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Influence of Jasmonic acid on Flavonoid Production in *Glycyrrhiza glabra* Callus Cultures

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Abstract

Glycyrrhiza glabra is one of the widely used medicinal plants and is a rich source of bioactive flavonoids. We investigated the effect of the abiotic elicitor jasmonic acid (JA) on the production of three flavonoids in *G.glabra* callus cultures grown in a previously standardized nutrient medium consisting of Murashige and Skoog's medium components supplemented with naphthalene acetic acid and benzyl amino purine. The JA elicitor was tested in the concentration range of 25–175 mg/L. At each elicitor concentration, a time-course analysis was also done to determine the effect of the duration of JA treatment on flavonoid production. The effect of YE on the key enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase, antioxidant enzymes, polyphenol oxidase, and peroxidase was also observed. The results revealed that more than two-fold increase in the production of liquirtigenin was obtained with 150 mg/L JA whereas the production of licoisoflavone B and licochalcone A was inhibited.

Introduction:

Glycyrrhiza glabra Linn. (Licorice) is a medicinal plant of family Fabaceae which possesses a wide range of phytochemicals and is well known for its pharmaceutical properties mainly attributed to the presence of flavonoids. Major flavonoids of *G.glabra* such as licochalcone A, liquirtigenin and licochalcone B possess distinguished medicinal properties like anti-inflammatory, antioxidant, antitumor, antiviral and antimicrobial activities which complement the age-long medicinal uses of licorice extracts. Wide occurrence, complex diversity and potential benefits of such flavonoids on human health have made it one of the well-studied groups of phytochemicals. However, most of the studies on licochalcone A, liquirtigenin, and licoisoflavone B are confined to their pharmacological properties. The chemical synthesis of most of these flavonoids is not feasible due to the complex structure and chirality exhibited by these compounds and the in vivo production is often not consistent due to several environmental factors. However, plant tissue cultures offer a good alternative for consistent production of target flavonoids and biotic or abiotic elicitation of tissue cultures extend an efficient strategy to increase the production of these metabolites. Plant defense against abiotic stresses is mediated through various signaling pathways that lead to

the production of many defensive proteins and non-protein compounds (War *et al.*, 2011; Rivas-SanVicente & Plasencia, 2011). Plant phytohormones such as abscisic acid, jasmonic acid, ethylene and salicylic acid are important components of different signaling pathways involved in plant defense (War *et al.*, 2011; Rivas-SanVicente & Plasencia, 2011). Thus, various plant hormones have been extensively used in in vitro elicitation studies for enhancing the production of secondary metabolites. Among these, the most studied hormonal abiotic elicitor is Jasmonic Acid (JA) because of its key role in the plant defense response. The exogenous application of JA manipulates various physiological, biochemical and molecular processes in plants including antioxidant enzyme activities.

JA and its methyl ester (MeJA) are widely distributed in the plant kingdom. JA and the related jasmonates are endogenous plant hormones that are involved in the stress response and are known as transducers of secondary metabolite production. They constitute an important class of elicitors for many plant secondary metabolic pathways, which are typically manifested by the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses. JA has been linked to the production of a range of secondary metabolites including flavonoids, terpenoids, and alkaloids. They have also been

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reported to play a key role in signal transduction processes that regulate defense responses in plants and shown effective to enhance the production of secondary metabolites in tissue cultures (Walker *et al.*, 2002; Zhao *et al.*, 2010). In this study, effect of these two abiotic elicitors on the production of flavonoids from *G.glabra* callus cultures has been evaluated.

Materials and Methods:

Callus culture: authentic *Glycyrrhiza glabra* plants were obtained from forest nursery, Faridabad in the month of August and its young leaves were used as explants. The callus culture was developed using previously standardized culture medium (Vijayalakshmi & Shourie 2016a) which was further referred to in this paper as GCC (*G.glabra* callus culture) medium. All the inoculations were carried out in a laminar airflow chamber and the cultures were incubated for 16 hours light and 8 hours dark photoperiod at $25\pm 2^\circ\text{C}$ under a controlled light intensity of 2000 Lux. Callus growth was assessed by measuring the weight of callus at the time of initiation and at the end of each week after inoculation for 8 weeks. Assessment of callus growth was done on the basis growth index (GI). $\text{GI} = (\text{Final fresh weight} - \text{initial fresh weight}) / \text{initial fresh weight}$.

