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Augmentation of major isoflavones in *Glycine max* L. through the elicitor-mediated approach

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Abstract – Isoflavone content in soybean seeds was enhanced by the elicitor-mediated approach under field conditions through the floral application of abiotic elicitors-salicylic acid, methyl jasmonate and biotic elicitors-*Aspergillus niger* and *Rhizopus oligosporus*. Among isoflavones, daidzein and glycitein were found to be highly responsive to elicitors, with an increase of 53.7% and 78.7%, respectively as compared to control. Highest total isoflavone content (1276.4 μg g^{-1} of seeds) was observed upon the administration of 0.1 mM salicylic acid, which is 92.7% higher than in control. This study would be valuable for augmentation of the isoflavone content in soybean seeds in field grown plants for better nutraceutical potential.

Keywords: *Glycine max* L, isoflavone, elicitation

Abbreviations: DPPH – 2,2-diphenyl-1-pycrilhydrazil hydrate

Introduction

Isoflavones belong to a broad class of phytoestrogens and are the naturally occurring and abundant bioactive components in soybean. They have antiestrogenic, antioxidant, anti-inflammatory activities and are also associated with lower incidence of chronic diseases such as cancers, heart and kidney diseases and in addition prevent bone loss (MESSINA 1999, PARR and BOLWELL 2000, ATKINSON et al. 2004). Estrogenic and anti-estrogenic effects of isoflavone depend on the natural levels of estrogen. At low level of natural estrogen, isoflavones mimic estrogen by activating the estrogen receptors (BARNES et al. 2000), whereas, at high estrogen levels, they bind with estrogen receptors, decrease the availability of estrogen receptors and act as anti-estrogens (KUIPER et al. 1998). In view of the various beneficial effects attributed to isoflavones, consumer interest in soybean products has been considerably enhanced, leading to the incorporation of soybean in a wide range of foods

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(SETCHELL and COLE 2003). Soybean and soy products contain twelve different isoflavone isomers including three principal aglycones (daidzein, genistein, and glycitein), their glycosides, and their corresponding acetyl and malonyl forms.

Several factors such as the genetic, the environmental, geographical location, storage and plant variety might influence the isoflavone content and composition in soybean seeds (WANG and MURPHY 1994, HOECK et al. 2000, LEE et al. 2003). Recently, variations in the isoflavone levels and antioxidant activity in the soybean cultivars of India and Bulgaria along with different soy products have been reported (SAKTHIVELU et al. 2008, AKITHA DEVI et al. 2009). Isoflavone content in soybean seeds (and other soybean tissues) is influenced by biotic and abiotic factors including physical and chemical damages, UV light, low temperature, wounding, pathogens and plant microbe interactions as well as elicitor treatment (WEGULO et al. 2005, CALDWELL et al. 2005, ZHANG et al. 2006, PHOMMALTH et al. 2008). It is also found that these factors are involved in the up-regulation or induction of the phenyl propanoid pathway genes for the biosynthesis of isoflavones.

Elicitation is an efficient strategy that by means of compounds or treatments induces plants to synthesize phytoalexins at enhanced levels (RAO and RAVISHANKAR 2002). Flavonoid production is stimulated when the plant recognizes certain elicitor molecules or structures that characterize a pathogen or a symbiont (TAHVONEN 1988, AL-TAWAHA et al. 2005). Elicitors thus, have been used to increase plant resistance to various pests (BEAUSEJOUR et al. 2003), to enhance the content of beneficial phytochemicals in various plant species (KIM et al. 2006, PEREZ-BALIBREA et al. 2011) as well in nutraceutical industries (ZHAO et al. 2001). The dynamic mechanisms of elicitors are considered to be complex and the effect of elicitors depends on numerous factors, such as the concentration and type of the elicitor and the growth stage of the culture at the time of elicitation.

