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Effect of additive supplementation and age of callus on the expression pattern of three key genes of phenylpropanoid pathway in *P. ovata*

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Abstract: Phenylpropanoid pathway is one of the most important metabolic pathways associated with plant secondary metabolism. This pathway leads to the formation of different types of polyphenolic compounds. Secondary metabolism in *Plantago ovata* involves various enzymes which are synthesized by different key genes of this pathway. *Phenylalanine ammonia lyase*, *chalcone synthase* and *dihydroflavonol-4-reductase* are the genes which were studied in this present investigation. Here for the first time partial sequences of coding regions of these three genes were sequenced and identified in this plant. There pivotal role in phenolic biosynthesis during callus culture was analyzed in this present study. Impact of ageing of callus, presence of plant growth regulators in the culture media and the effect of supplementation with organic additives on the expression of these genes were also analyzed. Alteration in expression of these genes were observed both in ageing callus tissue and also in callus grown in medium with additive supplementation. Among the three genes, expression of *DFR* and *CHS* enhanced significantly more than *PAL* in additive supplemented callus. Contrary to that *PAL* showed highest enhancement of expression during callogenesis. This study portrays the regulatory aspects of *PAL*, *CHS* and *DFR* induction for higher production of polyphenols using *in vitro* callogenesis.

Keywords: Callus, Chalcone synthase, Dihydroflavonol-4-reductase, *Plantago ovata*, Phenylalanine ammonia lyase, Phenylpropanoid pathway, Real time PCR.

INTRODUCTION

Plantago ovata Forsk is a medicinal plant which grows in arid and semi-arid tropical and temperate regions[1]. It is cultivated as a cash crop in western India. It produces different polyphenolic compounds which includes polyphenols comprise of phenolic acids, flavonoids, stilbenes, lignans, tannins etc. Polyphenols are the intermediate products of the phenylpropanoid pathway and their production is regulated by the differential expression of the genes involved in this pathway. Differentiation and dedifferentiation occur during callogenesis[2]. Callus culture is an established technique for large scale production of economically important plant metabolites [3]. Several recent studies have shown that combinatorial addition of different plant growth regulators (PGR) in the culture medium caused higher production of polphenolic compounds[4,5] .

Studies have documented that cytokinins could enhance polyphenol production by inducing PAL[6]. Studies on tobacco [7] and *Petunia*[8] also established the importance of PGR in influencing secondary metabolite production by inducing several key enzymes of polyphenol synthesis. In a recent study on phenylpropanoid pathway of willow importance of PAL in production of polyphenols, flavonoids and phenolic glycosides was established[9]. In a recent study on callogenesis from *P. ovata* explants our group observed that polyphenol and flavonoid accumulation increased till 3rd passage (63 days old callus). Significant enhancement in polyphenol content due to wounding of explants during sub culturing of callus tissue was also reported[10]. Influence of auxin and cytokinin on polyphenol accumulation and its antioxidant properties was reported in a recent study on Callus culture of *Senecio candicans* [11].