Elicitor Preparation: Jasmonic acid (JA) was dissolved in 96% ethanol to prepare a stock solution. These solutions were sterilized by microfiltration. The salicylic acid stock solution (10 mg/ml) was prepared by dissolving salicylic acid in distilled water, adjusted to pH 5.8 and sterilized by autoclaving for 20 minutes.

Elicitor Treatment: in all the experiments JA and salicylic acid were supplemented to the standardized GCC medium in the defined doses (25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L, 150 mg/L, and 175 mg/L). For the time-course study, untreated and elicited cultures were harvested at different time intervals (2, 4, 6, & 8 weeks).

Extraction, qualitative analysis and quantitative estimation of flavonoids: the elicited and non-elicited callus cultures were harvested, shade dried, powdered in a mechanical grinder and extracted and re-extracted using the same flask and partitioned using the following solvents: petroleum ether, diethyl ether, and ethyl acetate. Qualitative analysis and quantitative estimation of licoisoflavone B, licochalcone A and liquiritigenin was done in the extracts through GC-MS analysis. The samples were derivatized with trimethylsilyl (TMS) prior to injection in a chromatography column and separation was done using helium as a carrier gas at a flow of 1.21 ml/min, 85.4 kpa inlet pressure and 250°C temperature. Mass spectra were recorded at 70 eV with scan interval of 0.5 seconds (Vijayalakshmi & Shourie 2016).

Phenylalanine ammonia-lyase (PAL) assay: it was extracted from callus tissues by homogenizing it with 0.05 M Tris-HCl (pH 8.0), 0.8 mM β -mercaptoethanol and 1 %

w/v polyvinyl pyrrolidone. Substrate phenylalanine (10 mM) was added to the enzyme extract and incubated for 1 hour at 37°C . The reaction was stopped by the addition of 0.1 ml 1 N HCl, and absorbance was read at 290 nm in UV-VIS spectrophotometer. The enzyme activity was expressed in terms of the formation of 1 μM of trans-cinnamic acid per min per milligram of enzyme (Sykłowska-Baranek *et al.*, 2012). The protein content was determined according to the Bradford method (Bradford, 1976) with a standard curve prepared using bovine serum albumin.

Polyphenoloxidase (PPO) enzyme Assay: it was carried according to the method described by Soffan *et al.* (2014). The polyphenol oxidase activity was expressed as a change in absorbance per minute per mg of protein at 495 nm at 30 s intervals for 5 min. The protein content was determined according to the Bradford method (Bradford, 1976) with a standard curve prepared using bovine serum albumin.

Peroxidase (POD) Enzyme Assay: it was determined by measuring the appearance of brown colour resulting from oxidation of guaiacol as a substrate in the presence of hydrogen peroxide according to the method of Zieslin & Ben-Zaken (1993). The reaction mixture consisting of 0.02 M guaiacol, 0.38 M H_2O_2 and 0.2 M sodium phosphate buffer (pH 5.8), was added to 50 μl of enzyme extract. The absorbance was measured after every 1 min for 5 min at 470 nm, and enzyme activity was determined on the basis of an increase in absorbance by 1.0 at 470 nm per min at 25°C and the specific activity of POD was expressed as unit per mg protein. The protein content was determined according to the Bradford method (Bradford, 1976) with a standard curve prepared using bovine serum albumin.

Statistical Analysis: all the data were analyzed by one-way ANOVA. A Tukey's test was used to separate the means, where the effects of treatments were statistically significant ($p \leq 0.05$). Error bars of graphs represent standard deviation. Columns sharing the same letters are not significantly different.

Results and Discussion:

Effect of Jasmonic Acid on callus growth: exogenous supplementation of JA leads to a significant increase in the growth index of *G.glabra* callus as compared to that of control. Increase in growth up to certain level was observed in callus treated with all the concentrations of JA and the maximum growth index ($\text{GI}=8.67$) was observed at six weeks of the culture with 150 mg/L JA (Fig.-1) but when the cultures treated with different concentrations were compared, there was not a very high difference in the growth indices.