Both abiotic and biotic elicitors were previously studied for their efficacy in enriching isoflavones in mature seeds (AL-TAWAHA et al. 2005, BOUE et al. 2008). However, all these studies were confined to either matured stage beans or cotyledonary seedlings. Recently, isoflavones have attracted great interest due to their potent antioxidant activities through the stabilization of free radicals (GORDON, 1990). Significant losses of isoflavones (50% or more) occur during processing of the soybean in traditional soy foods (WANG and MURPHY 1996), which is a bottleneck in the maintenance of effective levels of isoflavones in soy products. This problem could be addressed by increasing the isoflavone content and reducing the variability in soybean seeds. A novel elicitor mediated approach thus seems possible for increasing isoflavone synthesis and content in mature soybean seeds.

In the present study, the efficacy of floral administration of selective elicitors to enhance the isoflavone content in *Glycine max* and antioxidant potential is explored.

Materials and methods

Plant materials

The soybean (*Glycine max* L.) breeding line JS-335 used in this study was selected because of its early maturity (90 days), maximum sown area in India and resistance to major diseases and pest. Authentic seeds were obtained from the National Seeds Corporation, Mysore. JS-335 soybean is derived from the cross of JS78-77 (Kohar × P.S. 73-22) × 71 – 05.

Preparation and application of elicitors

Biotic elicitors were prepared according to GIRIDHAR and PARIMALAN (2010), using two fungal cultures *Aspergillus niger* and *Rhizopus oligosporus* that were obtained from the microbial culture facility of Food Microbiology Department of CSIR-CFTRI. Fresh cultures of *A. niger* and *R. oligosporus* were grown on potato dextrose agar (HiMedia, Mumbai) slants and incubated for 7 days at 37 °C. Then the spores of the respective fungi were used to prepare a spore suspension in 0.1% sodium lauryl sulfate (w/v) and diluted with sterile distilled water under sterile conditions to obtain a spore density of $\sim 2.5 \times 10^6$ spores mL^{-1}. Later the same was inoculated into 150 mL Erlenmeyer conical flasks containing 40 mL of potato dextrose agar and the cultures were incubated in the dark for 10 days. After the incubation, the cultures were autoclaved and the mycelium was separated from the culture broth by filtration and its fresh weight was recorded. An aqueous extract was made by homogenizing with a mortar and pestle using neutralized sand. The extract was filtered through Whatman no. 1 filter paper, and kept as the stock solution from which the individual fungal mycelial extracts at a working concentration of 0.1, 0.25 and 0.5 % w/v (wet weight of fungal mycelium in 1000 mL of distilled water) were prepared in sterile water and used for the elicitation experiment. Abiotic elicitors, salicylic acid and methyl jasmonate dissolved in distilled water and diluted to three concentrations (0.1 mM, 0.25 mM, and 0.5 mM) were used for the study.

The field experiment was conducted at the Plant Cell Biotechnology Department of this institute. The soil type was red silt loam with fine-silty characteristics which is the best for soybean cultivation. JS-335 soybean was planted in complete random block design (CRBD) layout at the spacing of 1.5 m in length and 40 cm wide rows, with four replicates of control and each treatment. In each row, 5 plants were grown, with a plant to plant spacing of 20 cm. All the plants were maintained according to recommended management practice (ICAR (2006) throughout the growing season. All the elicitors were sprayed on the fully opened flower of JS-335 soybean plants between 10 and 11 am. An equal quantity of distilled H_2O was sprayed on control plants. A total 1000 mL of elicitor solution was sprayed in each treatment (four rows of total 20 plants)

Isoflavone extraction

After complete maturity, approximately 60 days after treatments, seeds were harvested and stored at ambient temperature for 30 days. Before extraction, seeds were dried at 37 °C for 48 hours. Seed samples with the seed coat (400 mg) were finely ground and extracted with 2 mL of concentrated HCl and 10 mL of absolute ethanol (99.9% pure) for 2 hours in a boiling water bath using a standard method (VYN et al. 2002), which relies on acid hydrolysis of 12 endogenous isoflavone isomers to their respective aglycone forms, mostly daidzein, glycitein and genistein. The resulting suspension were cooled and centrifuged at $10{,}000 \times g$ for 10 min. The supernatant obtained after centrifugation was filtered through a syringe filter (Whatman 0.5 μm, 13 mm diameter).

