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Cinnamic acid Supplementation Regulates the Production of Licochalcone A, Liquirtigenin and Licoisoflavone B in *Glycyrrhiza glabra* Callus Cultures.

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Abstract

Glycyrrhiza glabra is an ancient herbal medicine rich in large number of secondary metabolites which attribute to its therapeutic properties. These metabolites are usually obtained from field grown plants and their yields vary greatly on the basis of environmental conditions. Plant tissue culture can thus be a preferred method for consistent production of such metabolites. This study dealt with the enhancement of flavonoids through precursor feeding in callus cultures of G. glabra and investigated the influence of cinnamic acid on phenylalanine ammonia lyase (PAL) activity and production of licochalcone A, liquirtigenin and licoisoflavone B. Unorganized callus cultures were established from young leaf explants on Murashige and Skoog's (MS) medium supplemented with NAA (1mg/l), BAP (0.5 mg/l) and various concentrations of cinnamic acid. Flavonoids were obtained from calli through solvent extraction and were identified and quantified through Gas-Chromatography Mass spectrometry. Cinnamic acid supplementation at appropriate concentrations (50mg/100ml for licochalcone A and liquirtigenin, and 125mg/100ml for licoisoflavone B) significantly increased their production to 1.28, 1.2 and 9.76 folds respectively. However, prolonged treatment of cinnamic acid at concentration beyond 50mg/100ml led to decrease in the production of liquirtigenin and licochalcone A , but caused fair increase in licoisoflavone B. Also cinnamic acid concentrations higher than 50mg/100ml reduced the activity of PAL enzyme due to its feedback inhibition, but at the same time might have modulated other intermediate enzymes of the pathway like chalcone isomerase favoring the formation of licoisoflavone B. Therefore, this study provides clear evidences of enzymatic regulation of phenylpropanoid pathway by cinnamic acid in G. glabra callus cultures.

Keywords : Cinnamic acid*; Glycyrrhiza glabra* ; Precursor ; Callus culture ; Flavonoids.

Introduction

Glycyrrhiza glabra, an important perennial herb belonging to family Fabaceae is a rich source of secondary metabolites and is well known for its age long medicinal use. Flavonoids are one of the major classes of secondary metabolites in *Glycyrrhiza glabra* imparting it various medicinal properties for human ailments. Flavonoids such as liquirtigenin, licochalcone and licoisoflavones etc. have a wide range of biological effects such as anti-spasmolytic, anti-inflammatory, antioxidative, vascular, estrogenic, antitumor and antimicrobial activities [1-5]. Some of these are reported for their preventative activity in circulatory diseases and post climacteric osteoporosis [6].

The yields of such flavonoids, when extracted from field grown plants, vary greatly due to number of factors that are difficult to control such as climatic, edaphic and plant growth profile [7]. Plant cell and tissue cultures offer great opportunities for controlled production of myriad of such flavonoids and has been exploited as an efficient and useful tool for production of these metabolites. Callus as an unorganized mass of undifferentiated cells exhibit great biosynthetic potential, and is a good candidate for achieving higher degree of target metabolite production supported by media manipulation, elicitation and precursor feeding. Flavonoids are synthesized via the phenylpropanoid pathway, which also leads to the synthesis of other important secondary metabolites [8]. The synthesis of flavonoids starts with the deamination of phenylalanine to cinnamic acid by phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5). Hence, cinnamic acid, the product of PAL is an important precursor for the synthesis of flavonoids which acts as the signal molecule for regulating the flux into the pathway.

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There are ample opportunities for enhanced in-vitro production of valuable secondary metabolites by precursor feeding. A study on synthesis of various flavonoids in response to precursor feeding has been done by many researchers [9-11]. However, such reports in callus cultures of *Glycyrrhiza glabra* are meager. Therefore, the present study investigated the effect of cinnamic acid supplementation on production of medicinally useful flavonoids in Glycyrrhiza callus cultures, and ascertained its relationship with PAL gene expression and PAL enzyme activity.

Materials and Methods

Chemicals and Standards

Solvents for extraction (Ethanol, Methanol), for GC-MS analysis, were of chromatography grade, Cinnamic acid, Mercuric chloride, Petroleum ether, Diethyl ether, Sulphuric acid, ethyl acetate Trimethyl silyl, Tris-HCl, All other chemicals were of analytical grade Purchased from Quiagen, Merck, Scientific agencies Pvt. Ltd (INDIA).

Plant Material and callus induction

Glycyrrhiza glabra plants were collected from Forest nursery, Faridabad. The young leaves were excised from the field grown plants and used as explants for callus induction. The explants were washed thoroughly in running tap water for 10 minutes, were surface sterilized with 70% ethanol and 0.1% mercuric chloride and were placed in sterile petriplates for inoculation. Callus was grown on previously standardized medium [12] consisting of Murashige and Skoog (1962) basal salts and vitamins with 3% (w/v) sucrose, 1% agar and filter sterilized growth hormones NAA (1.0 mg/l) and BAP (0.5 mg/l). Callus cultures were maintained on this medium and sub cultured at frequent intervals and used for further studies.

