAPD technique allows the anthocyanin encoding gene Pr1 to attach to any base of the garden balsam DNA.

From the analysis of the total anthocyanin compounds revealed that the white color still showed the anthocyanin accumulation even though in the small amount. Although the red flowers (MR) as an indicator of genes coding for antibacterial properties does not provide a clear molecular difference using RAPD technique for purple (UG) and magenta (MG), however in this study showed that the type of anthocyanin for red is pelargonidin which generally encodes reddish-orange (To & Wang, 2006). This is similar to Fatmawati (2016) reported that the Pr1 anthocyanin coding gene is a resistance coding gene in maize that may be able to be detected in garden balsam as one of the molecular markers of the resistance trait gene. For this reason, further research needs to be carried out to explore more detail information related to the resistance characteristics and the genes responsible for antibacterial properties. By showing the results of electrophoresis in the form of a single band in Figure 3, it could be concluded that the Pr1 gene is a gene encoding anthocyanin pelargonidin which is detected in garden balsam at a DNA band length of 1000 bp for red (MR), purple (UG) and magenta (MG).

## CONCLUSION

The red garden balsam flower extract and pour plate media showed the strongest antibacterial properties characterized by the inhibitory zone of *R. solanacearum* by 33.46 mm that began appearing on day 3. The highest average total anthocyanin compound was in the purple flower of 88.83 ppm, followed by the red flower of 51.785 ppm. Pr1 is a gene encoding anthocyanin type pelargonidin detected in red (MR), purple (UG) and magenta (MG) garden balsam at a DNA base length of 1000 bp.

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