JA is a naturally occurring growth regulator found in higher plants. Several physiological roles have been described for this compound during plant development and in response to biotic abiotic stress. As per Creelman & Mullet (1995), JA influences several aspects of plant growth

and development. There are few studies supporting the role of JA in promoting culture growth. Lingakumar *et al.* (2014) reported that JA favoured callus induction in Vignamungo and a combination of NAA and BAP with salicylic acid or jasmonic acid is an efficient growth supplement for an in vitro culture of many agricultural plants. JA treatment significantly increased the callus fresh weight and dry weight as compared to that of control in callus cultures of *Catheranthus roseus* L (Al-Zuhairi & Ghanm, 2017) whereas according to Andrys *et al.* (2018), higher concentrations of JA inhibited the growth of *Lavandula angustifolia* cultures and lower concentrations didn't have any effect.

Effect of Jasmonic acid on flavonoid production: JA and its derivatives are a family of important signal transducers, that can efficiently stimulate secondary metabolism in plant cells (Wang *et al.*, 2005). Gundlach *et al.* (1992) firstly reported the effect of methyl jasmonate (MJ) on the accumulation of secondary metabolites in plant cell cultures. Since then, more than 100 plant species have been demonstrated to respond to the addition of jasmonates to the culture medium by accumulating secondary metabolites (Haider *et al.*, 2000).

In this study, JA supplementation surprisingly led to decrease in the production of licoisoflavone and licochalcone A with increase in its concentration. Whereas, liquiritigenin production was found to increase with increasing JA concentrations and more than two fold increase in its production was observed with 150 mg/L jasmonic acid (Table-1, Fig.-2). Licoisoflavone B production was found to increase with 25 mg/L to 75 mg/L JA concentration but the increase was insignificant as compared to that of control. There are several reports that deal with the positive effect of jasmonates on secondary metabolite production including flavonoids. Lucho-Constantino *et al.* (2017) reported that *Jatropha curcas* plants showed increased flavonoid and anthocyanin production in response to JA elicitation. The application of a two-stage culture system with a combined treatment of mannitol and JA also resulted in the optimum accumulation of resveratrol in the callus biomass of *Vitis vinifera* (Mihai *et al.*, 2011). JA and its more active derivative methyl jasmonate triggered the production of a wide range of plant secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid and plumbagin in various cell cultures (Krzyzanowska *et al.*, 2012; Almagro *et al.*, 2014; Silja *et al.*, 2014). In this study, The reason behind the negative action of JA on the flavonoids licoisoflavone B and licochalcone A is unknown and the reports regarding such negative effect of JA on flavonoid production is very rare but it can be hypothesized that jasmonic acid might be having some inhibitory effect on of the branch point enzymes of the phenylpropanoid pathway, chalcone synthase (CHS) which is responsible for the synthesis of licochalcone and further leads to the inhibition

licoisoflavone B production.

The time course analysis of flavonoid production in response to treatment with different concentrations of JA at different time intervals is shown in Fig.-3 which shows that the production of licoisoflavone B and licochalcone A increased with increase in the time and then maximized at six weeks both in case of control and treated samples and a drastic fall in the production of these flavonoids occurred after 6 weeks which might be due to the reason that the callus cultures used up all the nutrients during the exponential phase at 6 weeks and led to maximum production and then reached the lag phase leading to cell death which eventually led to decrease in the production.

Liquiritigenin production increased with increasing concentrations of the elicitor and the maximum flavonoid production was seen as early as 4 weeks irrespective of different JA concentrations when compared to that of control where maximum production was obtained at 6 weeks which shows that JA has a stimulating effect on liquiritigenin production but prolonged treatment of jasmonic acid beyond 4 weeks had some inhibitory effects on liquiritigenin production.

