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Research Article
Role of Elicitors Foliar Application in Increasing Isoflavone Content of Two Soybean Cultivars

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Abstract
Background and Objective: 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. The objective of the research was to determine the best elicitor for increasing isoflavone content in Anjasmoro and Wilis cultivar. **Materials and Methods:** The research design used in this study was a randomized block design factorial. The treatments are 3 factors and 3 replications. The first factor is soybean cultivars (Anjasmoro and Wilis). The second factor is foliar application of elicitors consisted of without elicitor; chitosan (1 mg mL^{-1}), methyl jasmonate (0.5 mM) and salicylic acid (0.5 mM). The third factor is the time of foliar application of elicitors consisted of V4 (four trifoliate leaves are fully developed) and R3 (early podding). The parameters of observations are genistein, daidzein, glycitein and total isoflavones. The data were subjected to two-way analysis of variance (ANOVA) procedures, the SAS version 12 computer program and comparison of means were tested for significance using Duncan's Multiple Range Test (DMRT) ($p = 0.05$). **Results:** The results suggested that in Wilis cultivar, the foliar application of chitosan elicitor at R3 is able to increase the content of genistein (25.10%), daidzein (42.76%), glycitein (76.50%) and total isoflavones (37.04%). **Conclusion:** The best treatment in increasing the content of genistein, daidzein, glycitein and total isoflavones in Wilis cultivar is foliar application of chitosan elicitor at R3.

Key words: Daidzein, genistein, glycitein, isoflavone, foliar application, soybean, chitosan elicitor

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Isoflavones produced from bio-resources is gaining attention for attractive components in food supplements. Isoflavone is a heterocyclic phenol with a very similar structure to estrogens. Genistein, daidzein and glycitein, as primary isoflavones in soybeans, are reported to have beneficial effects on atherosclerosis, chronic inflammatory diseases, displays like an estrogens and anti estrogen activity; influences sex hormone metabolism and their biological activity and prevents osteoporosis[1], anti atherosclerotic, antioxidative, antitumoral and antiestrogenic activities[2-5], dementia[6] and cancer[7,8].

The content of isoflavone in soybean seed is influenced by several factors such as abiotic and biotic factors. The temperature condition, water deficit and UV light were included in the abiotic factors that influenced soybean seed isoflavone[9,10]. The biotic factor which influenced the content of isoflavone such as pathogens, wounding and interaction of plant microbe[11]. These factors are involved in phenyl propanoid pathway genes for biosynthesis of isoflavone[12].

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. Previously field condition study has also demonstrated that foliar application of methyl jasmonate increased isoflavone content, whereas it decreased by increasing the concentration of salicylic acid, *Aspergillus niger* and *Rhizopus oligosporus*[13].

The objective of the present study was to determine the effects of foliar applications of elicitor compounds (i.e., methyl jasmonate, salicylic acid and chitosan) on isoflavone content (genistein, daidzein and glycitein) of soybean.

MATERIALS AND METHODS

Materials: Pot experiment has conducted in a screen house of Faculty of Agriculture, Universitas Sumatera Utara (Indonesia) on May-July, 2016. The soil content of nitrogen is low (0.14%), organic matter is 1.02%, with a pH of 4.5. The analysis of genistein, daidzein, glycitein and total isoflavones was conducted at Research Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara.

Methods

Experimental design and crop management: Treatments were arranged in a randomized block design with three factors and three replications. The first factor is two soybean cultivars (Anjasmoro and Wilis). The second factor is elicitor sources and consisted of no elicitor; chitosan; methyl jasmonate and salicylic acid. The third factor is time of application namely V4 (four trifoliate leaves are fully developed) and R3 (early podding). Selection of the type and concentration of elicitor referring to previously experiments[14]. Preparation of elicitor referred to standard procedures. Chitosan, methyl jasmonate and salicylic acid is a product of Sigma Aldrich. Autoclaved stock solution of 120°C for 20 min and sterile distilled water to obtain a final concentration of chitosan solution 1 mg mL^{-1}. Abiotic elicitor, salicylic acid and methyl jasmonate dissolved in distilled water and diluted to concentrations (0.5 mM). The determination of the concentration of methyl jasmonate and salicylic acid refers to previous research[16]. Parameters observed was the content of genistein, daidzein, glycitein and isoflavone total.