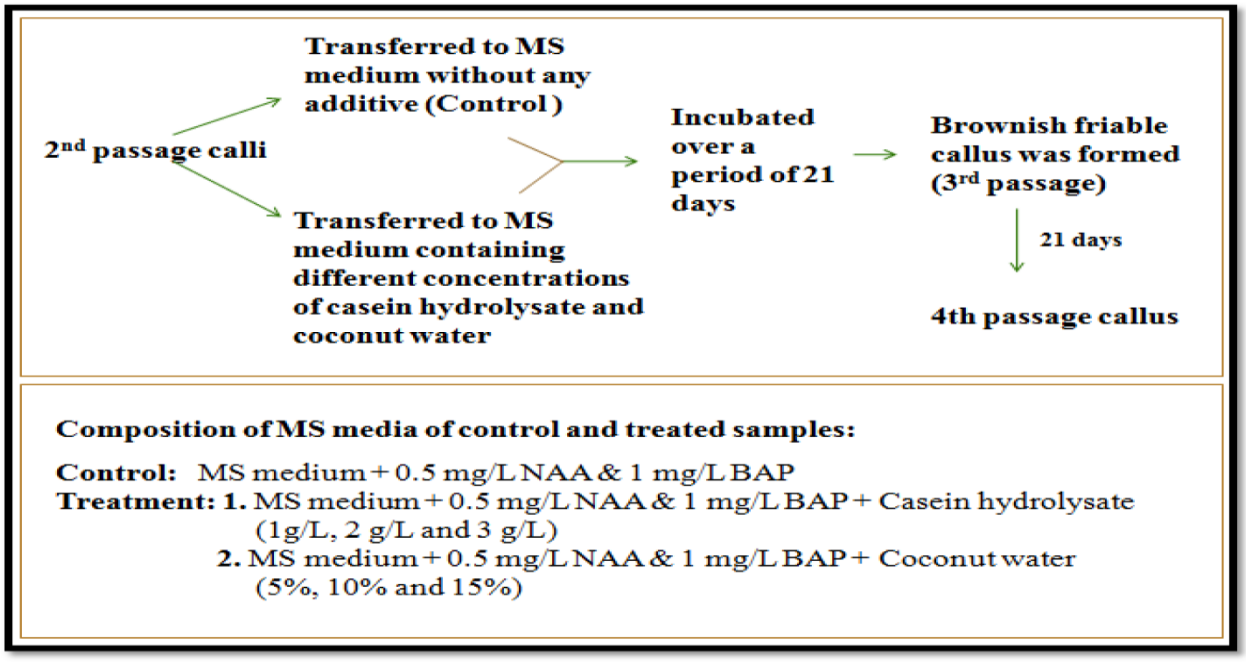
The elicitors and external additives stimulate the biosynthesis of plant metabolites. Some recent studies validated the use of casein hydrolysate (CH) and coconut water (CW) as potential external additives[12]. CH is prepared from the milk protein casein which is hydrolyzed to its component amino acids and hence, it provides various amino acids including phenylalanine which is the substrate of PAL. PAL converts phenylalanine to cinnamic acid, the first stable product of phenylpropanoid pathway[13]. In a recent study on *Pluchea lanceolata*, exogenous incorporation of cinnamic acid and also precursor feeding by addition of L- phenylalanine caused almost nine fold increase in quercetin production in callus tissue [14]. CH also causes enhancement of phytochemical production[15]. On the other hand, CW is the liquid endosperm of unripe coconut which contains a mixture of natural cytokinins, auxins and different micronutrients which are very important constituent of culture medium [16]. The addition of coconut water in nutrient media resulted in enhanced nutritional and carbohydrate content in the plants [17].

Thus supplementation of CW in the culture medium provides many essential components for callogenesis during tissue culture. Both CH and CW serve as sources of nutrients and amino acids in tissue culture medium [18]. Our recent study reported a positive influence of CH and CW in enhancing polyphenol accumulation during callogenesis[19]. A study on *Ephedra alalta* revealed that precursor feeding by the using CH and L- phenylalanine enhanced accumulation of phenolic compounds in callus culture[20]. Therefore, studying the genetic regulation of polyphenol accumulation with age of callus as well as in additive supplemented callus would be an important issue. Change in expression pattern of several genes occurs during callogenesis, some of these genes are involved in polyphenol synthesis via phenylpropanoid pathway. Phenylalanine is produced as a byproduct of primary metabolism of plants mainly by the shikimic acids pathway. PAL causes deamination of

L- phenylalanine to synthesize t- cinnamic acid, the precursor of several other polyphenolic compounds[21]. As PAL is situated at the junction of primary and secondary metabolism it is extensively studied to understand its importance in plant's metabolism [22]. Series of biochemical reactions occur in phenylpropanoid pathway which is essential for the production of important phenolic compounds. Flavonoid production is catalysed by chalcone synthase (*CHS*). It produces narginin which is the precursor of a large variety of secondary metabolites. In a study on *Freesia hybrid* it was documented that induction and expression of CHS is pivotal in flavonoid production[23]. Another important gene of phenylpropanoid pathway is Dihydroflavonol-4-reductase *(DFR)*. It produces a key enzyme which catalyses the synthesis of anthocyanins[24]. The important role of DFR in anthocyanin biosynthesis was studied in several plant species [25]. Positive effect of PGRs on callus induction and anthocyanin biosynthesis was reported in a study on *Solanum melongena* L. [26]. There is no previous report on the study of simultaneous expression of the key genes of phenylpropanoid pathway during *P. ovata* callus culture. So in this present study the impact of additives and age of callus on the expression profiles of three key genes of phenylpropanoid pathway were looked upon to explore the possibilities to scale up the production of phenolic compounds by *in vitro* callus culture.

MATERIALS AND METHOD

***In vitro* callus culture:** Callus culture was initiated from the shoots of 12 day old seedlings. It was established and maintained on MS medium[27] according to Talukder *et al.*[19] The scheme of callus culture and additive treatment is given below:



Primer designing: Gene specific primers used in this study for *P. ovata PAL, CHS, DFR* are listed in Table I. Primer express version 3.0 (Applied Biosystems, Foster City, CA, USA) was used to design the primers.