Preparation of precursor and treatment

To examine the effect of incorporation of precursor on flavonoid contents, callus tissue was transferred to fresh MS medium with above mentioned additives and supplemented with different concentrations of cinnamic acid. The cinnamic acid stock solution (10 mg/ml) was prepared, diluted to the required concentration with de-ionized water, adjusted to pH 5.8, and sterilized by autoclaving. The solutions of concentration ranging from 25mg/100ml to 150mg/100ml were added to the cultures. For a time course study, untreated and treated cultures were harvested at different time intervals (14, 21, 28, 35, 42, 49 and 56 days). Biomass was quantified by dry weight.

Extraction of flavonoids

The treated and non treated 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 [13]. 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.

Analysis of flavonoids

The extracted samples were derivatized with trimethylsilyl (TMS) to increase the volatility of compounds. The volume corresponding to 2 mg of the original dry weight from the each sample was subjected to 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 and 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 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. All the experiments were conducted using three replicates per treatment and the data were presented as mean± SD. The significant differences between control and treated samples were analyzed using Tukey' s post hoc test using one-way ANOVA for comparison of means at the level of significance = 0.05.

Phenylalanine ammonia-lyase (PAL) assay

PAL activity was measured according to [11]. The callus cells were homogenized in an ice-cold mortar with 5 ml 0.05 M Tris– HCl buffer pH 8.0 containing 0.8 mM β-mercaptoethanol and 1 % w/v polyvinyl pyrrolidone. The homogenate was centrifuged (18,000 g for 15 min at 4 C) and the supernatant was used to measure PAL

activity. The enzyme reaction mixture consisted of 1 ml 0.05 M Tris–HCl buffer pH 8.0, 0.1 ml of enzyme extract, 0.5 ml of 10 mM L-phenylalanine, and total volume was made upto 3 ml. After 1 h incubation at 37 C, the reaction was stopped by the addition of 0.1 ml 1 N HCl, and the absorbance was read at 290 nm on a NANODROP 2000C spectrophotometer. The enzyme activity was expressed in units, each representing the amount of enzyme catalyzed for the formation of 1 μM of trans-cinnamic acid (0 9,000 M ml 1) per min per milligram protein. The protein content was determined according to the Bradford method [14] with a standard curve prepared using bovine serum albumin. All Experiments were performed in triplicate and the results were expressed as mean value ± SD (n=3).

PCR analysis

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. PAL gene activity was tested using the PCR reaction to amplify the PAL sequence by the use of a specific designed primer. PCR 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 phenylalanine-ammonia lyase (PAL), and actin-encoding sequences were performed with primers sequences [15] listed below

PAL F: 5'-GCAATGGCTTGGTCCTCTTA-3'
R: 5'-CCATGCAAAGCCTTGTTTCT-3'

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

Transcript levels were calculated by using the standard curve method from triplicate data, with actin gene as internal control and non treated callus as reference sample. The significant differences between control and treated samples were analyzed using Tukey's post hoc test using one-way ANOVA for comparison of means at the level of significance = 0.05

Results and Discussion

Establishment of Callus Cultures

Plant tissue cultures usually require concerted and cooperative activities of growth regulators for tissue growth and development. Interactions of auxins and cytokinins are said to be highly influential for cell growth and division [16]. Callus cultures of *Glycyrrhiza glabra* were obtained by culturing leaf explants on MS media supplemented with 1 mg/L NAA and 0.5 mg/L BAP, since this combination exhibited fastest callus development with high growth index [12].The callus was soft, green and friable (Figure1) with a maximum growth index of 7.32 and was subcultured every 4 weeks on the same medium.

