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Research Article

Role of Elicitors in Chlorophyll Content and Stomatal Density of Soybean Cultivars by Foliar Application

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Abstract

Background and Objective: Elicitors could be used not only to increase isoflavone concentration of soybean seeds but also to influence the physiological characteristics of soybean. The objective of this research was to determine the effects of foliar applications of elicitor compounds (i.e., chitosan and salicylic acid) on the physiological characteristics of two soybean varieties. **Materials and Methods:** The research used a randomized block design with 2 factors and 3 replications. The first factor was soybean cultivar (Wilis and Devon). The second factor was foliar application consisting of without elicitor, chitosan at the four fully developed trifoliate leaves (V4), chitosan at the early podding (R3), chitosan at the V4 and R3, salicylic acid at the V4, salicylic acid at the R3 and salicylic acid at the V4 and R3. The parameters observed were chlorophyll a, chlorophyll b, total chlorophyll content and stomatal density. The data were analyzed by using analysis of variance (ANOVA) at $p = 0.05$. **Results:** The results suggest that the Devon cultivar had higher content of chlorophyll a and total chlorophyll, while Wilis cultivar had higher content of chlorophyll b and stomatal density. The foliar application with salicylic acid at the V4 resulted in the highest chlorophyll a and total chlorophyll content, while the chitosan application at the V4 and R3 resulted in the highest chlorophyll b content. The application of salicylic acid at the V4 at Wilis cultivar increased stomatal density, while the chitosan application at the V4 at Devon cultivar increased the stomatal density. **Conclusion:** It is concluded that in Wilis cultivar, the foliar application of salicylic acid at the V4 increased the chlorophyll a content, total chlorophyll content and stomatal density, while in the Devon cultivar, the foliar application of salicylic acid at the R3 resulted in the highest chlorophyll b content, while the application of chitosan at the V4 in Devon cultivar increased the stomatal density.

Key words: Chitosan, chlorophyll, cultivar, salicylic acid, soybean, stomatal density

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean isoflavones contains secondary metabolites that are highly useful for health. Genistein, daidzein and glycitein are the main isoflavones in soybeans reported to have beneficial effects on atherosclerosis, chronic inflammatory diseases, look like an estrogens and anti-estrogen activity, prevent osteoporosis¹, antiatherosclerotic, antioxidative, antitumoral and antiestrogenic activities²⁻⁵, dementia⁶ and cancer^{7,8}.

Isoflavone content is highly variable and regulated by genetic and environmental factors, including UV light, low temperature, wounding, pathogens and plant microbe interactions as well as elicitor application⁹⁻¹¹ and the qualitative-quantitative genetic character is controlled by several major and minor genes^{12,13}. In addition, it was also found that these factors were involved in the up-regulation or induction of the phenyl propanoid pathway genes for the biosynthesis of isoflavones. Increased accumulation of isoflavones in soybeans can be done by inducing a soybean with biotic or abiotic elicitor which stimulates production of phytoalexin in soybean.

Elicitors could be used not only to increase isoflavone concentration of soybean seeds but also to influence the physiological characteristics of soybean. Commonly used elicitors include chitosan and salicylic acid. Chitosan is a member of the polysaccharides that which is considered a useful natural polymer and is produced by alkaline N-deacetylation of chitin. Chitosan was first categorized as an elicitor in plants activating genes that underlie the biosynthetic pathways of secondary metabolites^{14,15}. The previous study results that chitosan increased the concentration of chlorophyll due to chitosan can enhanced the photosynthesis performance¹⁶. Salicylic acid is a phenolic compound involved in growth and development regulation of plants and their responses to biotic and abiotic stress factors¹⁷. Previous research suggested that salicylic acid is an important regulator of photosynthesis, photosystem II (PSII), photosynthetic pigments and the activity of enzymes such as Rubisco¹⁸.

The preliminary research by the author has shown that foliar application of chitosan at 80% of field capacity has resulted in the highest total chlorophyll and stomatal density in soybean (cv. Wilis) but foliar application of salicylic acid at 80% of field capacity has resulted in the lowest of stomatal density¹⁹.