Isoflavone standards

Standards of isoflavones, daidzein, genistein and daidzein were purchased from Sigma--Aldrich, Bangalore. Isoflavone stock solutions were prepared by dissolving the standards

in absolute ethanol at concentration of 1 mg mL^{-1}. Calibration curves were made for each standard in concentration range 0.01–0.1 mg mL^{-1}. The content of individual isoflavones in the sample was calculated manually on the basis of each peak area. Individual and total isoflavone content was expressed as micrograms per gram of dry weight.

Chromatographic conditions

The HPLC analysis of the respective extracts for isoflavones was performed using a Shimadzu chromatograph (LC 20-AD, HPLC), equipped with dual pump, UV detector (SPD 20A) and a C-18 column (Sunfire; 5 µm with dimension of 250 × 4.6 mm). The separation and elution of isoflavones was modified by employing a binary gradient mode using solvent A (10% acetonitrile) and solvent B (38% acetonitrile) with injection volume of 20 µL of the sample and at a flow rate of 0.8 mL min^{-1} for 40 min (KUMAR et al. 2009). The solvent system was run as follows (% solvent A/solvent B): 0 min (0/100), 5 min (10/90), 35 min (0/100), and 40 min (0/100). Isoflavones were monitored at 260 nm.

Free radical scavenging activity

DPPH (2,2-diphenyl-1-pycrilhydrazil hydrate) radical-scavenging activity of elicitor treated and untreated seed extracts were evaluated by using the method of KHAN et al. (2011). An aliquot of 0.2 mL of the methanolic seed extract (2 mg mL^{-1}) was added to a 3.8 mL absolute ethanol (99.9%) solution of DPPH (a stable free radical) to a final concentration of 0.1 mM. The mixture was shaken vigorously for 1 min by vortexing and left to stand at room temperature in the dark for 30 min. Subsequently, the absorbance of the sample (A_{sample}) was measured at 517 nm using the UV-visible spectrophotometer against ethanol as blank. A negative control ($A_{control}$) was taken after adding DPPH solution to 0.2 mL of the respective extraction solvent. The percent of DPPH inhibition and scavenging of the sample was calculated according to the following equation:

$$\text{Percent of inhibition and scavenging} = [1 - \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100]$$

The free radical-scavenging activity of the extracts was expressed as percent of inhibition at 100 µg mL^{-1} seed extract.

Total phenolic content

Total phenolic content of treated and untreated seed extracts was determined by Folin–Ciocalteu assay using gallic acid as standard (KIM et al. 2003). In brief, 50 µL of the sample was mixed with distilled water (3 mL), followed by 7% sodium carbonate (750 µl) solution and Folin– Ciocalteu reagent (250 µL). Subsequently the solution was vortexed and incubated for 8 min at room temperature. To this, 950 µL distilled water was added and the reaction mixture was allowed to stand for 2 hours at room temperature, and finally the absorbance was measured at 725 nm using a UV–Visible spectrophotometer (Shimadzu UV 160) against distilled water as blank. The total phenolic content was expressed as mg of gallic acid equivalents g^{-1} of seeds. Linearity range of the calibration curve was 50–1000 µg mL^{-1} (r = 0.98).

Statistical analysis

The isoflavone content, DPPH scavenging activity and total phenolic content from all four replicates were measured separately, and represented as mean ± standard deviation (S.D.). Data were analyzed statistically by SPSS 17.0 software by analysis of variance (one way ANOVA), least significant difference (LSD) was calculated between the mean values of different treatments. Different alphabetical letters were assigned to demonstrate significant difference between the treatments.

