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Elicitor induced flavonoid production in callus cultures of *Glycyrrhiza glabra* and regulation of genes encoding enzymes of the phenylpropanoid pathway

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ABSTRACT

Plant cell cultures have been shown as feasible systems for the production of secondary metabolites, elicitation with biotic or abiotic stimuli being the the most efficient strategy to increase the production of these metabolites. Glycrrhiza glabra is one of the well known medicinal plants rich in flavonoids. The potential benefits of Glycyrrhiza flavonoids on human health have made it one of the well studied grou of phytochemicals. However, reports on elicitation and gene expression studies of phenylpropanoid pathway enzymes in Glycrrhiza glabra callus cultures are meager. Therefore, the aim of this study was to evaluate the elicitation effect of chitosan on flavonoid production in callus cultures of Glycrrhiza glabra through Gas Chromatography-Mass Spectrometry and on the expression of flavonoid biosynthetic genes through real-time quantitative reverse transcription-polymerase chain reaction. Chitosan significantly induced the production of flavonoids licochalcone, liquirtigenin and Licoisoflavone B in G.glabra callus culture. Moreover, the production of Licoisoflavone was highly induced as compared to other flavonoids. This induction could be correlated with the higher expression of flavonoid biosynthetic gene, Isoflavone synthase. Expression of Chalcone synthase and chalcone isomerase was also induced by chitosan where as the expression of phenyl alanine ammonia lyase was not induced to a significant level. Therefore, it is hypothesized that the observed response to chitosan is the result of an induction or promotion of the phenylpropanoid pathway, mainly at the branch point leading to the activation of isoflavone synthesis and enhanced production of Licoisoflavone B in Glycyrrhiza glabra callus cultures.

Key words: Callus culture, Chitosan, Flavonoids, *Glycyrrhiza glabra*, Phenylpropanoid Pathway

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INTRODUCTION

Glycyrrhiza glabra is known as one of the most ancient herbal medicines. It has been extensively studied and widely used in many fields, such as confectionaries, cosmetics, and pharmaceuticals. Many biological activities, such as anti-mutagenic, anti-ulcer, anti-inflammatory, anti-tumor, anti-microbial, anti-oxidant and anti-viral activities have been reported in *Glycyrrhiza glabra* [1]. It was found that these biological activities of *G. glabra* were mainly attributed to the presence of major flavonoids of this plant like licochalcone, liquirtigenin, and licoisoflavone B which have tremendous therapeutic potential in curing various ailments [2]. Their wide occurrence, complex diversity and manifold functions have made flavonoids a very attractive system for research on a molecular-biological level.

Flavonoids are synthesized via the phenylpropanoid pathway (Fig. 4), which also leads to the synthesis of other important secondary metabolites [3]. The synthesis of flavonoids starts with the deamination of phenylalanine to

cinnamic acid by phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5), followed by two other reactions leading to coumaroyl-CoA. This compound is then transformed to Licochalcone by the enzymes chalcone synthase (CHS; E.C. 2.3.1.74) and chalcone reductase (CHR; E.C.2.3.1.170), respectively. Liquiritigenin is then produced by chalcone isomerase (CHI; E.C. 5.5.1.6). The synthesis of licoisoflavone from liquiritigenin is finally catalyzed by the enzyme isoflavone synthase (IFS; E.C.1.14.13.86) [4]. Genes encoding enzymes of this pathway are regulated by developmental and environmental factors leading to the change in flavonoid concentrations. Flavonoid contents in Glycyrrhiza are mainly affected by certain biotic stresses and abiotic stresses [5]. Exposing plant cells to such biotic/abiotic stresses in the form of elicitors under controlled conditions might highly induce flavonoid production.

Chitosan is a natural biopolymer derived by deacetylation of chitin, which is a major component of crustaceans, has several agricultural, food, and biomedical applications [6]. Studies revealed that chitosan is a promising elicitor for inducing flavonoid production in plant tissue cultures. The recent development of real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) allows accurate expression profiling of RNA transcripts and has become the most useful method for characterizing gene expression in plants during their interaction with biotic and abiotic factors [7-9]. Studies aimed at measuring transcript levels of genes encoding enzymes in the phenylpropanoid pathway in other plants treated with elicitor compounds using PCR methodologies are available [10-12]. But, reports on the use of transcriptional studies to characterize the response of callus cultures to chitosan are rarely available. Non-embryogenic callus cultures, containing more or less homogenous clumps of dedifferentiated cells, are used for secondary metabolite production. Of the tissue culture means, this approach is relatively frequently used for production of flavonoids [13-16].

Therefore, The present study investigated gene expression alteration of genes encoding major flavonoids in Glycyrrhiza callus cultures, as well as concentrations of flavonoids following treatment with chitosan. The effect of chitosan was studied by measuring transcript levels of key encoding genes in the phenylpropanoid pathway using real-time QRT-PCR, and by measuring flavonoid contents with Gas chromatography- Mass Spectrometry (GC-MS). The objective was thus to evaluate the potential use of chitosan as a means of increasing flavonoid concentrations in G. glabra callus cultures, and to correlate such an increase with quantitative transcript abundance of their encoding genes.

MATERIALS AND METHODS

Reagents and Standards

Solvents for extraction and GC-MS analyses (methanol, deionized water) were HPLC grade and Elicitors Chitosan and Jasmonic acid were purchased from Sigma Aldrich Chemicals Pvt. Ltd (Barakhamba Road, New Delhi). All other chemicals used were of analytical grade.