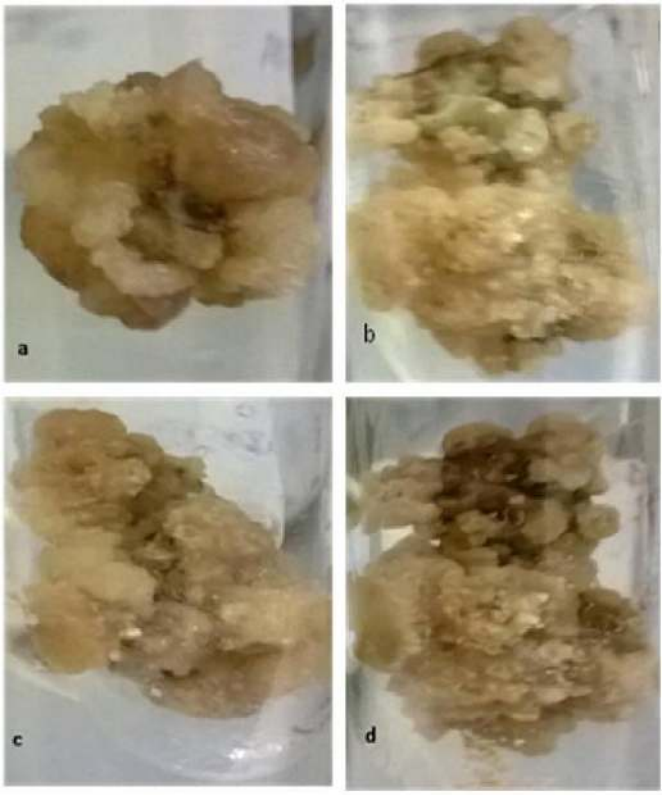

Figure 1: Callus established in MS medium supplemented with 1mg/L NAA and 0.5 mg/L BAP at a) 2 weeks b) 4 weeks c) 6 weeks and d) 8 weeks after inoculation.

Effect of Cinnamic acid on flavonoid production

Glycyrrhiza glabra callus was induced in an optimized medium, further supplemented with different concentrations of cinnamic acid and its effect on flavonoid biosynthesis was investigated. Tissue culture-derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues and overall the concentrations can be significantly enhanced in callus cultures through micro environmental control and precursor feeding to provoke metabolite production [17].

Samples obtained from the control and treated callus cultures at maximum growth index were subjected to GC-MS analysis. The time-course analyses were performed for each concentration of cinnamic acid used for precursor feeding and triplicate instrumental analysis of individual biological replicates were performed on samples throughout each time-course to provide an estimate of instrumental variability. Flavonoid composition of the samples was determined through highly sensitive Mass Spectral analysis. The respective flavonoids were identified on the basis of their retention

time (RT) and the respective mass spectra (Table 1) matching with authentic compounds. Identifications were further confirmed through spectral matching against the National Institute of Standards and Technology (NIST) library. Three pharmaceutically important flavonoids of *Glycyrrhiza glabra* namely licoisoflavone B, licochalcone A, liquirtigenin (Figure 2) were studied in the treated and untreated callus samples at maximum growth index, for their response to in-vitro cinnamic acid supplementation ranging from 25mg/L to 150mg/ (Table 2). Further higher concentrations of cinnamic acid could not be studied as the survival rate of callus cultures fed with concentrations beyond 150mg/L was very low.

Table 1: Retention time, fragmentation pattern (m/z) and significant ions from mass spectra of each flavonoid.

Compound	RT(min)	Molecular ion [M+]	Significant ions (m/z)
Licoisoflavone B	27.1	352	139, 151, 165, 193, 222, 250, 27, 280, 352
Licochalcone A	30.3	338	121, 177,189, 277,307, 308, 323, 338
Liquirtigenin	30.7	256	39, 51, 69, 104, 124, 152, 179, 238, 256