Results

Effect of elicitors on isoflavones in soybean seeds

The effect of floral administration of several abiotic and biotic elicitors on the isoflavone levels of soybean seeds was determined in the present study. Aglycone forms as well as total isoflavones were evaluated in elicitor-treated and untreated soybean seeds (Tab. 1). The isoflavone content increased with an increase in the concentration of methyl jasmonate whereas it decreased with an increase in the concentration of salicylic acid, *Aspergillus niger* and *Rhizopus oligosporus*. Among the major isoflavones analysed, daidzein and glycitein showed higher responses to elicitors with a mean increase of 53.7% and 78.7%, while, genistein increased negligibly (2.9%) compared to control. A maximum of 54.7% increase in total isoflavone content was observed in the experiment and a minimum of 27.3% in case of *A. niger,* compared to control.

Elicitors at different concentrations had a varying influence on isoflavone levels in soybean seeds. The total isoflavone response to salicylic acid was 14.7% greater then average.

Tab. 1. Isoflavone content (μg g^{-1}) in mature seeds of soybean plants (var. JS-335) treated with biotic and abiotic elicitors. Values are mean ± SD of four independent replicates. Different letters indicate statistically significant differences between the means ($P < 0.05$) for total and individual isoflavone content.

Treatment	Concentration	Daidzein	Glycitein	Genistein	Total isoflavone
Salicylic acid	0.1 mM	503.5 ± 25.1^{a}	389.0 ± 35.7^{a}	384.0 ± 35.0^{c}	1276.4^{a}
	0.25 mM	283.6 ± 15.2^{g}	191.3 ± 9.0^{e}	438.1 ± 25.5^{b}	913.1^{c}
	0.5 mM	393.3 ± 16.0^{de}	270.2 ± 18.1^{c}	221.1 ± 18.7^{g}	884.7^{c}
Methyl jasmonate	0.1 mM	331.9 ± 9.8^{f}	173.5 ± 12.5^{ef}	178.0 ± 14.7^{h}	683.4^{e}
	0.25 mM	369.1 ± 16.9^{e}	138.0 ± 15.9^{gh}	256.5 ± 24.0^{f}	763.5^{d}
	0.5 mM	442.9 ± 20.9^{bc}	296.2 ± 25.1^{b}	313.0 ± 39.8^{de}	1052.0^{b}
Aspergillus niger	0.10%	422.2 ± 20.7^{cd}	161.3 ± 13.5^{f}	524.0 ± 26.5^{a}	1107.4^{b}
	0.25%	376.9 ± 44.8^{e}	229.0 ± 12.5^{d}	239.5 ± 8.9^{fg}	845.5^{c}
	0.50%	201.7 ± 10.6^{i}	123.3 ± 17.6^{h}	342.6 ± 20.0^{d}	667.7^{de}
Rhizopus oligosporus	0.10%	465.3 ± 16.6^{b}	271.7 ± 16.6^{c}	335.5 ± 17.5^{d}	1072.6^{b}
	0.25%	308.4 ± 17.2^{fg}	225.0 ± 22.5^{d}	225.2 ± 22.5^{g}	$758.7b^{d}$
	0.50%	228.0 ± 13.0^{hi}	155.7 ± 14.8^{fg}	314.0 ± 21.0^{de}	697.6^{de}
Control	–	234.6 ± 12.5^{h}	122.4 ± 10.6^{h}	305.5 ± 10.3^{e}	662.5^{e}

Highest total isoflavone content (1276.4 μg g^{-1} of seeds) was observed following the floral application of 0.1 mM salicylic acid, which represented a 92.7% increase in comparison to the untreated control. Maximum daidzein and glycitein content (i.e. 503.5 and 389.0 μg g^{-1} of seeds, respectively) was observed in the same treatment, whereas maximum genistein content (i.e. mean of 524.0 μg g^{-1} of seeds) was observed in 0.1% *A. niger* treated seeds. In this study, salicylic acid as abiotic elicitor, and *A. niger* as biotic elicitor were found to be most effective for enhancement of isoflavone content in soybean seeds.