Table I: Details of primers

PAL	PLAF1- AGCAACCAGAGCAGCAATGC 20 mer, Tm=53.8°C, GC content- 55%	PALR1- TATTTGGCCCGGGTGATGCT 20 mer, Tm=53.8°C, GC content- 55%
CHS	CHSF1- CAGCCCAAATCCAAGATCACC 21 mer, Tm=54.4°C, GC content- 52.4%	CHSR1- GGCCTTCCTCATCTCATCCA 20 mer, Tm=53.8°C, GC content- 55%
DFR	DFR F1- CGTTGATGCAGAAACTGAGG 20 mer, Tm=49.7°C, GC content- 45%	DFR R2- GCACATTGCTGTGAACACCT 20 mer, Tm=49°C, GC content- 47%

PCR conditions for amplification of the genes
PAL: 94°C for 1 min, 53.5°C for 1 min, 72°C for 1 min
CHS: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min
DFR: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min
Followed by a single cycle at 72°C - 10 mins.

} 35 cycles

Analysis of gene expression by quantitative PCR: RNA was obtained from callus culture following manufacturer's protocol (Qiagen Plant RNeasy Mini Kit, New Delhi, India). The reaction condition was: 10 minutes at 95 °C; 40 cycles- [95 °C at 30 second, 60 °C at 1 min.] *Plantago* β-actin served as endogenous control. Three biological replicates and negative controls were kept for each reaction and calibration was done with the expression in a control untreated callus tissue. Negative control was maintained in each reaction. The experiments were carried out in an Applied Biosystem Step one plus system (Applied Biosystem, Foster city, USA). $2^{-\Delta\Delta CT}$ method[28] was used for data analysis.

Table II: List of primers used for Real Time PCR

PAL	PALRTF- AAGAACGGCGAACATGAGAAG 21mer,Tm=52.4°C, GC content- 47.6%	PALRTR- GATCCGATTCGCGATTGCT 19 mer, Tm=51.1°C, GC content- 52.6%
CHS	CHSRTF- TCGATGGTCACTTGAGGGAAGT 22 mer, Tm=54°C, GC content- 50%	CHSRTR- GGTGCGCGATCCAGAAAA 18mer, Tm=50.3°C, GC content- 55.6%
DFR	DFRRTF- TGCCAAAAGCGGATACAAACT 21 mer, Tm=51°C, GC content- 43%	DFRRTR- GGTGTGGCCAAGTGAAACACT 21 mer, Tm=54°C, GC content- 52%
ACTIN	ACTINF- CACGAGACCACCTACAACTCG 21 mer, Tm=56.3°C, GC content-57.1%	ACTINR- CAACCTTAATCTTCATGCTGCTC 23mer, Tm=53.5°C, GC content- 44%

RESULTS

Induction of callus: *P. ovata* seeds were found to germinate within 2-3 days of inoculation in agar-sucrose medium. Callus culture was established from shoot explants.

Sequence analysis of *Plantago PAL* gene: 401 bp fragment of *PAL* was sequenced and submitted to GenBank (Accession No.KM198971.1). Upon blastn analysis this partial cds showed similarity with almost all the *PAL* sequences of order Lamiales, reported in the Genbank. It showed significant high similarity with the following sequences (Accession No. mentioned with each sequence). It has highest similarity with *Salvia miltiorrhiza* (DQ408636.1), *Prunella vulgaris* (KJ010815.1), *Digitalis lanata* (AJ002221.1) *Agastache rugose* (AF326116.1), *Scutellaria baicalensis* (HM062777.1); all of which belongs to Lamiales. Sequence homology confirmed it belongs to *PAL* super family.

Sequence analysis of Plantago CHS gene: A 316 bp fragment of *CHS* was sequenced (Accession No.KP198546.1). Upon BLAST it showed high similarity with majority of the *CHS* sequences, enlisted in the Genbank. The sequence showed maximum similarity (97%) with *Digitalis lanata* (AJ002526.1), *Misopates orontium* (AM162205.1), *Incarvillea lutea* (GU587725.1), *Mimulus aurantiacus* (EU305683.1) *CHS* sequences. All these plants belong to order Lamiales. Hence the *CHS* is evolutionarily quiet related within the closely related species.