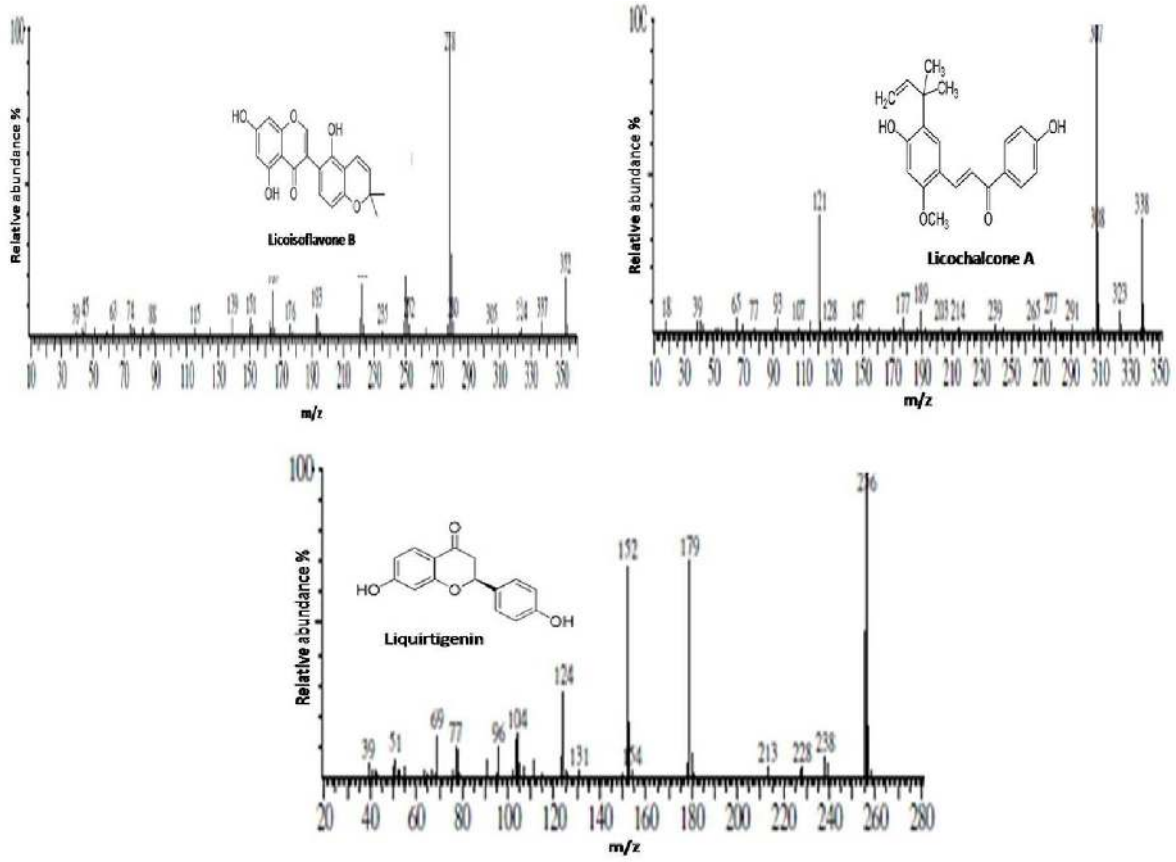

Figure 2: The mass spectra of licoisoflavone B, licochalcone A and liquirtigenin with respective molecular structure.

Biosynthesis of licoisoflavone B was enhanced most remarkably with increasing concentrations of cinnamic acid and nearly 5.1 fold to 9.76 fold increase in its contents was noticed as compared to control, the licoisoflavone B content was found to be significantly different from that of control at all concentrations of cinnamic acid at =0.05. However, the production of licochalcone A and liquirtigenin followed a different pattern where in the content of both the flavonoids maximized on supplementing 50mg/100ml cinnamic acid and thereafter decreased with its increasing concentrations.

Table 2: Influence of different concentrations of cinnamic acid on Peak area% of flavonoids licoisoflavone B, licochalcone A and liquirtigenin in callus culture of *Glycyrrhiza glabra* at maximum growth index.

Treatment	Concentration(mg/100ml)	Callus Growth Index	Licoisoflavone B (Peak area%)	Licochalcone A (Peak area%)	Liquirtigenin (Peak area%)
Control	0	7.32±2.41	0.18±0.013^{a}	8.43±2.74^{a}	2.05±0.43^{a}
	25	7.82±3.01	0.922±0.35^{b}	10.65±1.45^{b}	2.13±0.35^{a}
	50	7.81±1.36	1.035±0.87^{c}	10.83±2.34^{c}	2.46±0.18^{b}
Cinnamic acid	75	7.78±2.11	1.15±0.07^{c}	10.42±2.83^{c}	1.89±0.21^{c}
	100	6.78±1.76	1.539±0.23^{d}	8.83±1.79 ^{c}	1.76±0.24^{bd}
	125	6.59±1.86	1.679±0.11^{e}	7 .54±1.12^{bc}	1.43±0.19b^{a}
	150	6.27±1.28	1.75± 022^{e}	5.04±1.00 ^{b}	0.95±0.06^{a}

Values represent means ± S.D for 20 replicates per treatment. Means followed by different letters are significantly different at =0.05
Induction % = No. of explants initiating Calli / Total number of explants inoculated x 100
GI= (Final dry weight-initial dry weight) / initial dry weight

Licochalcone A and liquirtigenin levels were increased to 1.28 and 1.2 folds respectively as compared to that of control at 50mg/100ml cinnamic acid concentrations. Several other studies report the elevation of target secondary metabolites on cinnamic acid supplementation in tissue cultures of sweet potato [18], *Pluchea lanceolata* [19], and soybean [20]. In this study, it was clearly inferred that feeding of cinnamic acid up to certain concentrations (50mg/L) to the callus cultures of *G. glabra* increases the accumulation of flavonoids, while its higher concentrations seriously inhibit the flavonoid production. This may be attributed to the fact that higher concentrations of precursor leads to the feedback inhibition of the metabolic pathway [21].

Flavonoid production is not only influenced by the type and concentration of precursors but also depends on growth period of cell culture and duration of precursor feeding. Therefore time course of production of each flavonoid at each concentration of cinnamic acid was also monitored and a dose dependent response to cinnamic acid by the callus cultures of *Glycyrrhiza glabra* was observed for flavonoid production. The time course followed for feeding of different concentrations of cinnamic acid for each of the flavonoids is shown in figures 3-5.