Plant Material

Glycyrrhiza glabra plants were collected from Jamia Humdard University, New Delhi and Forest nursery, Faridabad.

Callus Induction

From the field grown plants, the young leaves were excised and used for callus induction. The collected explants were washed thoroughly in running tap water for 10 minutes. The explants were rinsed with 0.1% mercuric chloride solution for 8 minutes and again washed with sterile distilled water for several times. Then the explants were washed with 70% alcohol for 10 seconds followed by washing with distilled water and were placed in sterile petriplates for inoculation.

Murashige and Skoog (1962) medium with various combinations of auxins and cytokinins was used for callus induction. The MS medium consists of macronutrients, micronutrients, iron source and vitamins supplemented with sucrose (3%) as a carbon source and agar (1%) as a solidifying agent. A cytokinin, 6-Benzyl amino purine (6-BAP) and two auxins 2,4 –Diphenoxy acetic acid(2,4-D) and Naphthalene-3-acetic acid (NAA) in different concentrations were also supplemented in to the MS medium for callus induction. Stock solution of growth hormones were prepared and filter sterilized using Whatman filter paper and a syringe filter. Callus cultures were maintained on solid MS medium and sub cultured at frequent intervals and used for further studies.

Elicitor preparation and treatment

Elicitation was carried out with Chitosan. The Chitosan stock solution (10mg/ml) was prepared by adding glacial acetic acid drop wise to chitosan at 60˚C for a period of 15minutes.Then the mixture was diluted with de-ionised water, adjusted to pH 5.8 and sterilized by autoclaving and the solution of final concentration ranging from 25mg/L to 150mg/L was added to the cultures. For a time course study, untreated and elicited cultures were harvested at different time intervals (14, 21, 28, 35, 42, 49 and 56 days) and then frozen immediately at -80 ºC for the next biochemical assays. Biomass was quantified by dry weight. All treatments were performed in triplicate.

Extraction of Flavonoids

The Elicited and non elicited callus cultures were harvested, shade dried and subjected to extraction. The dried calli were pulverized to powder in a mechanical grinder. One gram of the powder was transferred into a flask and extracted with three volumes of 70% ethanol at 85 °C for 4 hours with constant agitation. The extract was filtered and re-extracted two times under same conditions. Each time the filtrate was collected in the same flask and was partially purified using solvent separation method, 50 ml of the extract was mixed with 10ml of petroleum ether in a separating funnel and the upper layer was discarded to remove fatty acids, lower layer was mixed with diethyl ether and was shaken well, free flavonoids present in the upper layer are retained the lower layer is extracted with 20ml of ethyl acetate in a separating funnel, lower layer is discarded and upper layer is hydrolyzed in 7% H_2SO_4, boiled for 1-2 hours and filtered. The filtrate was re extracted in 10ml of ethyl acetate and the lower layer was discarded; upper layer containing flavonoid glycosides was washed with water until it gets neutral. Finally, the samples were derivatized with trimethylsilyl (TMS) to increase the volatility of studied compound. The volume corresponding to 2 mg of the original dry weight from the each sample was transferred to the Teflon-lined screw-capped vials and taken for further GC-MS analysis.

GC-MS Analysis

GC-MS analysis of the derivatized samples was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS). The sample (2 µl) was injected into a RTX-5 column (60 m x 0.25 mm internal diameter, film thickness 0.25 µm) of GC-MS (model GC-MS-QP-2010 plus, Shimadzu Make). Helium was used as carrier gas at a constant column flow of 1.21 ml/min at 85.4 kpa inlet pressure. Temperature programming was maintained from 80°C to 250°C with constant rise of 5°C/min and then held isothermal at 250°C for 10 min; further the temperature was increased by 30°C/min up to 310°C and again held isothermal at 320°C for 22 min. The injector and ion source temperatures were 270°C and 230°C, respectively. The crude extract dissolved in methanol (Chromatography grade, Merck, India) was injected with a split ratio of 1:20. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and the total GC/MS running time was 50 minutes.

PCR analysis

Primer Design: Based on Glycyrrhiza protein sequences or corresponding mRNA sequences published in Genbank primer pairs for PAL, CHS, CHI and IFS genes were designed using online software Primer 3 in biology workbench (http://workbench.sdsc.edu). The primers were evaluated by alignments with the original contigs from online standard nucleotide-nucleotide BLAST search (blastn in http://www.ncbi.nlm.nih.gov/BLAST/). For normalization purposes of quantification of target genes expression, the housekeeping genes (HKG) encoding actin was used. All amplifiable products were sequenced to ensure that the right sequence of the gene is amplified. Isolation of RNA and Reverse Transcription and Quantitative PCR: Total RNA was isolated using the RNeasy Plant Mini Kit and treated with RNase-free DNase I and reverse transcribed to cDNA using QuantiTect Reverse Transcription kit following the manufacturer's recommendations. Quantitative reverse transcription (QRT)-PCR: The reactions (20 IL) were performed for each of the target gene and for the HKG, actin using Mx3000P and SYBR Green QPCR master mix. The PCR amplifications of the chalcone synthase (CHS), chalcone isomerase (CHI), phenylalanine-ammonia lyase (PAL), isoflavone synthase (IFS) and actin-encoding sequences were performed with primers listed below

PAL F: 5'-GCAATGGCTTGGTCCTCTTA-3'
R: 5'-CCATGCAAAGCCTTGTTTCT-3'
CHS F: 5'-GGTGCACGTGTTCTTGTTGT-3'
R: 5'-GCTTGGCCCACAAGACTATC-3'
CHI F: 5'-TGGAGAAGGTGATTAGGCTTG-3'
R: 5'-TTTGTCATCAGCAGCCAAAC-3'
IFS F: 5' -GGGCCCTCAAGGACAAATA- 3'