Antioxidant activity

Antioxidant activities of the elicitor-treated soybean seed extracts were evaluated by free radical scavenging assay and also by total phenolic content (Tab. 2). In the present study, there was no significant difference in free radical scavenging activities between the control and the treated soybean seed extracts ($P < 0.05$). On the whole, the average free radical scavenging activity of elicitor treated and control seeds were approximately 80.0 %. The DPPH scavenging activity did not correlate significantly with total isoflavones or with total phenolic content ($R^2 = 0.153$).

The total phenolic contents of elicitor-treated and control seed extracts are shown in table 2. The total phenolic content correlated well with total isoflavone ($R^2 = 0.767$), as expected. Thus, the trend in total phenolic content upon treatments is in agreement with trend in total isoflavone content. Salicylic acid treatment (0.1 mM) produced the highest total phenolic content reaching 9.73 mg of gallic acid equivalent g^{-1} of seeds.

Tab. 2. Free radical scavenging activity and total phenolic contents of seed extracts from different treatment. Free radical scavenging activity was represented as percent of DPPH inhibition and scavenging at 100 μg mL^{-1} seed extract. Different letters indicate statistically significant differences between the means ($P < 0.05$) for total phenolic content.

Treatment	Concentration	DPPH (% free radical scavenging activity)	TPC (mg of GAE/g)
Salicylic acid	0.1 mM	82.22 ± 1.7	9.73 ± 0.23^{a}
	0.25 mM	82.22 ± 2.0	8.42 ± 0.17^{c}
	0.5 mM	82.44 ± 2.4	8.22 ± 0.23^{cd}
Methyl jasmonate	0.1 mM	80.18 ± 1.8	7.79 ± 0.2^{ef}
	0.25 mM	78.93 ± 1.4	7.89 ± 0.26^{de}
	0.5 mM	80.18 ± 1.6	8.42 ± 0.3^{c}
Aspergillus niger	0.10%	80.41 ± 1.4	8.42 ± 0.29^{c}
	0.25%	80.52 ± 1.4	8.18 ± 0.21^{cd}
	0.50%	80.52 ± 1.2	8.0 ± 0.35^{de}
Rhizopus oligosporus	0.10%	77.78 ± 1.4	9.32 ± 0.20^{b}
	0.25%	76.16 ± 1.3	8.17 ± 0.29^{cd}
	0.50%	80.41 ± 0.3	7.8 ± 0.23^{ef}
Control	–	80.52 ± 1.6	7.5 ± 0.17^{f}

DPPH – 2,2-Diphenyl-1-picrylhydrazyl, TPC – Total phenolic contents, GAE – Gallic acid equivalent.

Discussion

In this study, a floral spray of various elicitors was found effective in increasing the isoflavone content in mature seeds of soybean plants. Both abiotic (i.e. salicylic acid and methyl jasmonate) and biotic elicitors (*A. niger* and *R. oligosporus*) caused an increased level of aglycones and total isoflavone in soybean seeds and this result is analogous to the studies of ZHANG et al. (2006), that aglycone could be enhanced by selecting the appropriate and optimal concentration of elicitor. Upon elicitation, daidzein were more responsive than genistein and this observation was similar to the results of AL-TAWAHA et al. (2005). Several researchers observed that, genistein is the least responsive to elicitors (SEGUIN et al. 2004, SHINDE et al. 2009). However, ZHANG et al. (2006) demonstrated that salicylic acid treatment significantly affected malonyl-daidzein and glycitein levels at different developmental stages in soybean plant. Moreover, our findings were in contrast to the result of KNEER et al. (1999), who demonstrated that salicylic acid, soluble chitosan and potassium cyanide stimulated production of genistein in yellow lupin roots (*Lupinus luteus* L.). In the present study, we found that the *A. niger* elicitor proved to be better for the induction of isoflavones than *R. oligosporus*. However, LEE et al. (2010) suggested that *Rhizopus oligosporus* was the best elicitor for the induction of glyceollins in Korean soybean varieties. Similarly, the studies of PRASAD et al. (2006) suggested that *R. oligosporus* mycelial-extract-treated cultures of *Capsicum frutescens* showed a 6-fold elicitation of capsaicinoids. Interestingly, soybean isoflavones act as inducers for infection and nodulation of *Rhizobium* spp. (PHILLIPS and TSAI 1992) and also induce disease resistance to pathogens, especially in *Phytophthora sojae* (RIVERA-VARGAS et al. 1993). In general, concentration is a more important factor than treatment period in elicitation, where a lower concentration is more effective on most isoflavones (ZHANG et al. 2006). Applied concentration of elicitor and content of elicited molecule obtained generally showed a distinct bell-shaped dose–response curve (KNEER et al. 1999), which means that elicitor molecules are effective at optimum concentration while higher doses are toxic to the plants by inducing cell death and apoplastic oxidative stress (MUR et al. 2006). Some studies also have demonstrated that the timing of floral administration of elicitors havs a significant effect on isoflavone content in soybean (ZHANG et al. 2006).