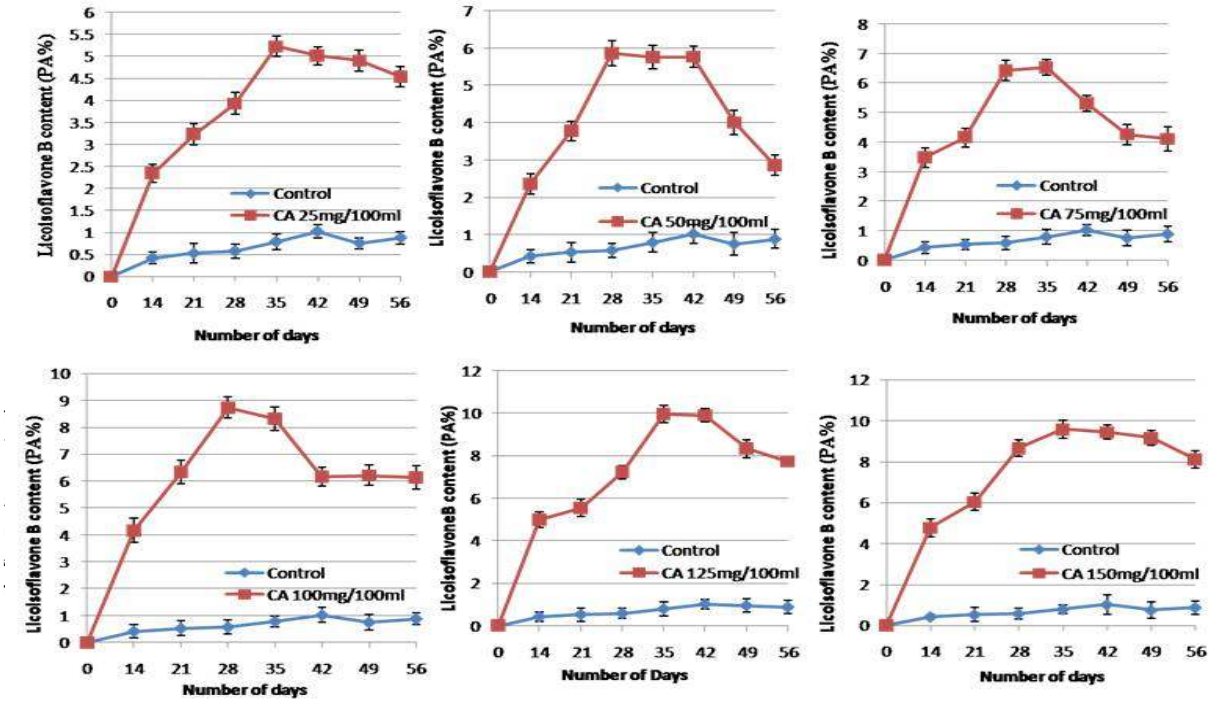

Figure 3: Time course analysis of Cinnamic acid feeding time on licoisoflavone B production at different concentrations a) 25mg/100ml b) 50mg/100ml, c) 75mg/100ml, d) 100mg/100ml, e) 125mg/100ml and f) 150mg/100ml.

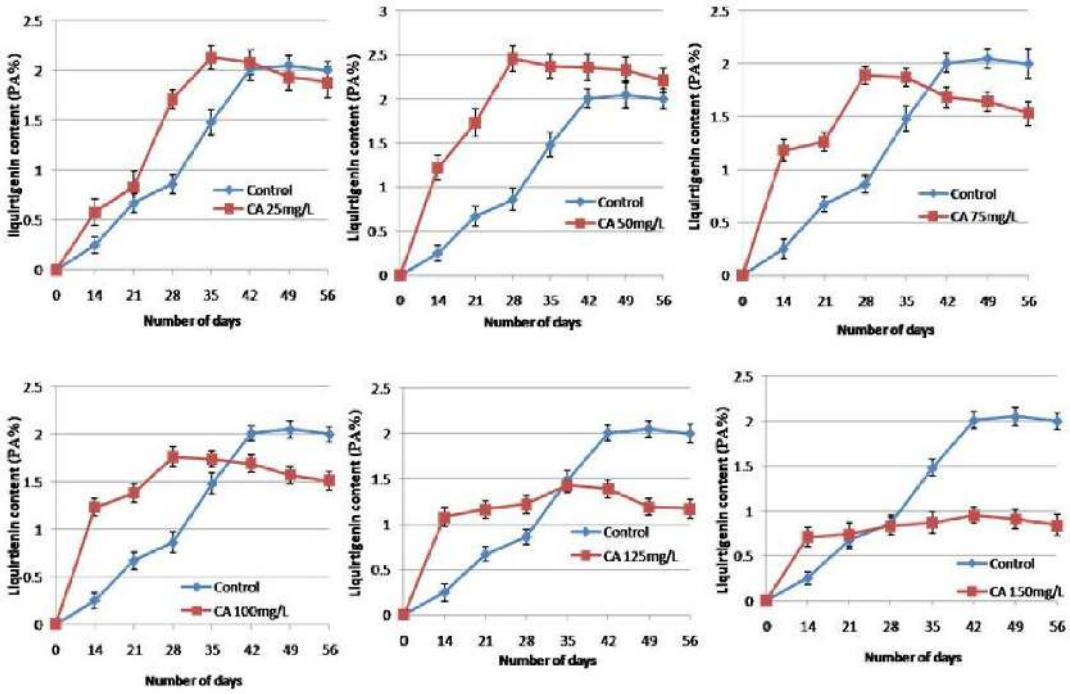

Figure 4: Time course analysis of Cinnamic acid feeding time on liquirtigenin production at different concentrations a) 25mg/100ml b) 50mg/100ml, c) 75mg/100ml, d) 100 mg/100ml, e) 125mg/100ml and f) 150mg/100ml.