Statistical analysis: The data were subjected to two-way analysis of variance (ANOVA) procedures, the SAS version 12 computer program and comparison of means were tested for significance using Duncan's Multiple Range Test (DMRT) ($p = 0.05$).

Isoflavone extraction and HPLC analysis: Following harvest, seeds were stored at room temperature and within 1 month, isoflavones were extracted for determination of isoflavone composition and content. Concentration of genistein, daidzein and glycitein were determined using a Ultra High Performance Liquid Chromatography (UHPLC) method from Vyn *et al.*[15]. The duplicate sample of soybean seeds was finely ground and weighed 0.50 g each and dispersed in 10 mL of ethanol plus 2 mL of concentrated HCl. In the sand bath, the resulting solution was hydrolyzed by heating to 125°C for 2 h. The sample was cooled and centrifuged at 3000 rpm for 10 min. The clear aliquot is filtered through a 0.45 μm PTFE filter. Individual hydrolyzed genistein, daidzein and glycitein are separated in Ultra High Performance Liquid Chromatography (UHPLC) equipped of Ultraviolet (UV) and visible light detectors. This type of detector shows high sensitivity for many compounds. Nevertheless, a compound must absorb light in the UV or visible region of 190-600 nm to be detected.

UHPLC column, SB C18 column (4.8×50 mm, 3.55 mm particle size) with C18 guard column; UHPLC mobile phases, solvent A was 4% aqueous acetic acid and solvent B was 100% UHPLC grade methanol; flow rate was 1.5 mL min^{-1} and injection volume, 5 mL. The solvent system was as follows (% solvent A and % solvent B): 0 min (70/30), 12.5 min (65/35), 13 min (50/50), 15 min (30/70), 22.5 min (25/75) and 23 min (70/30).

RESULTS

Genistein content: Result showed that interaction of cultivar type and type of elicitor application give significance effect on genistein content (Table 1). Wilis cultivar has higher genistein content than Anjasmoro cultivar. In Wilis cultivar, application of chitosan at R3 produced the highest of genistein content (2318.06 μg g^{-1} seed dry weight), while application of salicylic acid at R3 produced the lowest genistein content (1105.10 μg g^{-1} seed dry weight). Otherwise in Anjasmoro cultivar, application of salicylic acid at R3 produced the highest of genistein content (1272.33 μg g^{-1} seed dry weight). Application of methyl jasmonate at V4 produced the lowest of genistein (677.2 μg g^{-1} seed dry weight).

Daidzein content: The research result showed that interaction of cultivar type and type of elicitor application give significance effect on daidzein content (Table 2). Wilis cultivar has higher daidzein content than Anjasmoro cultivar. In Wilis cultivar, application of chitosan at R3 produced the highest of daidzein content (2463.65 μg g^{-1} seed dry weight); while application of salicylic acid at R3 produced the lowest of daidzein content (791.02 μg g^{-1} seed dry weight). Otherwise, in Anjasmoro cultivar, application of without elicitor at V4 produced the highest of daidzein content (884.94 μg g^{-1} seed dry weight). Application of methyl jasmonate at V4 produced the lowest of daidzein (419.23 μg g^{-1} seed dry weight).

Glycitein content: The interaction of cultivar type and type of elicitor application gives significance effect on glycitein content (Table 3). Wilis cultivar has higher glycitein content than Anjasmoro cultivar. In Wilis cultivar, application of chitosan at R3 produced the highest of glycitein content (347.04 μg g^{-1} seed dry weight); while application of chitosan at V4 produced the lowest of glycitein content (64.62 μg g^{-1} seed dry weight). Otherwise, in Anjasmoro cultivar, application of methyl jasmonate at R3 produced the highest of glycitein content (139.96 μg g^{-1} seed dry weight. Both of two cultivars,

Table 1: Genistein content of Anjasmoro and Wilis cultivars with foliar application of elicitors