Sequence analysis of *Plantago DFR* gene: A 639 bp fragment of *DFR* gene was obtained (Accession No.KU668976). The *DFR* sequence showed high homology (80-90%) with the other reported *DFR* sequences of Lamiales. High similarity was observed with *Antirrhinum majus* (X15536.1), *Penstemon barbatus* (KM388827.1), *Penstemonneo mexicanus* (.KM388826.1), *Scutellaria viscidula* (FJ605512.1), *Perilla frutescens* (AB002817.1). It can be concluded from the sequence homology of *Plantago DFR* with other *DFR* sequences of Lamiales that this gene do possess high sequence similarity with in this order.

Expression analysis of *PAL* by Real-Time PCR: *PAL* expression was observed in callus tissue of all the passages and also in additive supplemented callus issue. During initial callus induction (1st passage-21 days) expression level was quite low. It increased significantly (1.5 fold) during 2nd passage of sub culturing. Highest expression (3 fold compared to that of 1st passage) of *PAL* was observed in 3rd passage of callus culture.

Expression analysis of *PAL* expression in callus tissue grown in CH added medium gave an idea of its role in induction of *PAL* expression. Highest expression (3.2-fold) was recorded in 2 g $^{L-1}$ CH-supplemented callus.

On the other hand supplementation of CW in the media proved to have more impact on enhancing *PAL* expression. Maximum expression was recorded in 5% CW treated samples (5.1-fold). 10 % and 15% CW treated samples also exhibited higher expression (3.8 and 1.8 fold**)(Fig. 1a, Fig. 1b, Fig. 1c).**

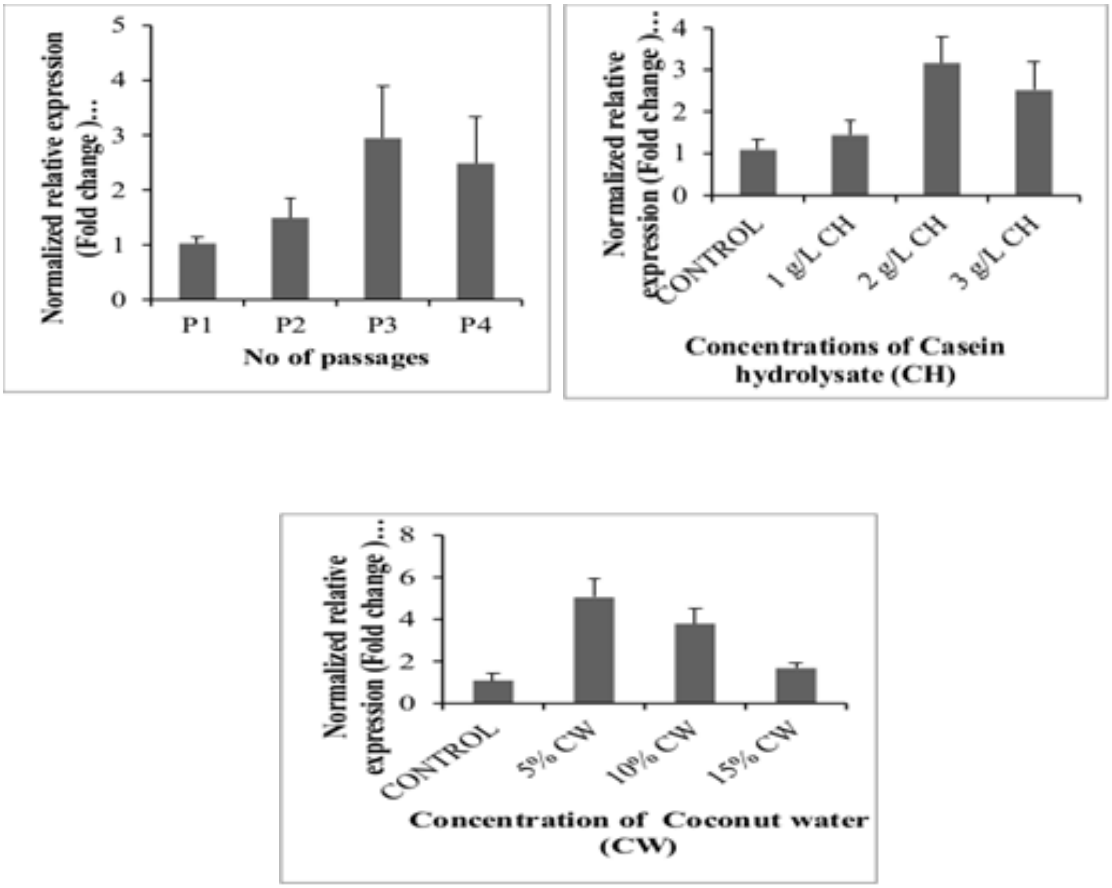


Fig 1: Expression profile of *Phenylalanine ammonia lyase* (*PAL*) Expression profile of *PAL* at different stages of callus culture.