R: 5'-TGCGATGGCAAGACACTAC-3'
Actin F: 5'-TCAAGACGAAGGATG-3'
R: 5'-TTGGATTCTGGTGAT-3'

RESULTS AND DISCUSSION

Callus culture

Callus initiation is the primary stage in plant tissue culture studies. Callus induction and growth is highly determined by the type of growth regulator, their concentration and combination in culture medium. Various plant growth regulator (PGRs) treatments have been used for these purposes in different plant cultures [17]. In this study, *Glycyrrhiza glabra* Leaf explants were cultured on MS medium with different concentrations of NAA, 2, 4 –D alone or in combination with BAP for callus induction. Various PGR concentrations used in this study produced different types of callus morphology. The calli were yellow, hard and compact in medium with 0.5mg/L BAP and relatively soft with yellow appearance in medium containing 1mg/L NAA where as their combination produced cream friable callus (Figure 1). Moreover, in MS (Murashige and Skoog, 1962) medium supplemented with 1 mg/L NAA and 0.5mg/L BAP, cultured explants were swollen after five days of inoculation and callus initiated at the cut edges within 12 days of culture. The best result in terms of percentage of callus induction (96%), and High growth index (9.86) was obtained in media containing1 mg/L NAA and 0.5mg/L BAP (Table 1). Therefore, this GR combination can be reported to be effective in producing rapidly proliferating friable callus in *G.glabra* leaf explants and the same was used in further studies.

Table 1. Effect of different concentrations and combinations of Plant growth hormones explants on callus induction from *Glycyrrhiza glabra* leaf explants on MS medium

PLANT GROWTH HORMONE CONCENTRATIONS (mg/L)			CALLUS	
NAA	2,4-D	BAP	Induction (%)	Growth Index (GI)
0	0	0.5	0	-
0	0	1	24 ± 0.98	5.69 ± 1.45
0.5	0	0	21 ± 1.21	6.03 ± 0.98
1	0	0	36 ± 1.65	6.87 ± 2.16
0	0.5	0	0	-
0	1	0	0	-
0.5	0	0.5	83 ± 3.12	8.61 ± 3.78
1	0	0.5	96 ± 3.87	9.86 ±3.56
1.5	0	0.5	89 ± 2.99	7.17 ± 2.98
2	0	0.5	77 ± 3.56	7.98 ± 1.99
0.5	0	1	62 ± 2.9	5.95 ± 0.89
0	0.5	0.5	11 ±0.19	5.87 ± 1.54
0	1	0.5	8 ±0.11	4.58 ± 1.2
1	0.5	1	0	-
2	1	1	0	-

Values represent means ± standard deviation (S.D) for 20 cultures per treatment
Induction % = No. of explants initiating Calli / Total number of explants inoculated x 100
GI= (Final dry weight-initial dry weight) / initial dry weight

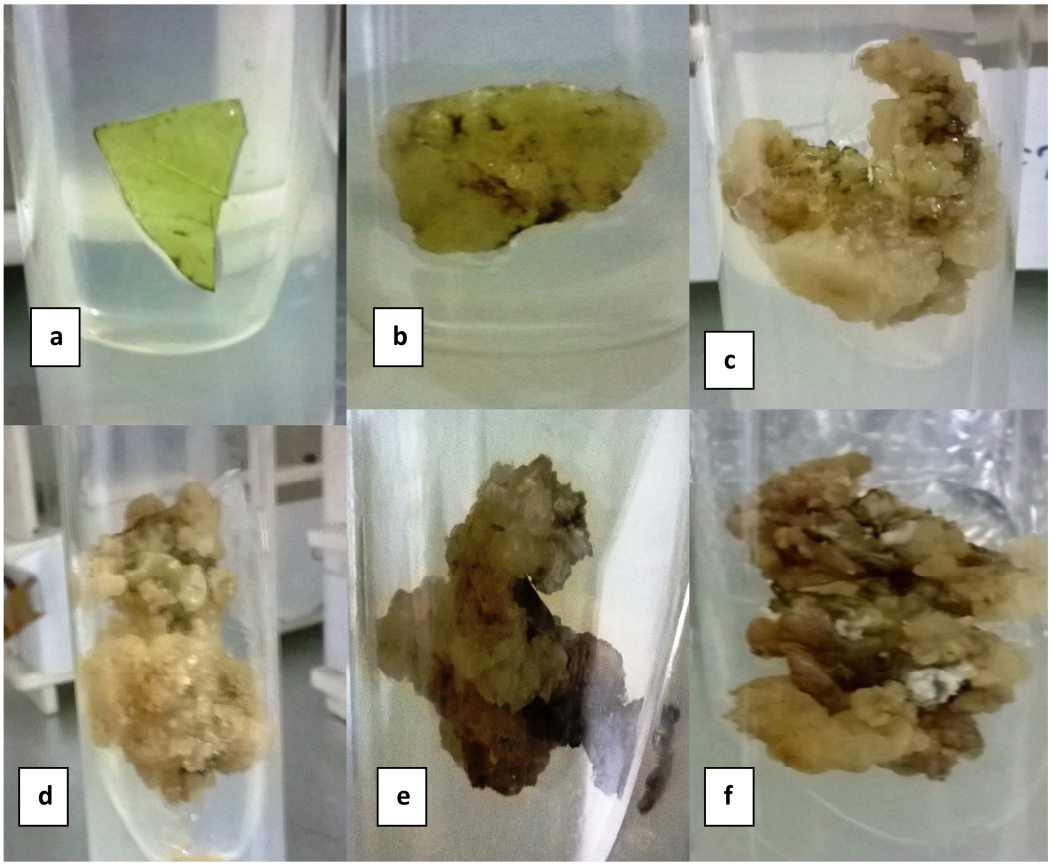

Figure 1. Callus induction in MS medium supplemented with 1mg/L NAA and 0.5 mg/L BAP. A. During inoculation B. after 2weeks C. After 3 weeks D. After 4 weeks E. after 5 weeks and F. after 6 weeks