Based on the background, the objective of the present study was to determine the effects of foliar applications of

elicitor compounds (salicylic acid and chitosan) on chlorophyll content and stomatal density of soybean.

MATERIALS AND METHODS

The research was conducted in the farming land of Medan Selayang, Sumatera Utara (Indonesia) from July-September, 2017. The soil used contained low Nitrogen (0.14%), organic matter of 1.02%, with a pH of 5.5.

Experimental design and crop management: The treatments were arranged in a randomized block design with 2 factors and 3 replications. The first factor was two soybean cultivars (Wilis and Devon) referring to previous experiment^{20,21}.

The second factor was elicitor and time of elicitor, application of non-elicitor, chitosan at four fully developed trifoliolate leaves (V4), chitosan at early podding (R3), chitosan at V4 and R3, salicylic acid at V4, salicylic acid at R3 and salicylic acid at V4 and R3. The selection of the type and concentration of elicitor referred to the previous experiments²². The preparation of elicitor referred to standard procedures. Chitosan and salicylic acids were the products of Sigma Aldrich. The stock solution was autoclaved at 120°C for 20 min and sterile distilled water was added to obtain a final concentration of chitosan solution of 1 mg g⁻¹ of fresh leaf. Abiotic elicitor, salicylic acid was dissolved in distilled water and diluted up to the concentration of 0.5 mM. The determination of the concentration of salicylic acid referred to previous research²³. The parameters observed were chlorophyll content and stomatal density.

Chlorophyll content analysis: The determination of chlorophyll content used 0.1 g of fully expanded leaflets 1 week after the R3 of each plant was collected and macerated in a mortar with 10 mL of 80% aqueous acetone (v/v). Extracts were used to measure the absorbance at 649 and 665 nm. The chlorophyll a, chlorophyll b and total of chlorophyll contents were determined using equations based on the specific absorption coefficients as reported by Hendry and Grime²⁴:

$$\text{Chlorophyll a} = \{(12.7 \times A_{663}) - (2.69 \times A_{645})\} / 10$$

$$\text{Chlorophyll b} = \{(22.9 \times A_{645}) - (4.68 \times A_{663})\} / 10$$

$$\text{Total of chlorophyll} = \{(8.02 \times A_{663}) + (20.2 \times A_{645})\} / 10$$

The chlorophyll contents were expressed as mg g⁻¹ of fresh leaf.

Measuring stomatal density: Determination of stomatal density used clear nail varnish. Preparing the epidermal impression was done by coating the leaf surface with nail varnish. Peeling off the dried layer of nail varnish was done by using sellotape and placing it under the microscope.

Statistical analysis: The data of chlorophyll content and stomatal density were recorded and subjected to two-way analysis of variance (ANOVA) at the significant level $p = 0.05$.

RESULTS

Chlorophyll a, chlorophyll b and total chlorophyll content:

The chlorophyll a content and the total chlorophyll content in the Devon cultivar tended to be higher than in the Wilis. However, the chlorophyll b content in the Wilis is higher than Devon (Table 1). The salicylic acid application at the V4 tended to result the highest chlorophyll a content (2.34 mg g^{-1} of fresh leaf) and the total chlorophyll content (3.43 mg g^{-1} of fresh leaf) than the other applications. However, the chitosan application at the V4 and R3 tended

to result the highest chlorophyll b content. The application of salicylic acid at the V4 on the Wilis cultivar tended to increase the chlorophyll a content (2.39 mg g^{-1} of fresh leaf) and the total chlorophyll content (3.51 mg g^{-1} of fresh leaf) but the application of salicylic acid at the V4 and R3 on the Wilis tended to increase the chlorophyll b content (1.84 mg g^{-1} of fresh leaf). Without application at Devon tended to increase the chlorophyll a content (2.41 mg g^{-1} of fresh leaf) and the total chlorophyll content (3.84 mg g^{-1} of fresh leaf), however, the application of salicylic acid at V4 on the Devon tended to increase the chlorophyll b content (2.41 mg g^{-1} of fresh leaf).