(a) Change in expression of *PAL* in callus formed in MS medium supplemented with different concentrations of CH.
(b) Change in expression of *PAL* in callus formed in MS medium supplemented with different concentrations of CW.

Expression analysis of *CHS* gene by Real-Time PCR: During initial callus induction (1st passage-21 days) expression level was quite low. It increased significantly (1.7 fold) during 2nd passage of sub culturing. Highest expression (2.6 fold compared to that of 1st passage) of *CHS* was observed in 3rd passage of callus culture.

Expression analysis in additive supplemented callus gave an idea of their role in induction of *CHS* expression. Highest expression (6.2-fold) was recorded in 2 g $^{L-1}$ CH-supplemented callus among the CH treated samples.

On the other hand supplementation of CW in the media proved to have more impact on enhancing *CHS* expression. 5% CW treated samples showed the highest expression (13.2 fold). It can be concluded from the analysis that additive supplementation was more effective in enhancing *CHS* expression compared to that of *PAL* expression **(Fig 2a, Fig 2b, Fig 2c).**

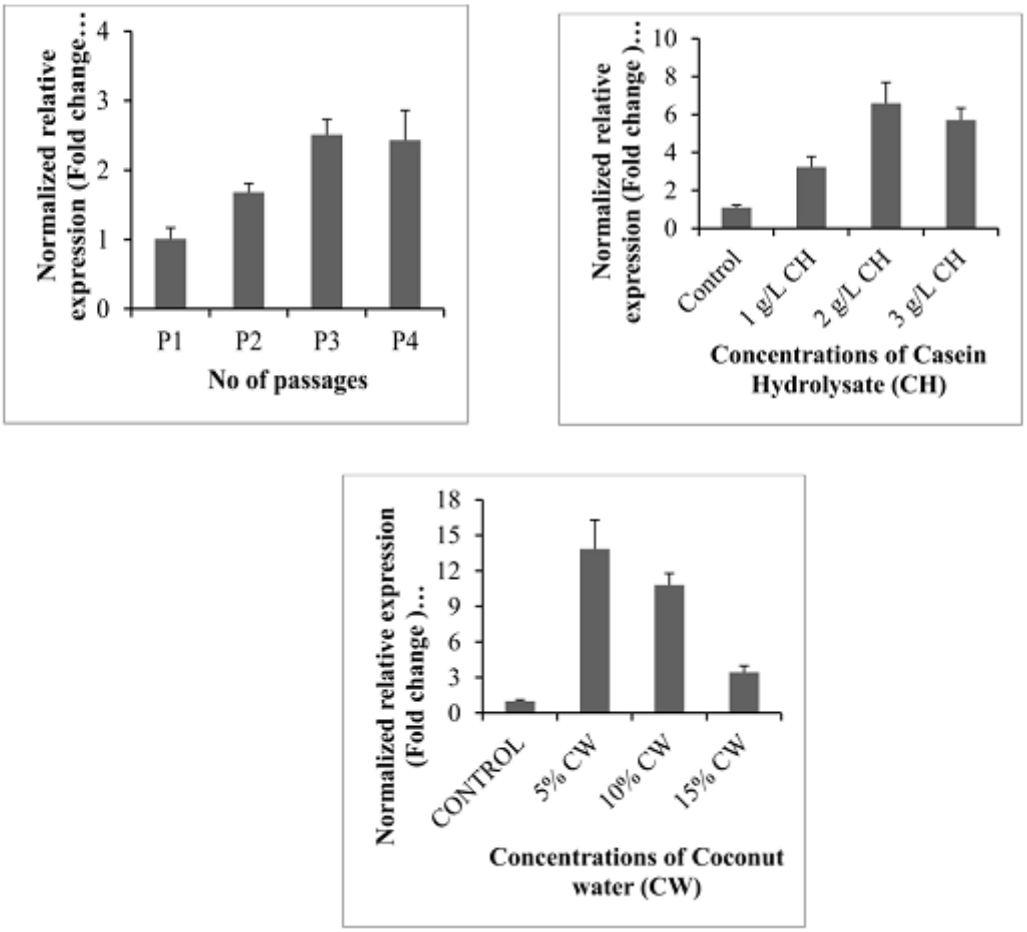


Fig 2: Expression profile of *Chalcone synthase* (*CHS*)

(a) Expression profile of *CHS* at different stages of callus culture.
(b) Change in expression of *CHS* in callus formed in MS medium supplemented with different concentrations of CH.
(c) Change in expression of *CHS* in callus formed in MS medium supplemented with different concentrations of CW.