Elicitation

Callus was sub-cultured 3 times on the same fresh media supplemented with 0.5mg/L BAP and 1mg/L NAA for inducement before sub-culturing on the medium containing different concentrations of biotic elicitor, Chitosan. The effect of different concentrations of elicitors on and flavonoid contents were investigated.

Samples were prepared from control callus as well as from the ones subjected to elicitation with different concentrations of Chitosan. The samples obtained from the control and treated callus cultures with a maximum growth index were submitted for GC-MS analysis. Duplicate GC-MS analysis were performed for each sample of chitosan time-course and were analyzed for peak area variation associated with replicate analysis. This variability was less than 3% for majority of the metabolites and only 10% of peaks varied by more than 5%. Based on these results single GC-MS analysis was performed all subsequent samples. However, biological triplicates were still utilized and triplicate instrumental analysis of individual biological replicates were performed on samples throughout each time-course to provide an estimate of instrumental variability.

The high resolution MS analysis helped to establish the flavonoid composition present in the samples and to determine the effect of chitosan on the flavonoid content. The respective flavonoids were identified on the basis of their RT and the respective mass spectra matching with authentic compounds. Identifications were further confirmed through spectral matching against the National Institute of Standards and Technology (NIST) library. Three major flavonoids namely Licoisoflavone B, , Licochalcone A, Liquirtigenin identified in the treated and untreated samples of *Glycyrrhiza glabra* callus were taken into consideration for this study and their response to elicitor concentration and time course was observed.

Elicitor induced accumulation of secondary metabolites is of great importance because of its ability to improve the productivity of the plant cell systems significantly. Our results show that *G.glabra* callus cultures showed a dose

dependent response for flavonoid production on elicitor treatment. As shown in figure 1, a linear increase in levels of Licoisoflavone B was observed at maximum Growth index of callus, as an effect of chitosan treatment with increase in concentration from 25mg/L up to 150 mg/L. The most dramatic effect of elicitation by chitosan was shown by Licochalcone and Liquirtigenin. Their level first decreased and then increased by approximately 1.5 folds on elicitation. Licoisoflavone B elicitation was much more pronounced with even lower concentration (25mg/L) of Chitosan and a 9.83 fold increase in the contents could be observed. This was followed by a moderate increase with increasing concentrations of Chitosan culminating into 36.72 Fold increase in Licoisoflavone B content which was the highest among other compounds.

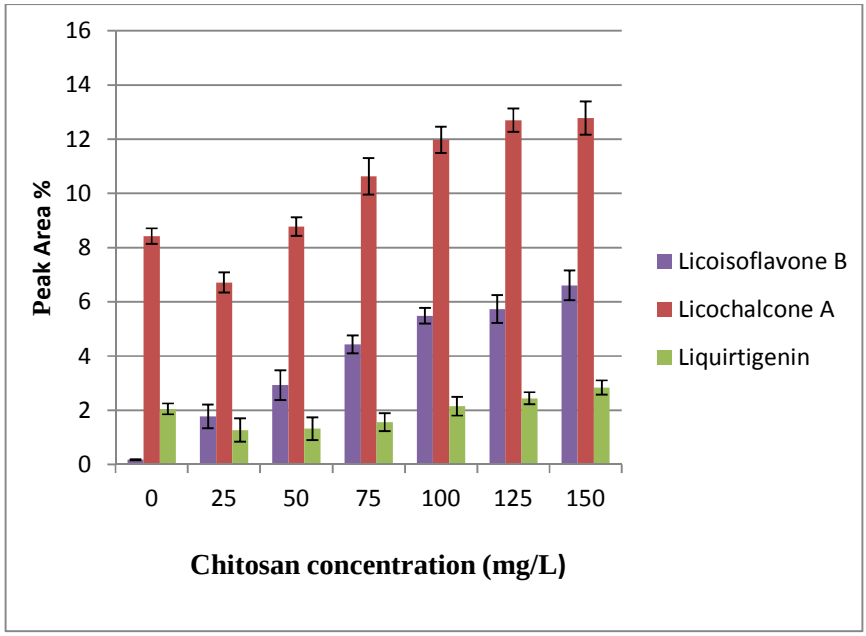

Figure 2. Effect of Chitosan treatments on Licoisoflavone B, Licochalcone & Liquirtigenin Contents at maximum growth index

At Chitosan concentrations higher than 150mg/L, the survival rate of callus was very poor. Either the cells deteriorated soon after 2-3 weeks of treatment or the explants didn't respond at all. Therefore further analysis could not be done in cultures treated with higher concentration of elicitor. The response of elicitation is dependent on the growth period of cell culture as well as on the duration of exposure of elicitor which affects the quantitative response and product accumulation pattern in general. Cultures treated with 150mg/L of chitosan showed maximum response in terms of flavonoid content.