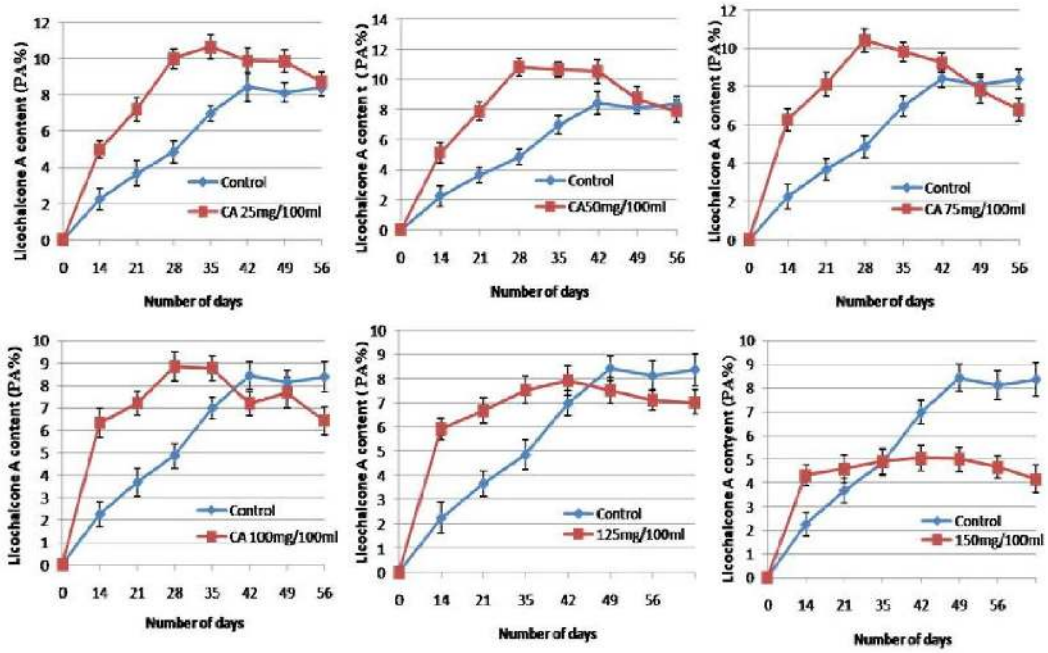

Figure 5: Time course analysis of Cinnamic acid feeding time on licochalcone A production at different concentrations a) 25mg/100ml b) 50mg/100ml, c) 75mg/100ml, d) 100 mg/100ml, e) 125mg/100ml and f) 150mg/100ml.

It revealed that the onset of the accumulation of all the three compounds occurred after 14 days. licoisoflavone B was found in higher amounts at all studied time points in comparison to that of control which showed that cinnamic acid significantly enhanced the levels of licoisoflavone B at all studied time points and at all Although the licoisoflavone B content increased with increasing concentrations, at concentrations 75mg/100ml, 100mg/100ml and 125mg/100ml, maximum response directed to its synthesis was recorded either at 28 days or 35 days beyond which there was a decline in the production, as was obvious in its time course analysis (Figure 3). Time course analysis of licochalcone A and liquirtigenin also revealed that both the flavonoids followed a similar pattern of accumulation in response to different doses and time points of cinnamic acid treatment. Their levels of production decreased relative to control at prolonged treatments with concentrations beyond 50mg/100ml (Figure 4, 5).

In the presence of 25mg/100ml and 50mg/100ml cinnamic acid, increase in licochalcone A and liquirtigenin productions was noticed as compared to control. At 50mg/100ml comparatively higher production of these flavonoids was observed which reached their maximum at 28th day whereas in control the maximum production was seen only after 42 days. With increase in time period beyond 35 days and concentrations higher than 50mg/100ml, gradual decrease in the production of these flavonoids was observed. Such pattern of flavonoid accumulation suggested that the optimum concentration of cinnamic acid for obtaining higher content of licochalcone A and liquirtigenin is 50mg/100ml and the inhibition of flavonoid production at prolonged treatments of high doses of cinnamic acid is due to disturbances in cell permeability, osmotic condition, changes in membrane potential and cell damage caused by longer contact with the precursor [22].

concentrations of cinnamic acid as compared to other flavonoids. However, maximum production of licoisoflavone B was observed at 35 days of culture at 125mg/100ml cinnamic acid treatment whereas in untreated callus, maximum production occurred at 42 days.

Effect of cinnamic acid on PAL activity

The analysis of flavonoid production in the *G. glabra* callus cultures supplemented with cinnamic acid clearly complies with the fact that if the metabolic machinery is fed with the compound, which is an intermediate or is produced at the beginning of a secondary metabolite biosynthetic route, it stands a good chance of increasing the yield of the final product.