Expression of *DFR* gene by Real-Time PCR: *DFR* expression was observed in callus tissue of all the passages and also in additive supplemented callus issue. During initial callus induction (1st passage-21 days) expression level was quite low. It increased significantly (1.4 fold) during 2nd passage of sub culturing. Highest expression (2.6 fold compared to that of 1st passage) of *DFR* was observed in 3rd passage of callus culture. Expression level decreased in 4th passage callus (1.5 fold as compared 1st passage callus). Additive supplementation was found to be most effective on *DFR* compared to that of the other two genes. Highest expression (4.5 fold) was recorded in callus tissue grown in 2 g $^{L-1}$ CH added culture medium. On other hand supplementation of CW in the media proved to have more impact on enhancing *DFR* expression. 5% CW treated samples showed the

highest expression (15.1-fold). 10 % and 15% CW treated samples also exhibited higher expression (5 and 2 fold respectively) **(Fig 3a, Fig 3b, Fig 3c).**

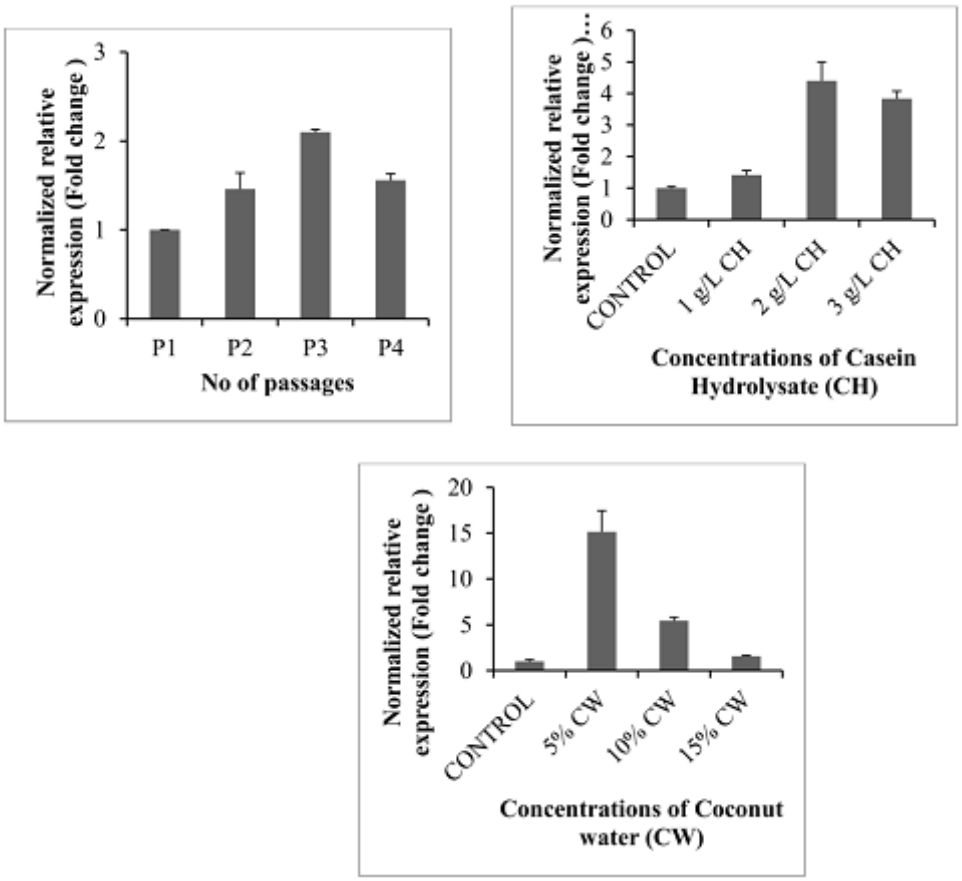


Fig 3: Expression profile of *Dihydroflavonol - 4 -Reductase (DFR)*

(a) Expression profile of *DFR* transcript at different stages of callus culture.
(b) Change in expression of *DFR* in callus formed in MS medium supplemented with different concentrations of CH.
(c) Change in expression of *DFR* in callus formed in MS medium supplemented with different concentrations of CW.