Stomatal density: There was no significant difference in elicitor treatment in both soybean cultivars. However, Wilis cultivar had higher stomatal density ($2.00 \text{ unit cm}^{-2}$) than Devon ($1.99 \text{ unit cm}^{-2}$). The salicylic acid application at the V4 resulted in higher stomatal density ($2.45 \text{ unit cm}^{-2}$) than the other applications. The application of salicylic acid at the V4 on the Wilis cultivar increased the stomatal density ($2.76 \text{ unit cm}^{-2}$). However, the application of chitosan at the V4 on the Devon cultivar increased the stomatal density (Table 2).

Table 1: Chlorophyll a, chlorophyll b and total of chlorophyll content of two soybean cultivars with application of elicitor

		Cultivars		
		Wilis	Devon	
Variable observed	Elicitor	(mg g ⁻¹ of fresh leaf)		Mean
Chlorophyll a	E ₀ (without elicitor)	1.80	2.41	2.11
	E ₁ (Chitosan at V4)	2.22	2.28	2.25
	E ₂ (Chitosan at R3)	1.89	2.11	2.00
	E ₃ (Chitosan at V4 and R3)	1.80	1.88	1.84
	E ₄ (Salicylic acid at V4)	2.39	2.29	2.34
	E ₅ (Salicylic acid at R3)	1.99	1.96	1.98
	E ₆ (Salicylic acid at V4 dan R3)	2.13	1.79	1.96
	Mean	2.03	2.11	
Chlorophyll b	E ₀ (without elicitor)	1.53	1.47	1.45
	E ₁ (Chitosan at V4)	1.06	1.20	1.13
	E ₂ (Chitosan at R3)	1.37	1.21	1.29
	E ₃ (Chitosan at V4 and R3)	1.84	1.54	1.69
	E ₄ (Salicylic acid at V4)	1.23	1.60	1.41
	E ₅ (Salicylic acid at R3)	1.12	1.30	1.21
	E ₆ (Salicylic acid at V4 dan R3)	1.74	1.21	1.48
	Mean	1.41	1.35	
Total of Chlorophyll	E ₀ (without elicitor)	2.68	3.84	3.26
	E ₁ (Chitosan at V4)	2.91	3.22	3.07
	E ₂ (Chitosan at R3)	3.27	3.10	3.18
	E ₃ (Chitosan at V4 and R3)	3.19	2.79	2.99
	E ₄ (Salicylic acid at V4)	3.51	3.35	3.43
	E ₅ (Salicylic acid at R3)	2.75	3.05	2.90
	E ₆ (Salicylic acid at V4 dan R3)	3.00	2.76	2.88
	Mean	3.04	3.16	

The numbers are not followed by letters because the effect is not significant based on two-way analysis of variance (ANOVA) at the significant level $p = 0.05$, V4: The four fully developed trifoliolate leaves, R3: Early podding

Table 2: Stomatal density of two soybean cultivars with application of elicitor

Elicitor	Cultivars		Mean
	Wilis	Devon	
	(unit cm ⁻²)		
E ₀ (without elicitor)	2.23	1.79	2.01
E ₁ (Chitosan at V4)	2.14	2.54	2.34
E ₂ (Chitosan at R3)	1.57	1.93	1.75
E ₃ (Chitosan at V4 and R3)	2.07	2.19	2.13
E ₄ (Salicylic acid at V4)	2.76	2.14	2.45
E ₅ (Salicylic acid at R3)	1.60	1.69	1.64
E ₆ (Salicylic acid at V4 dan R3)	1.66	1.69	1.68
Mean	2.01	1.99	

The numbers are not followed by letters because the effect is not significant based on two-way analysis of variance (ANOVA) at the significant level $p = 0.05$, V4: The four fully developed trifoliate leaves, R3: Early podding

DISCUSSION

The results showed that the Devon cultivar had higher chlorophyll a and total chlorophyll contents than Wilis. The results of the research found that the ability of each cultivar to form chlorophyll was different. One of the factors influencing the formation of chlorophyll was the gene. The chlorophyll formation was carried by a particular gene within the chromosome. A number of studies have reported the ability of genetic elements to control chlorophyll accumulation in photosynthetic tissues²⁵⁻²⁹.