The time course followed for the elicitation of these compounds at 150 mg/L chitosan is shown in Figure 3. Peak areas were normalized by dividing each peak area value by mean peak area for that compound, with each time course treated independently. It revealed that the onset of the accumulation of these compounds was observed after 14 days and a maximum response directed to the synthesis of flavonoids licochalcone A, liquirtigenin and licoisoflavone B was recorded after 49 days. Further increase in incubation period significantly reduced their accumulation. This may be due to the fact that longer elicitor contact leads to disturbances in cell permeability, osmotic condition, and changes in membrane potential [18]. Licoisoflavone B was found in much increased amounts at all studied time points in comparison to those observed in control which showed that chitosan most efficiently enhanced the levels of licoisoflavone B as compared to other flavonoids.

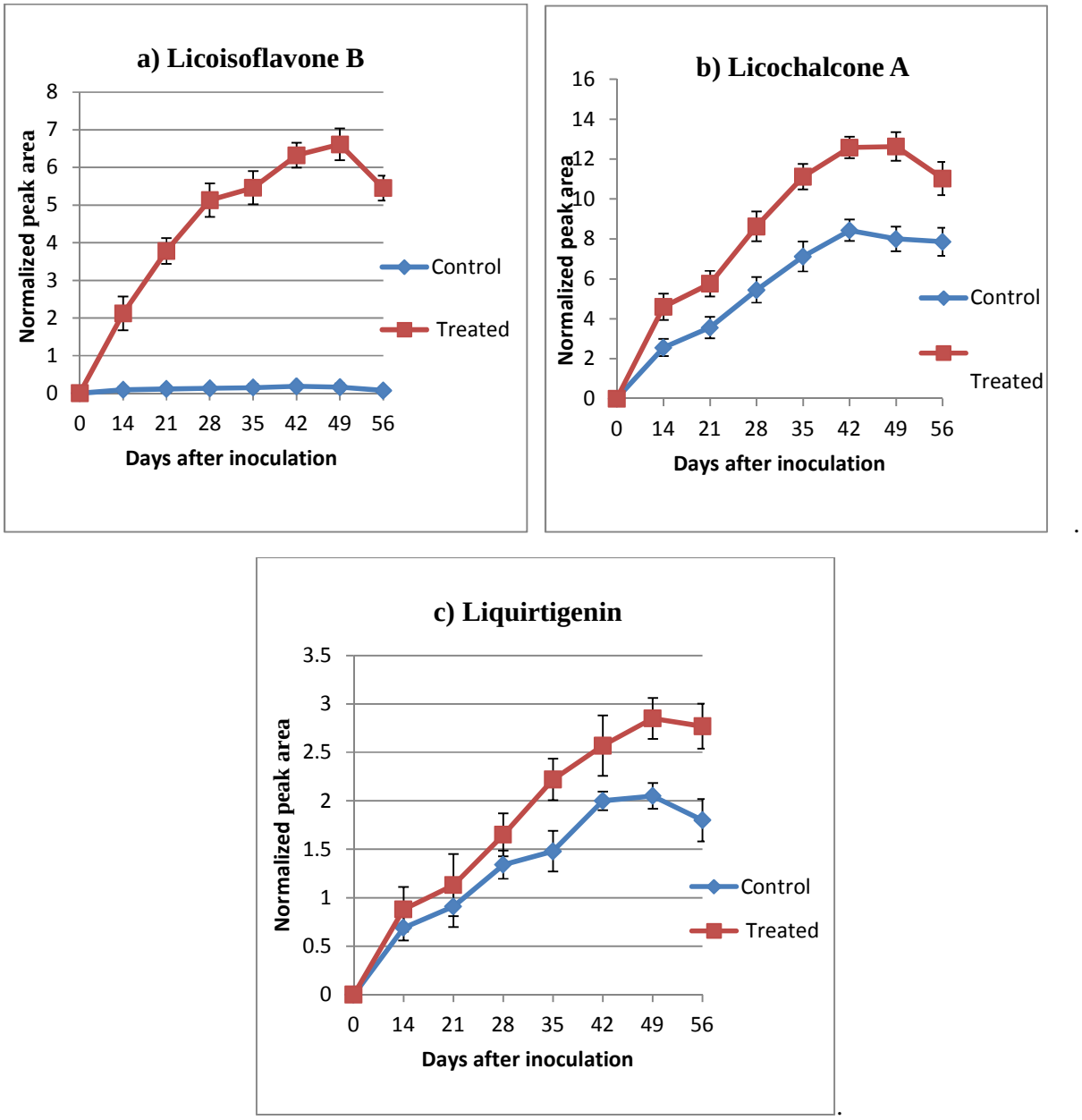

Figure 3. Time course analysis of response of selected flavonoids a) Licoisoflavone B, b) Licochlcone, c) Licochalcone to elicitation with chitosan

Analysis of plant reactions during elicitation with different biotic/abiotic elicitors may indicate that different reactions at various molecular levels (RNAs, Proteins and SM) are developed in studied plants. These reactions may differ in various plants due to numerous defense mechanisms developed by plants during their evolution at perception and recognition levels of natural products signaling the stress reaction. That is why the response of *G.glabra* callus to elicitation with defined elicitors may involve expression of various proteins and further synthesis of different secondary metabolites, due to activation of different enzymes in metabolic pathways. The induction of phenylpropanoids, such as isoflavones and phytoalexins, in response to bacterial and fungal infections as well as elicitors of biological origin has been studied and documented for a few plant model systems [19-22]. The majority of these findings were derived from single gene studies and global gene expression studies [23-27]. Chitosan originating from cell walls of fungi or from marine organisms has been also reported to elicit a variety of defense reactions in higher plants, including the stimulation of enzymes and the accumulation of phytoalexins [28-31].