In general, methyl jasmonate and salicylic acid are key signaling molecules, modulating several physiological events such as defense response to environmental stresses in plants (CREELMAN and MULLET 1997, DRAPER 1997). LU et al. (2006) reported that the activation of a set of defense genes resulted after exogenous application of salicylic acid and methyl jasmonate, which has been shown to move systematically through plants. Significant evidence showed that exogenous supply of methyl jasmonate led to an increase in various classes of secondary metabolites of interest in several plants (MODOLO et al. 2002, WEI 2010, PEREZ-BALIBREA et al. 2011). As suggested by earlier reports, the use of methyl jasmonate might activate the respective genes in the phenylpropanoid (PP) pathway (DIXON and PAIVA 1995). The varied expression of *IFS* genes (IFS1 and IFS2) is responsible for the changes in level of isoflavones in seeds under elicitor treatment (DHAUBHADEL et al. 2007, CHENG et al. 2008). Also, yeast extract elicited the accumulation of isoflavones via elevated level of L-phenylalanine ammonia lyase and chalcone synthase transcripts in cell suspension of *Medicago truncatula* (SUZUKI et al. 2005) and transcripts of flavanone 2-hydro-

xylase, isoflavone 2ž-hydroxylase, and isoflavone synthase in *Glycyrrhiza echinata* cell suspension cultures (NAKAMURA et al. 1999). In contrast, long-term drought stress in soybean plants has been established to result in the down-regulation of *IFS2* gene coinciding with a decrease in isoflavone content (GUTIERREZ-GONZALEZ et al. 2010). Herein may be explanation for the increased production of isoflavones. KOLEVA et al. (2002) described the DPPH method as a simple, rapid and convenient method for screening the free radical scavenging activity of various samples. In the present study, free radical scavenging activity was not correlated with isoflavone content, which suggests that isoflavones are less potent in the free radical scavenging activity compared to some phenolic acids present in soybean seeds (COS et al. 2003). However, soybean isoflavones are potent antioxidants and free radical scavengers, as shown by several reports (LEE et al. 2002, LIN and LAI 2006, BOUE et al. 2008, SAKTHIVELU et al. 2008, AKITHA DEVI et al. 2009).

The soybean contains many phenolic acids such as syringic, ferulic, sinapic, coumaric, gentisic, vanillic, hydroxybenzoic, caffeic, and chlorogenic acids (KOZLOWSKA et al. 1983). Out of these, caffeic and chlorogenic acids are strong free radical scavengers (SROKA et al. 2003). In the present study, we are able to show the improvement in the isoflavone content in soybean seeds through an elicitor-mediated approach. Significant losses of isoflavones (50% or more) occur during processing of the soybean in traditional soy foods, which is a bottleneck in the maintenance of effective levels of isoflavones in soy products. Accordingly, a higher level of isoflavones in the harvested seeds is advantageous in that it will retain the seeds' nutraceutical benefits in processed foods.

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