Regarding the inducible mechanisms of jasmonates-stimulated secondary metabolite biosynthesis in plant cell cultures, to date, most efforts have been focused on the induction of plant defense responses including oxidative and nitric oxide bursts by the addition of jasmonates (Chong *et al.*, 2005). But, in those studies, there was no information on the alteration of secondary metabolic gene expression. There is a lack of reports on a causal link of the signal cascade from defense signal response to gene activation and secondary metabolite accumulation in jasmonates-induced cell cultures.

Research on JA signaling has become vital because of the primary reason that they are involved in the regulation of tolerance against different environmental stresses (Wasternack & Hause, 2013) as well as their integration with the signaling pathways generated by salicylic acid, abscisic acid (ABA), ethylene, and other such molecules. In fact, most of the information on JA signaling has been derived from studies on two widely researched plants *Arabidopsis* and tomato (Turner *et al.*, 2002; Browse & Howe, 2008). One could expect considerable variation among JA signaling pathways operative in other species with complex genomes exposed to different stresses.

Although, mitogen-activated protein kinases (MAPKs) were said to be the important components of JA signaling and biosynthesis pathways, nitric oxide, ROS, calcium, abscisic acid, ethylene, and salicylic acid are also important mediators of JA signal transduction and synthesis. Jasmonates are involved in a diversity of functions but there is limited information available in the literature on how these plant processes vary from species to species with jasmonate application. A diverse range of

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components and genes of JA including its receptors have been identified, but their appropriate functions still need to be explored. Much work is to be done in the near future to find out the proper answers of questions like the action of JA on the production of secondary metabolites, and identification of universal JA receptors, etc. Complete signaling pathways are yet to be studied and to understand the complete mechanism of action of it. The most important aspect is that the extent of the effectiveness of jasmonates like other hormones is plant species dependent. Effect of JA on Enzymes of Phenylpropanoid Pathway: the changes in enzyme activity of any specific phytochemical biosynthesis pathway are often related to the secondary metabolite accumulation. JA is known to induce enzymes involved in flavonoid biosyntheses such as CHS, PAL and other enzymes (Creelman & Mullet., 1995). For a better understanding of the elicitation effect of jasmonic acid, the activities of three important enzymes (PAL, PPO and POD) affecting phenylpropanoid pathway under JA treatment were determined.

PAL activity of *Glycyrrhiza glabra* callus cultures treated with JA was significantly induced at almost all concentrations of JA when compared to control except for 25 mg/L concentration wherein there was no change in the PAL activity. The enzyme activity was increased with increasing concentrations of JA (Fig.-4a). The time-course analysis showed that there was a rapid increase in the PAL activities with an increase in the time duration at higher concentrations of JA (75 mg/L-150 mg/L) and maximum PAL activity was observed at 150 mg/L jasmonic acid. The PAL activity became constant after six weeks at all the concentrations of JA except for 175 mg/L concentration where a considerable decline in PAL activity was observed after 6 weeks (Fig.-4b). Zhao *et al.* (2010) and Wang *et al.* (2015) have observed similar results in *Salvia miltiorrhiza* cell cultures and in cell suspension cultures of *Hypericum perforatum* respectively which indicated that jasmonates elicitation resulted in the up-regulation of PAL activity. JA had a remarkable effect on the polyphenol oxidase activity as well. The PPO activity was more than two times higher than that of the control at 75 mg/L to 150 mg/L concentrations of jasmonic acid (Fig.-5a). In case of control, the enzyme activity increased up to 4 weeks followed by a decrease in the activity afterward whereas in treated samples there was a shoot up in the PPO activity between 4 to 6 weeks (Fig.-5b). Similar effects of JA were also observed in several other studies that evaluated the stimulating effect of JA on PPO activity (Jaiti *et al.*, 2009; Bosch *et al.*, 2014).

It is now well known that the secondary products and the respective enzymes, particularly those of the phenylpropanoid pathway are significantly enhanced under the influence of elicitors. Peroxidase (POD) is one such enzyme associated with the plant defense pathway and is elicited when challenged with elicitors (Gomez-Vásquez

Table 1. Influence of different concentrations of JA on the production of flavonoids licoisoflavone B, licochalcone A and liquiritigenin in callus culture of *G. glabra* with maximum growth index. Means $\pm$ SD (n=3). Means with different superscripts are significantly different at $\alpha=0.05$.