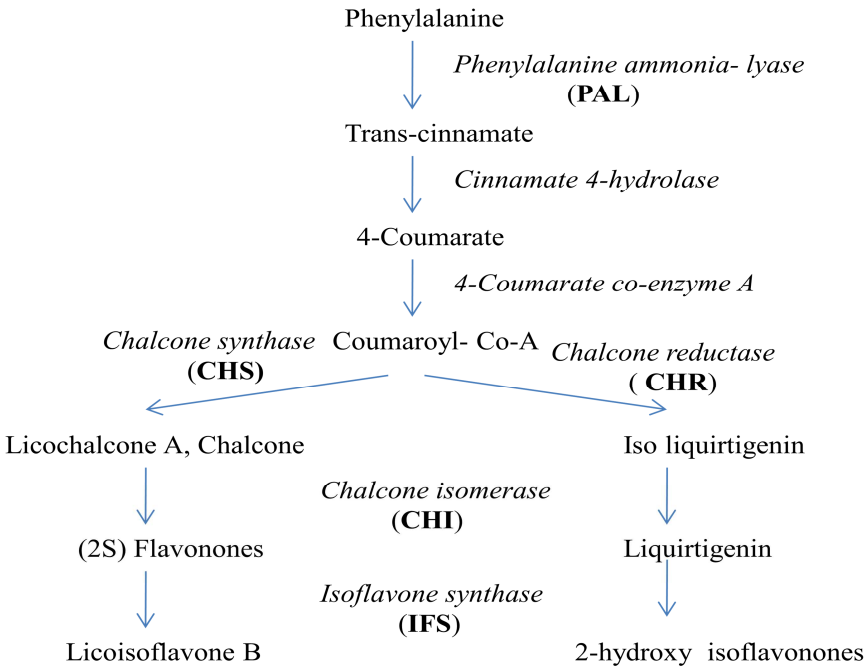

Figure 4. A proposed diagram of the phenyl propanoid pathway, with the key enzymes involved in the synthesis of flavonoids

Despite these advances, very little has been reported on gene activities of enzymes of the pathway leading to the synthesis of flavonoids mainly flavones and isoflavone in response to chitosan [32-34]. Therefore, the induction of expression of genes involved in flavonoid biosynthesis pathway. i,e genes encoding enzymes at key flux control points (i.e., *PAL*, *CHS* and *CHI*) and at a branch-point enzyme in isoflavone biosynthesis (i.e., *IFS*) in *Glycyrhiza* callus cultures following chitosan treatments was studied here in.

Limited genomic information on Glycyrrhiza glabra restricts its research and thus biosynthetic mechanisms of many of its phytochemicals are still poorly understood. Therefore the gene expression studies of the enzymes were carried out based on the mRNA sequence data of phenyl propanoid enzymes of Glycyrrhiza uralensis available in genbank as it is a licorice plant closely related to Glycyrrhiza glabra. Changes in the transcription genes responsible for the key enzymes participating in flavonoid biosynthesis pathway in response to chitosan treatment (150mg/L) were studied at two different time points. The transcription of the studied genes was monitored after 42 and 49 days of elicitor treatment. Significant differences in the expression patterns of different genes were observed. Figures 5, demonstrates the relative transcript levels of PAL, CHS, CHI and IFS gene expression. From the gene expression profiling it was observed that the expression of CHS, CHI and IFS was up regulated by Chitosan treatment. However, greatest response was observed in the expression of IFS as compared to others. Expression of PAL was neither increased nor decreased on treatment with chitosan and remained at the the same level.

Isoflavone synthase is a key enzyme in the synthesis of isoflavones and defense-inducible phytoalexins. Higher accumulation of *IFS* after elicitation with chitosan supports previous fi nding that these genes are highly induced in Fabaceae plants in response to biotic stress [24, 34, 35, 36]. Induction of a key checkpoint enzyme in the phenyl propanoid pathway (i.e., *CHS*) in response to a pathogen has also been reported previously in many plant systems [37,38]. Ferri et al. [39] have reported the upregulation of CHI and CHS transcript expression levels and accumulation of the flavonoid anthocyanins in response to chitosan treatment. Results obtained through gene expression analysis can be well correlated with the results obtained through time course analysis and with those showing the effect of chitosan, where in the increased expression of genes CHS,CHI, IFS was found to be proportional to accumulation of their corresponding flavonoid end products, which was in the order, Licoisoflavone (IFS) > Chalcones (CHS)> Liquirtigenin (CHI).