Cultivar	Elicitor	Time of application: V4 (T1)	Time of application: R3 (T2)	Mean
		μg g^{-1} seed dry weight		
Anjasmoro (V1)	Without elicitor (E0)	921.55^{fgh}	960.93^{fgh}	941.24
	Chitosan (E1)	820.28^{ghi}	738.10^{hi}	779.19
	Methyl jasmonate (E2)	677.21^{i}	1023.80^{fg}	850.51
	Salicylic acid (E3)	895.18^{fgh}	1272.33^{de}	1083.76
Mean of V1×T		828.56	998.79	
Mean of V1				913.67
Wilis (V2)	Without elicitor (E0)	1549.43^{bc}	1736.95^{b}	1643.19
	Chitosan (E1)	1518.87^{bcd}	2318.06^{a}	1918.47
	Methyl jasmonate (E2)	1298.58^{de}	1445.18^{cd}	1371.88
	Salicylic acid (E3)	1364.55^{cd}	1105.10^{ef}	1234.83
Mean of V2×T		1432.86	1651.32	
Mean of V2				1542.09

Different letters represent significant differences at Duncan's multiple range test (p = 0.05)

Table 2: Daidzein content of Anjasmoro and Wilis cultivars with foliar application of elicitors

Cultivar	Elicitor	Time of application: V4 (T1)	Time of application: R3 (T2)	Mean
		μg g^{-1} seed dry weight		
Anjasmoro (V1)	Without elicitor (E0)	884.94^{def}	615.72^{fghi}	750.33
	Chitosan (E1)	573.68^{ghi}	519.59^{ghi}	546.63
	Methyl jasmonate (E2)	419.23^{i}	702.69^{fgh}	560.96
	Salicylic acid (E3)	487.72^{hi}	477.38^{hi}	482.55
Mean of V1×T		591.39	578.85	
Mean of V1				585.12
Wilis (V2)	Without elicitor (E0)	1781.27^{b}	1410.16^{c}	1595.71
	Chitosan (E1)	1544.26^{bc}	2463.65^{a}	2003.95
	Methyl jasmonate (E2)	1051.59^{de}	1099.40^{d}	1075.50
	Salicylic acid (E3)	1026.67^{de}	791.02^{efg}	908.85
Mean of V2×T		1350.95	1441.06	
Mean of V2		884.94	615.72	750.33

Different letters represent significant differences at Duncan's multiple range test (p = 0.05)

Table 3: Glycitein content of Anjasmoro and Wilis cultivars with foliar application of elicitors

Cultivar	Elicitor	Time of application V4 (T1)	R3 (T2)	Mean
		μg g^{-1} seed dry weight		
Anjasmoro (V1)	Without elicitor (E0)	69.69^{e}	59.80^{e}	64.74
	Chitosan (E1)	67.87^{e}	60.48^{e}	64.17
	Methyl jasmonate (E2)	47.98^{e}	139.96^{cd}	93.97
	Salicylic acid (E3)	59.56^{e}	64.03^{e}	61.79
Mean of V1×T		61.27	81.07	
Mean of V1				71.17
Wilis (V2)	Without elicitor (E0)	76.49^{de}	81.53^{de}	79.01
	Chitosan (E1)	64.62^{e}	347.04^{a}	205.83
	Methyl jasmonate (E2)	170.25^{bc}	222.05^{b}	196.15
	Salicylic acid (E3)	115.72^{cde}	183.09^{bc}	149.41
Mean of V2×T		106.77	208.43	
Mean of V2				157.60

Different letters represent significant differences at Duncan's multiple range test (p = 0.05)

Table 4: Total isoflavone content of Anjasmoro and Wilis cultivars with foliar application of elicitors

Cultivar	Elicitor	Time of application V4 (T1)	R3 (T2)	Mean
		μg g^{-1} seed dry weight		
Anjasmoro (V1)	Without elicitor (E0)	1876.18^{fg}	1636.44^{fgh}	1756.31
	Chitosan (E1)	1461.83^{ghi}	1318.18^{hi}	1390.00
	Methyl jasmonate (E2)	1144.42^{i}	1866.46^{fg}	1505.44
	Salicylic acid (E3)	1442.46^{ghi}	1813.74^{fg}	1628.10
Mean of V1×T		1481.22	1658.71	
Mean of V1				1569.96
Wilis (V2)	Without elicitor (E0)	3407.19^{b}	3228.64^{b}	3317.92
	Chitosan (E1)	3127.76^{bc}	5128.75^{a}	4128.25
	Methyl jasmonate (E2)	2520.42^{de}	2766.64^{bc}	2643.53
	Salicylic acid (E3)	2506.94^{de}	2079.21^{ef}	2293.08
Mean of V2×T		2890.58	3300.81	
Mean of V2				1756.31

Different letters represent significant differences at Duncan's multiple range test (p = 0.05)

the application of elicitor at R3 produced glycitein content of soybean higher than the application of elicitor at V4.