This involves expression of different genes and regulation of various enzymes in metabolic pathways. The levels of enzymes of the phenylpropanoid pathway are tightly and coordinately regulated during responses of plants to the changes occurring in their environment [23-25]. Phenylalanine ammonia-lyase is first enzyme of phenylpropanoid pathway which plays a pivotal role in phenolic synthesis, and many reports emphasize the correlation between increase in the corresponding PAL gene expression and increase in biosynthesis of phenolic compounds in response to different stimuli [26-28]. Therefore, the study of effect of cinnamic acid treatment on the activity and expression of PAL was found imperative.

Under our experimental conditions, the highest PAL activity was attained in control cultures on day 21 with a small second peak observed at day 35 followed by a constant activity throughout the time course (Figure 6).

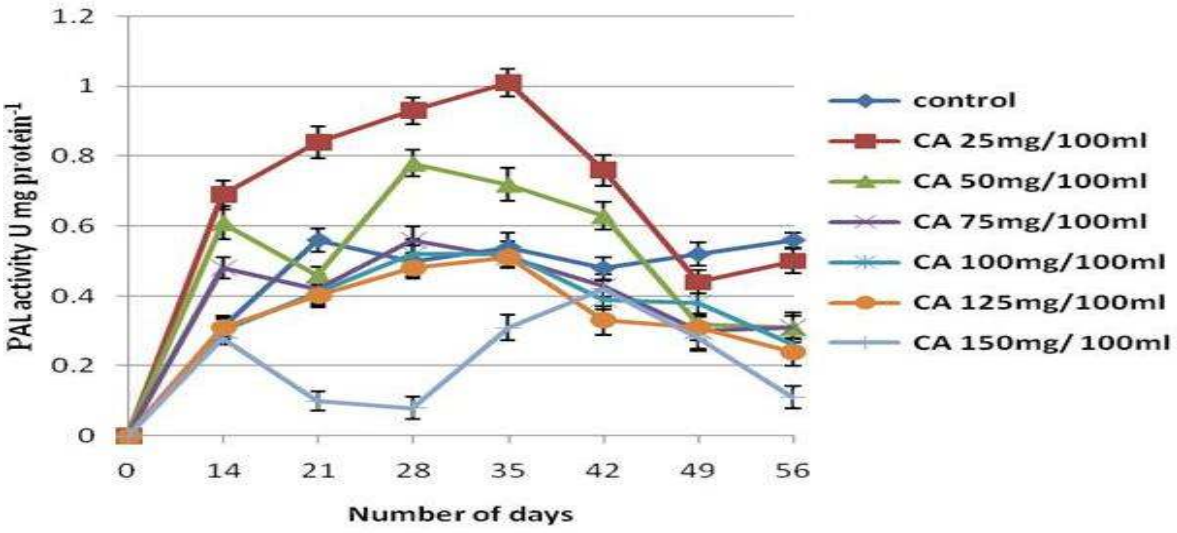

Figure 6: Time course of PAL activity at different concentrations of Cinnamic acid.

The very first peak of PAL activity was observed at day 14 in all callus cultures after they were transferred to the fresh media supplemented with different concentrations of cinnamic acid, which represented an immediate stress response of the cells as soon as they were transferred to fresh medium. A transient considerable increase in PAL activity as compared to that of control was observed as a result of treatment with 25mg/100ml and 50mg/100ml cinnamic acid in which much pronounced activities were observed at 28th day and 35th day respectively. In case of cinnamic acid treatments higher than 50mg/100ml, the PAL activity followed a similar trend to that of control till 35th day beyond which there was a drop in the activity as compared to control. These results are consistent with the results of time course of licochalcone A and liquirtigenin accumulation in treated cultures.

Trans-cinnamic acid is a feedback modulator of the expression and enzymatic activity of L-phenylalanine ammonia-lyase [29]. The exogenously applied cinnamic acid inhibits PAL activity and transcription of PAL genes, and induces the synthesis of a proteinaceous inhibitor of PAL [24, 30-31]. However, an increase in amount of flavonoids and PAL activity obtained in our results at optimum concentrations (25mg/100ml and 50mg/100ml) of cinnamic acid suggests that a significant increase in the amount of flavonoids is obtained with optimum concentrations and appropriate feeding time of the precursor.

Adding concentrations of cinnamic acid beyond certain levels and prolonged treatments led to the feedback inhibition of the PAL in *G. glabra* callus cultures leading to a decrease in PAL activity, thereby decreasing the production of licochalcone A and liquirtigenin. However, greater accumulation of licoisoflavone B observed even at higher concentrations (125mg/100ml) of cinnamic acid, despite the inhibition of PAL, might be due to the modulatory effect of cinnamic acid on the activity of chalcone isomerase [32], which catalyses the further step in the formation of Licoisoflavone B favoring its accumulation.

In most studies, the effects of cinnamic acid have also been related to its action on the plasma membrane and related processes, including the induction of oxidative stress [33], an increase in reactive oxygen species (ROS) levels [34], a disturbance in Ca^{2+} homeostasis [35], which might also be the reason for increase in flavonoid accumulation.