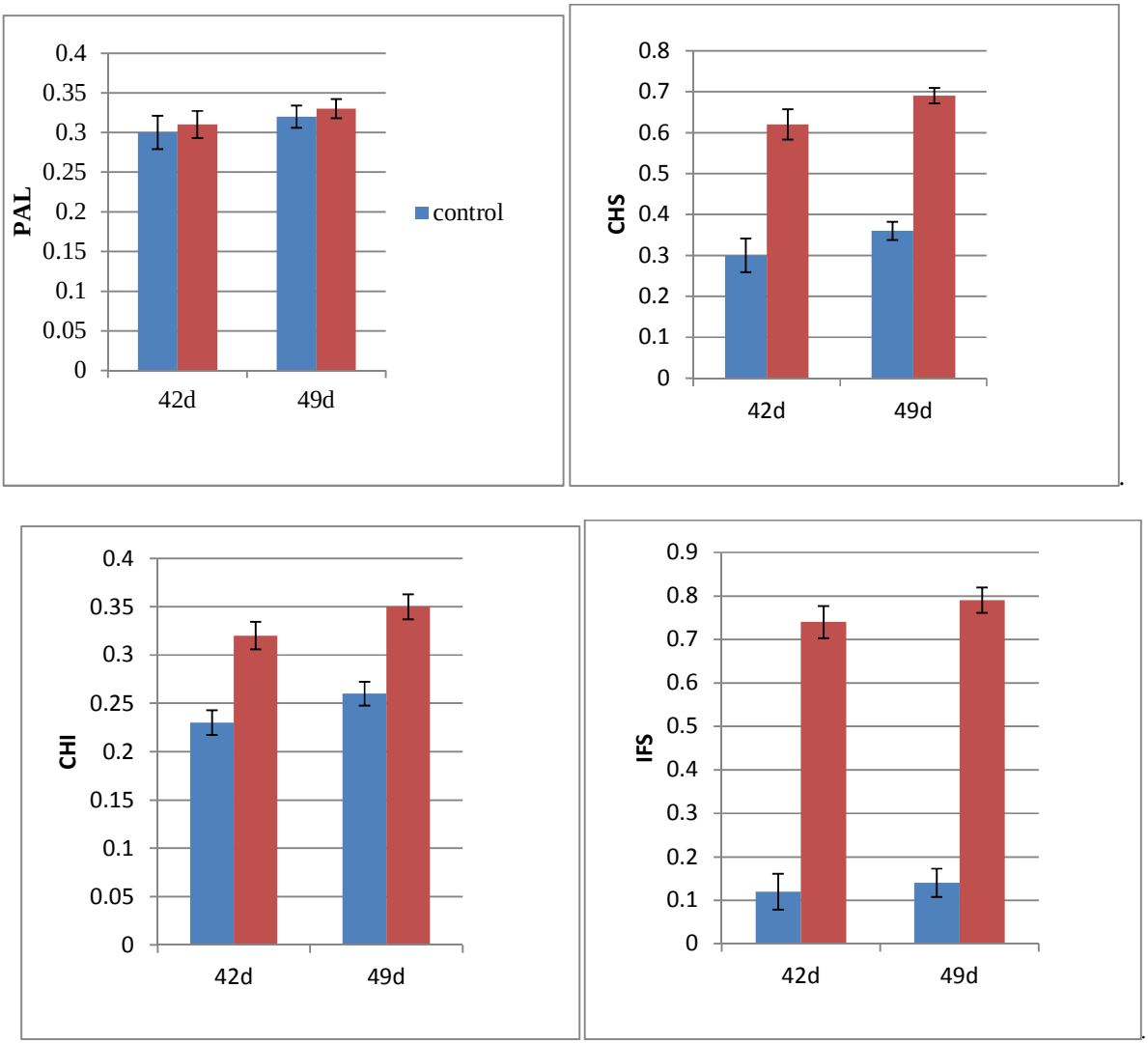

Figure 5. Relative transcript level ratio of PAL, CHS, CHI, and IFS at selected time course of chitosan treatment. (Analyses were performed by real-time quantitative polymerase chain reaction. Transcripts levels were calculated by using the standard curve method from triplicate data, with actin gene as internal control. Results are mean standard deviations of three experiments).

On the basis of these data we can conclude that the regulatory mechanisms of G.*glabra* callus cells activated during chitosan treatment might involve the induction of isoflavone biosynthesis pathaway. When chitosan is supplied exogenously, the interaction between the chitosan oligosaccharins and receptors located in plant cell membranes activate a cascade of reactions that results in accumulation of many secondary metabolites. Chitosan receptors were identified [40] but the signaling pathaway from the point at which oligosaccharin contacts the cell surface to that at which the final response is realized is not yet confirmed. Amborabe and coworkers (2008) have investigated the early events induced by chitosan in plant cells. It was found that chitosan triggers, in a dose dependent manner, the rapid transient depolarization of cell membrane with consequent modification of the proton fluxes and alteration of the cell permeability with the plasma membrane H^+-ATPase as primary site of action. However, the oligosaccharin receptor seems to be followed by an unknown step that is thought to activate more than one defense response, among which specific gene transcription activities that lead to synthesis of phenylpropanoid enzymes.

CONCLUSION

In conclusion, It can be said that the enhanced expression of IFS as compared to other phenyl propanoid enzyme genes (CHS, CHI, PAL) suggests that chitosan upregulates specific target branches of phenyl propanoid pathway

leading to isoflavone synthesis and is a suitable elicitor for enhancement of Chalcones, Liquirtigenin and most importantly Licoisoflavone B.