Total isoflavone content: The interaction of cultivar type and type of elicitors application give significance effect on total isoflavone content (Table 4). In Wilis cultivar, application of chitosan at R3 produced the highest total isoflavone content (5128.75 μg g^{-1} seed dry weight); while application of salicylic acid at R3 produced the lowest total isoflavone content (2079.21 μg g^{-1} seed dry weight). Otherwise in Anjasmoro cultivar, application of methyl jasmonate at V4 produced the lowest of total isoflavone content (1144.42 μg g^{-1} seed dry weight). Both of two cultivars, the application of elicitor at R3 produced total isoflavone content of soybean higher than the application of elicitor at V4.

DISCUSSION

Wilis cultivar with application of Chitosan at R3 application produced a higher content of genistein, daidzein, glycitein and total isoflavones content compared to other treatments (Table 1-4). This study confirmed the results from previously green house studies, demonstrating that elicitors hold potential as a means of increasing isoflavone concentrations in soybean seed[16], hairy root cultures of *Pueraria candollei*, trigonelline content[17]. Previous research by the author that conducted with different sources of nitrogen under dryland conditions also reported that Wilis cultivar has higher genistein, daidzein and glycitein than Anjasmoro and Sinabung cultivars[18].

It was previously hypothesized that soybean response to foliar application of elicitor compounds is the result of an induction or promotion of the phenyl-propanoid pathway leading to increase in isoflavones synthesis[19,20]. Increased activity of some enzymes or genes regulating this pathway and related ones were previously reported as a plant response to elicitors[21]. It is likely that responses to elicitors in field-grown plants are affected by prevailing environmental conditions, which have also been demonstrated to greatly affect isoflavone synthesis and accumulation in soybean[22].

The elicitor application time of R3 in both cultivars produces a higher isoflavone than V4. This is related to the soybean growth phase where R3 is the early phase of pod formation, whereas in soybean, V4 is in the rapid vegetative growth phase. This also suggested that the phase of R3 is the best time of the elicitor application to stimulate the formation of isoflavones which rapidly increase from R5 (beginning seed development) to R7 (onset of physiological maturity). This was supported by previously research that application of elicitor on R3 increased the isoflavone content of soybean[23] and also the total isoflavone concentrations rapidly increased as growth stages transitioned from R5 to R7[24]. Several factors may influence isoflavone content and composition, including soybean variety, growing conditions and stage of soybean growth[21].

There was a difference in the response of soybean cultivars to the type and timing of elicitor applications. The application of chitosan elicitor when soybean plant is in phase, R3 increased the content of genistein, daidzein, glycitein and total isoflavones, otherwise in Anjasmoro cultivar, the application of salicylic acid when soybean plant is in phase R3 increased the content of genistein and total isoflavones.

CONCLUSION

The content of genistein, daidzein, glycitein and total isoflavone in Wilis cultivar is higher than Anjasmoro. The elicitor application time of R3 is able to increase the total isoflavone around 12% in Wilis cultivar and 14% in Anjasmoro cultivar than application time of V4. The content of genistein, daidzein, glycitein and total isoflavones in Wilis cultivar with application of Chitosan at R3 application were highest compared to other treatments.

SIGNIFICANT STATEMENT

This study discovered differences in response of each cultivar to the type and time of elicitors' foliar application, which are beneficial to increase the isoflavone content, which is very important for human health. This study will help researchers and farmers about the type of elicitor and the best time for elicitor applications on soybean cultivar. Thus, the new theory found that the foliar application of chitosan at R3 in Wilis cultivar increased genistein, daidzein, glycitein and total isoflavone.

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