Simultaneous Expression of *PAL, CHS, DFR* gene by Real-Time PCR: Level of expression of *PAL, CHS and DFR* during 3rd passage callus culture and in 2 g/L CH, 5% CW treated callus were also studied to get an idea of the interactive expression of these genes. Among these, *PAL* expression was found to be highest (2.9 fold) in 3rd passage callus and *CHS* and *DFR* expression was also quite high (2.5 and 2.2 fold respectively). Among the two additives used CW was more effective in enhancing expression of these genes. Addition of 2 g/L CH in callus caused a 6.5 fold increase in *CHS* expression; it was highest among the three genes. DFR expression enhanced about 4.5 fold due to 2 g/L CH treatment but *PAL* expression remained almost the same (3.1 fold). 5% CW added callus

showed huge increase in *DFR* expression (15.1 fold) and *CHS* also showed a similar pattern (13.8 fold increase). Even *PAL* expression increased (5 fold) due to CW treatment **(Fig 4a, 4b, 4c).** Data analysis revealed that among the three genes, expression of *DFR* was highly induced by the additive supplementation. *CHS* expression also increased but the increment in expression was comparatively low in case of *PAL*.

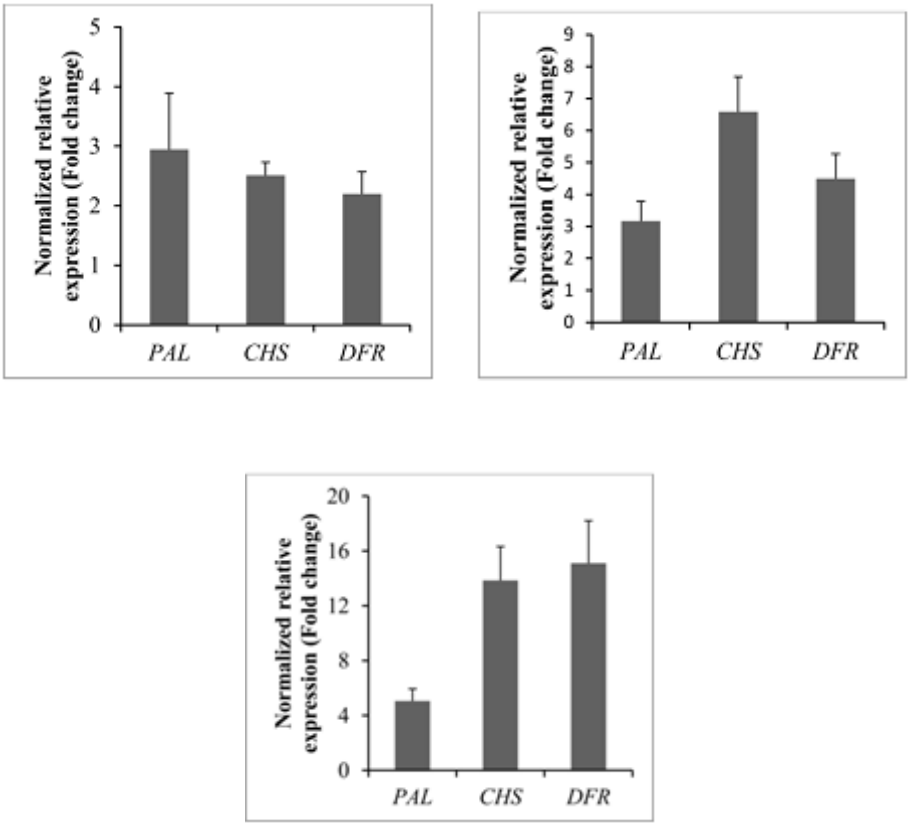


Fig 4: Simultaneous expression of *PAL, CHS, DFR*

(a) Comparative expression profile of *PAL, CHS, DFR* transcript at 3rd passage (63 days old) callus culture.
(b) Effect of 2g/L CH in the expression profile of *PAL, CHS, DFR* transcripts.
(c) Effect of 5% CW in the expression profile of *PAL, CHS, DFR* transcripts.

DISCUSSION

Plant phenolics constituted of phenolic acids, flavonoids, lignans, and tannins which are structurally diverse[29]. Transcriptional regulation is one of the main regulatory mechanisms of secondary metabolite production[30, 31]. Alteration in the rate of transcription of the key genes of phenylpropanoid pathway brings about change in polyphenol production. In this present study some major aspects of this genetic regulation of polyphenol production during callogenesis was studied. The genetic