Jasmonic acid Conc. (mg/L)	Licoisoflavone B (Peak area%)	Licochalcone A (Peak area%)	Liquiritigenin (Peak area%)
0	0.18 $\pm$ 0.013 ^a	8.43 $\pm$ 1.131 ^a	2.05 $\pm$ 0.417 ^a
25	0.231 $\pm$ 0.042 ^a	8.10 $\pm$ 0.923 ^b	2.54 $\pm$ 0.535 ^b
50	0.230 $\pm$ 0.045 ^a	8.15 $\pm$ 0.713 ^b	3.08 $\pm$ 0.981 ^c
75	0.183 $\pm$ 0.074 ^a	7.86 $\pm$ 0.994 ^c	4.16 $\pm$ 1.343 ^d
100	0.178 $\pm$ 0.031 ^a	7.71 $\pm$ 1.062 ^c	5.13 $\pm$ 1.425 ^e
125	0.144 $\pm$ 0.026 ^d	7.13 $\pm$ 1.446 ^d	5.41 $\pm$ 1.378 ^f
150	0.137 $\pm$ 0.053 ^d	7.15 $\pm$ 1.546 ^d	5.49 $\pm$ 2.526 ^f
175	0.124 $\pm$ 0.041 ^e	7.00 $\pm$ 1.147 ^d	4.98 $\pm$ 1.287 ^e

Figure-1: Effect of different concentrations of JA on *G. glabra* callus growth. Means $\pm$ SD (n=20).

Figure-2: Comparative GC-MS profile of untreated and JA treated (150 mg/L) samples of *G. glabra* calli. Peaks 1, 2 & 3 represent licoisoflavone B (RT=27.1), licochalcone A (RT= 30.3) and liquiritigenin (RT=30.7)

Figure-3: Time course analysis of a) licoisoflavone B, b) licochalcone A, c) liquiritigenin production at different concentrations of JA.

Figure-4: a) Effect of different concentrations of JA on PAL activity b) Time course of changes in PAL activity at different concentrations of JA. Means $\pm$ SD (n=3). Values with different letters are significantly different from each other & from the control

Figure-5: a) Effect of different concentrations of JA on PPO activity b) Time course of changes in PPO activity at different concentrations of JA. Means $\pm$ SD (n=3). Values with different letters are significantly different from each other & from the control.

Figure-6: a) Effect of different concentrations of JA on POD activity b) Time course of changes in POD activity at different concentrations of JA. Means $\pm$ SD (n=3). Values with different letters are significantly different from each other and from the

et al., 2004) As shown in the Fig.-6a the POD activity of callus cultures of *G.glabra* elicited with JA also increased to a certain extent. At lower concentrations of JA the peroxidase activity was not significantly different from that of the control. However, JA concentrations of 100 mg/L, 125 mg/L and 150 mg/L significantly increased the flavonoid concentrations when compared to that of control (Fig.-6a). In Fig.-6b it could be noted that the time course of the changes in PAL activity under JA elicitation with all concentrations followed a similar trend showing a gradual increase with an increase in time till 6 weeks whereas in case of control there was a decrease in the POD activity after 6 weeks. The results of this study were in accordance with Nafie *et al.* (2011) where JA elicited melon cells exhibited enhanced activities of PAL, PPO and POD.

Conclusion:

There are many reports about the enhancement in metabolite production by JA elicitation. However, JA had negative effects on the licoisoflavone B and licochalcone A production and had positive effects on Liquirtigenin production in *G.glabra* callus cultures. It was probably due to some change or inhibition of the enzymes of phenylpropanoid pathway leading to the decline in

production of these two flavonoids caused by JA signalling. This shows that elicitation treatments may have also had varied effects among different plant species. Therefore, further studies devoted to the role of these abiotic elicitors in triggering the phenylpropanoid pathway is required for the complete understanding as these results have thrown up many questions in need of further investigations related to the genes encoding the enzymes of phenylpropanoid pathway that regulates the flavonoid production.

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