Effect of cinnamic acid on PAL gene expression

Changes in the transcription levels of gene responsible for PAL enzyme participating in flavonoid biosynthesis pathway in response to cinnamic acid treatment (25mg/100ml 50mg/100ml, 75mg/100ml, 125mg/100ml and 150mg/100ml) were studied at three different time points. The transcription of the studied genes was monitored after 21, 28, 35 days as maximum flavonoid accumulation and PAL activity were obtained at these time points. Significant differences in the expression patterns of PAL gene were observed. Figure 7 demonstrates the relative transcript levels of PAL gene expression.

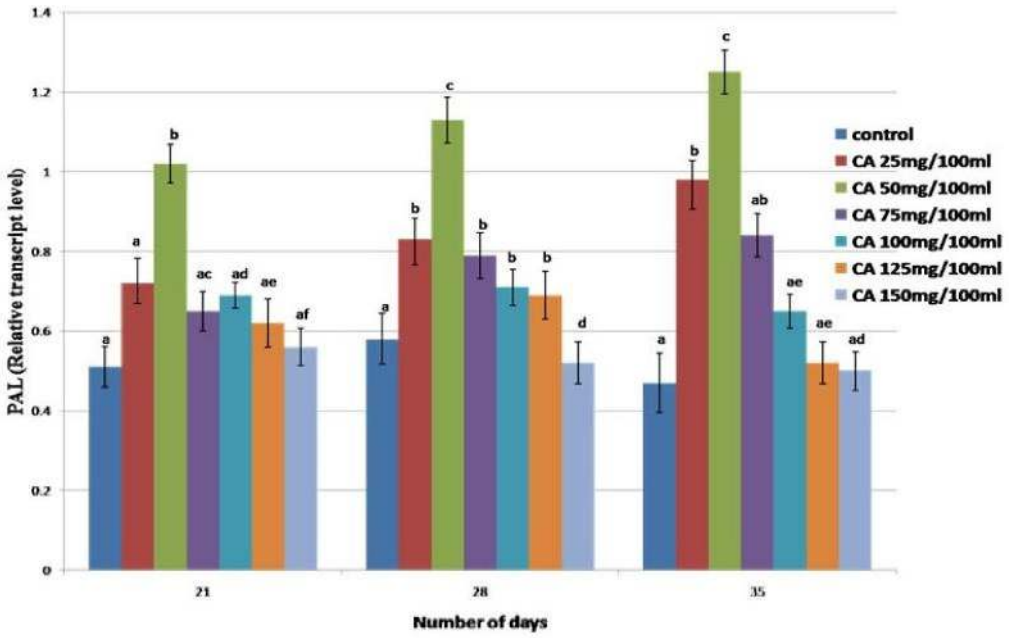

Figure 7: Relative transcript level ratio of PAL at selected concentrations and time course of chitosan treatment. Results represent the mean fold increase of mRNA level over untreated samples. Results are mean standard deviations of three experiments, Values marked with different letters are significantly different at =0.05.

From the gene expression profiling at selected time points, it was obvious that the expression of PAL was up regulated by cinnamic acid treatment as compared to that of control at cinnamic acid concentrations 25mg/100ml and 50mg/100ml . However, greatest response was observed in the PAL expression at 50mg/100ml cinnamic acid treatment as compared to others, where expression was found to be increasing with the time points 21day, 28days, 35days as compared to that of control, where a slight decrease in expression levels was observed at 35days. In this study, PAL gene expression was in complete accordance with the PAL enzyme activity and flavonoid accumulation at these selected time points, which further confirms the feedback inhibition of PAL by excess of cinnamic acid fed in the cultures.

Conclusion

The regulatory mechanisms of *G. glabra* callus cells activated by cinnamic acid treatment at particular concentrations and time duration involve the induction of phenylpropanoid biosynthesis, through feed-forward modulation of PAL. Cinnamic acid acts as a substrate for enzymes catalyzing follow up steps of the pathway leading to flavonoid synthesis. However, in this study, higher concentrations of cinnamic acid and longer time durations of its exposure led to decrease in the production of flavonoids liquirtigenin and licochalcone A due to the feedback inhibition of PAL. Moreover, cinnamic acid regulates the expression of genes, other than that of PAL, which encode intermediate enzymes of phenylpropanoid biosynthesis pathway. Cinnamic acid is also known to have modulatory effect on the activity of chalcone isomerase (CHI) which catalyzes the production of intermediate products favoring the production of licoisoflavone B, leading to its higher accumulation. However, the cellular response towards the stress caused by cinnamic acid treatment cannot be denied and probably number of mechanisms together comes into play in a complex manner, altering the secondary metabolism in plant tissue cultures. This study provides sufficient evidences to support the use of cinnamic acid for production of medicinally useful flavonoids licochalcone A, liquirtigenin and licoisoflavone B in callus cultures of *G. glabra*.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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