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REFERENCES

[1] M Damle**,** *Int J Herb Med.*, **2014**, 2, 2, 132-136.
[2] G Rusak, HO. Gutzeit, J Ludwig-Müller, *Food Technol. Biotechnol*., **2002,** 40, 267–273.
[3] O Yu, B McGonigle, *Adv. Agron*., **2005**, 86,147–190.
[4] W Jung, O Yu, SMC Lau, DPO Keefe, J Odell, G Fader, B McGonigle, *Nat. Biotechnol*., **2000**, 18, 208–212.
[5] P Seguin , WJ Zheng, DL Smith, WH Deng, *J.Sci. Food Agric*., 2004, 84,1327–1332.
[6] J Synowiecki, NAA Khateeb, Crit. Rev. *Food Sci. Nutr*., **2003**, 43,145–171.
[7] SJ McMaugh, B.R. Lyon, Biotechniques, **2003**, 34, 982–986.
[8] S Restrepo, KL Myers, O del Pozo, GB Martin, AL Hart,CR Buell, W Fry, CD Smart, *Mol.Plant Microbe Interact*., **2005**, 18,913–922.
[9] K Wen, P Seguin, M St-Arnaud, S Jabaji-Hare, *Phytopathology*., **2005**. 95, 345–353.
[10] HJ Cools, H Ishii, Physiol. *Mol. Plant Pathol*., **2002**, 61,273–280.
[11] AA Borges, HJ Cools, JA Lucas, *Plant Pathol*, **2003**, 52,429–436.
[12] S Subramanian , X Hu, G Lu, JT Odelland, O. Yu, *Plant Mol. Biol*, **2004** ,54,623–639.
[13] M Masoumian , A Arbakariya, A Syahida, M Maziah, *African J. Biotechnol*., **2011**, 10,32 , 6021-6029.
[14] G.Sudha, R. Gokare, *Acta Physiologiae Plantarum*., **2003**, 25,3, 249-256.
[15] **R**. Mihai, S. Cristina, FA Helepciuc, A Brezeanu, G Stoian**,** *Rom Biotech Lett*., **2011**,16, 6, 6683-6689**.**
[16] M.Ram, KV Rasad, SK Singh, B surendra kumar, *Plant Cell Tiss Org* , **2013**,113,3.
[17] RT Afshari, R Angoshtari, S Kalantari, Plant Omics J., **2011** 4,2, 60-67.
[18] A Vasconsuelo , R Boland , Plant Science., **2007**, 172, 861–875.
[19] RA Dixon, L Achnine, P Kota, CJ Liu, MSS Reddy, and L Wang, Mol. Plant Pathol., **2002**, 3, 371–390.
[20] R Goâ mez-vaâ squez, R Day , H Buschmann , S Randles , JR. Beeching, R Ichard M. Cooper , *Ann Botany*., **2004**, 94, 87-97.
[21] A. Farag, D V. Huhman, R A. Dixon, LW. Sumner, Plant Physiol., **2008**,146, 387–402.
[22] SG Simic, O Tusevski, S Maury, A Delaunay, C Joseph, D Hagège, The Scientific World Journal., **2014**, 3, 649-659.
[23] P Moy, D Qutob, BP Chapman, IAtkinson, M Gijzen, *Mol. Plant Microbe Interact*., **2004**, 17,1051–1062.
[24] G Zabala, J Zou, J Tuteja, DO Gonzalez, SJ Clough, LO. Vodkin, *BMC Plant Pathol*., **2006**, 6, 1–28.
[25] JL Zhang, DR Pan, YF Zhou, ZC Wang, SM Hua, LL Hou, FF Sui, *Acta Horticulturae Sinica*, **2009**, 36, 1, 73-80.
[26] Y Zhang, H Xia, M Yuan, C Zhao, A Li, X Wang, Electronic Journal of Biotechnology,**2011**,15, 1-6.
[27] JH Ahn, JS Kim, S Kim, HY Soh , H Shin, H Jang, *PLoS ONE*, **2015**,10, 4,121-129.
[28] GK Agrawal, R Rakwal, S Tamogami, M Yonekura, A Kubo, H Saji, *Plant Physiol. Biochem.*, **2002** ,40,1061–1069.
[29] G Romanazzi, E Nigro, A Ippolito, D Di Venere, M Salerno, *J. Food Sci*, **2002**, 67,1862–1867.
[30] W Khan, B Prithiviraj, DL Smith, *J. Plant Physiol*., **2003**, 160,859–863.
[31] B Alejandro, F Rodríguez, J C Cabrera, E Ortega, Miguel ÁM Téllez, Am. J. Agric. *Biol Sci*., **2009**,4, 3, 192-200,
[32] A Zuppin, B Baldan, R Millioni, F Favaron, L Navazio, P Mariani, *New Phytol*., **2004**, 161:557–568
[33] ME Mason, JM Davis, *Mol. Plant Microbe Interact*. **1997**,10, 135–137.
[34] HP Chen, LL Xu, J Integr Plant Biol., **2005**, 47, 452–456.
[35] D Qutob, PT Hraber, BWS Sobral, M Gijzen, *Plant Physiol*., **2000**, 123, 243–253.
[36] S Dhaubhadel, BD McGarvey, R Williams, M Gijzen, *Plant Mol. Biol*, **2003**, 53:733–743.
[37] Dhawale, S., G. Souciet, and D.N. Kuhn, Plant Physiol., **1989**, 91,911–916.
[38] R Shoemaker, P Keim , L Vodkin ,E Retzel , SW Clifton , RWaterston ,D Smoller , V Coryell , A Khanna , J Erpelding , X Gai , V Brendel ,C Raph-Schmidt , EG Shoop , CJ Vielweber , M Schmatz , M Pape , M Bowers , B Theising , J Martin , M Dante , T Wylie ,C Granger, *Genome*., **2002,** 45, 2, 329-38.
[39] M Ferri, A Tassoni, M Franceschetti, LRighetti, M J Naldrett, N Bagni , *Proteomics*., **2009**, 9, 610–624.

[40] HP Chen, LL Xu, *J Integr Plant Biol*., **2005**, 47, 452–456.
[41] BE Ambrobe, J Bonmort, F-Lessard, P Robin, *J.exp.Bot*, **2008**, 59, 2317-2324.