regulation of *in vitro* callogenesis and polyphenol accumulation during callus culture is still needed to be fully understood. Elicitation by the use of different combinations of PGRs and external additives can positively influence various metabolite productions during *in vitro* callus culture. *P. ovata* is a medicinally important herbaceous plant; hence *in vitro* callogenesis is very beneficial from the aspect of its medicinal value. This study reports the partial sequences of coding regions of the *PAL*, *CHS* and *DFR* are important enzymes of this pathway which leads to the formation of the three major compounds. *PAL* expression was observed in non-organ-forming cultures in rice[32]. In our study we also observed an alteration in *PAL* expression with increase in age of callus. A similar pattern was observed in *Capsicum annuum* L. callus culture supplemented with 2, 4- D and kinetin[33]. These data indicate that PAL activity and its expression is essential for the initiation of secondary metabolism from the products (Phenylalanine) of primary metabolism. In a recent study on *Tecoma stans* callus culture it was observed that use of different PGR showed a positive effect on callus induction and also in enhancing antioxidant activity[34.] These results present a link between additives induced *PAL* regulation and secondary metabolite production during callus culture. In a study on *Taxus sp* higher expression of PAL was observed when treated with methyl jasmonate[35]. Real time PCR analysis showed that PAL activity in *Picrohia kurrooa* increased significantly under the influence of PGR and additives[36]. Among the constituents of CH and CW it was observed that phenylalanine is one of the major constituent and it is the substrate of PAL which converts it to cinnamic acid. Hence in this study additive supplementation showed a positive impact in enhancing *PAL* expression. Flavonoids are a major class of plant secondary metabolites[37]. One of the most important genes involved in flavonoid synthesis is chalcone synthase (*CHS*). Several factors such as UV[38], organic additives and wounding[39] induce the expression of *CHS* genes. Other studies[40] also reported that initiation of flavonoid biosynthesis is coupled with CHS induction. In a study by our group it was observed that an increase in flavonoid accumulation occurred in aging as well as in callus induced and maintained in additive rich medium, so we examined the pattern of *CHS* expression in callus. Another study on Cassava cell culture documented that PAL activity induced maximally under the influence of elicitors[41]. Importance of *CHS* in flavonoid accumulation was studied in citrus cell culture and it was observed that *CHS* expression was crucial in flavonoid synthesis[42]. The positive effect of additive supplementation in *CHS* gene expression resulted in enhanced synthesis of flavonoids in *P. ovata* callus. It was observed that the additives played a positive role in *CHS* expression in *Silybum marianum*[43]. In this present study, results showed external additives such as CH and CW were effective in enhancing CHS expression during callus culture. Real time PCR analysis of *CHS* in *Lamiophlomis rotata* showed that it is a elicitor responsive gene which can be up regulated under the influence of methyl jasmonate, UV light to produce higher amount of flavonoids[44]. Real time PCR analysis of CHS gene in *Silybum marianum* showed that highest transcript level achieved during flavonoid biosynthesis[45]. Study on etiolated *Sorghum bicolor* it was documented that enhanced anthocyanin production occurred under the influence of light by inducing dihydroflavonol 4-reductase[46]. Isolation, sequencing and characterization of *DFR* genes have been carried out in many plants[47.] In this paper, a novel *DFR* gene, exhibiting strong homology with previously reported *DFR*s, was partially sequenced. The real-time PCR analyses showed the expression increment of *DFR* with callus age and further increase in *DFR* expression was recorded in additive supplemented callus. Another study on additive supplemented callus culture of *Vitis vinifera* it was observed that anthocyanin production increased under the influence of additive treatment[48]. Synchronized transcriptional regulation of these genes was also documented[49]. A common pattern of transcription

regulation among *PAL, CHS* and *DFR* was observed n this study. In a study on grapevine cell culture it was concluded that external elicitors induced several key genes of phenylpropanoid pathway[50]. The factor which play pivotal role in perception of stress and induce specific genes of phenylpropanoid pathway is not yet clearly known. Few studies suggested that ROS and wounding might play important role in it [51]. In our study wounding during callogenesis and increased ROS accumulation with age of callus may be the important factors which enhance the transcription level of the major genes of phenylpropanoid pathway. In this study we also observed that there is a relation between age of callus and expression profile of these three genes. Apart from that both the additives (CW and CH) had positive impact in their expression. It can be concluded from the results of the real time data that among the three genes, expression of *DFR* was highly up regulated by the additive supplementation. *CHS* expression also increased but the increment in expression was comparatively low in case of *PAL* in additive supplemented callus. The results showed that the expression profile of these three key genes get altered with age of callus and additive supplementation showed positive impact in their expression. It suggests that the transcription regulation of these three genes- *PAL, CHS* and *DFR* is closely related and coordinated in *P. ovata*.

Thus results of this study would provide key insights into the genetic regulation of polyphenol production by *PAL, CHS and DFR* induction during *in vitro* callogenesis and elicitation using external additives. This would help us to adopt a new strategy for industrious production of polyphenols using *in vitro* callus culture.

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