That chlorophyll b content was higher in Wilis cultivars than in Devon showed a difference in the formation of chlorophyll b in each cultivar. Chlorophyll b in photosynthesis played its role in the photosystem I, whereas chlorophyll a played its role in the photosystem II. This is supported by Ohmiya *et al.*³⁰, who found that the chlorophyll metabolic pathway could be divided into three distinct phases, namely synthesis of chlorophyll a from glutamate, interconversion between chlorophyll a and b (chlorophyll cycle) and degradation of chlorophyll a into a non-fluorescent chlorophyll catabolite.

The results also showed that the application of salicylic acid at the V4 on Wilis cultivar increased the chlorophyll a and total chlorophyll contents but the application of salicylic acid at the V4 and R3 on Wilis increased the chlorophyll b content. It can be explained that each cultivar has different responses to the timing and type of elicitor. In Wilis cultivar the salicylic acid treatment at the V4 tended to increase the chlorophyll a and total chlorophyll contents because foliar application of salicylic acid played a vital role in plant growth, whereas at the V4 soybean, it played a vital role in the vegetative growth phase, in which photosynthesis was active, so that the addition of salicylic acid increased the chlorophyll content. This is supported by previous research findings that the

exogenous salicylic acid application also enhanced the growth and photosynthetic rate and the effect of exogenous salicylic acid depended on various factors, including the species and developmental stage, the mode of application and the concentration of salicylic acid used³¹⁻³⁵. Other researchers demonstrated that salicylic acid participated in the regulation of physiological processes in plant such as stomatal closure, nutrient uptake, chlorophyll synthesis and was supposed to increase the functional state of the photosynthetic machinery in plants either by the mobilization of internal tissue nitrate or chlorophyll biosynthesis³⁶⁻³⁸. The previous study has also reported that the effects of exogenous and endogenous salicylic acid on the photosynthetic processes under optimal and stress conditions³⁹. However, the effectiveness of salicylic acid in stress tolerance depended on the type of species and experiment conditions such as the concentration of the salicylic acid applied, the type of stress and the level and duration of stress⁴⁰⁻⁴². This study is in the contrary with the previous researches finding that chitosan produced higher chlorophyll content. This might occur because of differences in location and microclimate characteristics of the research sites.

In this study, the foliar application of salicylic acid treatment at the V4 resulted in higher stomatal density. This is supported by the previous research findings showing that salicylic acid had an effect on the photosynthetic activity and stomatal conductance of tomato under salt stress⁴³. The present study shows that stomatal limitation is involved in salicylic acid that induces alleviation of the negative effects of drought stress on photosynthesis in barley leaves.

CONCLUSION

It is concluded that in Wilis cultivar, the foliar application of salicylic acid at the V4 increased the chlorophyll a content,

the total chlorophyll content and the stomatal density, while in the Devon cultivar, the foliar application of salicylic acid at the V4 resulted in the highest chlorophyll b content, while the application of chitosan at the V4 on Devon cultivar increased the stomatal density.

SIGNIFICANCE STATEMENT

This study discovers a difference in the response of each cultivar to the elicitor application, that are beneficial to increase the chlorophyll content and stomatal density that are very instrumental in the process of photosynthesis and also found that the foliar application of salicylic acid at V4 in Wilis cultivar increased the chlorophyll a, total chlorophyll content and stomatal density, while in Devon cultivar, the treatment of chitosan at V4 increased stomatal density. This study will help researchers to cope the best elicitor that can be used for certain soybean cultivars to increase chlorophyll content and stomatal density when associated with photosynthesis and specific environmental stress such as drought stress.

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