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Enhancement of silymarin accumulation using precursor feeding in *Silybum marianum* hairy root cultures

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Abstract

This study focused on the effect of different concentrations of L- phenylalanine (0, 1, 10 and 100 µM) as the precursor, on the phenylalanine ammonia-lyase activity, naringenin content, root biomass and silymarin production in *Silybum marianum* hairy roots. Precursor was added to the medium of hairy roots on the 30th day of culture and the roots were harvested on 0, 24, 48, 72, 96 and 120h after feeding. Detection and identification of flavonolignans was carried out by high performance liquid chromatography method. Cultures supplemented with phenylalanine 10 µM increased the root biomass (0.43 g) 1.89 fold compared to control (0.23 g) 72h after feeding. Phenylalanine 100 µM after 72h was found optimal feeding condition for production of silymarin (1.84 fold higher than that of the control). However, the stimulatory effect of 100 µM phenylalanine could be attributed to the taxifoline (0.143 mg g^{-1} DW), silydianin (0.117 mg g^{-1} DW) and silybin (0.031 mg g^{-1} DW) contents were greatly rose up to 4.16, 2.44 and 1.58 fold greater than that of untreated cultures (0.03, 0.05 and 0.02 mg g^{-1} DW) after 72h, respectively. Furthermore, the content of naringenin, precursor of flavonolignans indicated the similar pattern at 100 µM concentration 72h after treatment which was 4.64 fold greater than untreated cultures (0.005 mg g^{-1} DW). All three concentrations of added phenylalanine stimulated phenylalanine ammonia-lyase activity suggest utilization of phenylalanine for the initial step of phenyl-propanoid metabolism. This is the first report on PHE feeding as the precursor for silymarin accumulation in *S. marianum* hairy root cultures.

Key words: Hairy root; Naringenin; Phenylalanine; *Silybum marianum;* Silymarin.
Abbreviations: DW- dry weight; ISB - isosilybin; NAA-L-naphthaleneacetic acid; PAL - phenylalanine ammonia-lyase; PHE-L-phenylalanine; SB- silybin; SC- silychristin; SD- silydianin; TXF- taxifolin.

Introduction

Silymarin, a mixture of seven distinct flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin; and one flavonoid taxifolin isolated from milk thistle (*Silybum marianum,* a member of the Asteraceae family) (Davis-Searles et al., 2005; Kim et al., 2003) . Meanwhile, this safe herbal product was used as the therapy for liver diseases, the anticancer activity and favorable effects on Hepatitis C virus (HCV) infection and alcoholic liver disease were proved for this metabolite as well (Agarwal et al., 2006; Singh and Agarwal 2006; Singh and Agarwal 2004; Bonifaz et al., 2009; Song et al., 2006). Genetically transformed hairy roots, caused by infection of *Agrobacterium rhizogenes,* suggest attractive properties for secondary metabolite production such as fast growth, greater biosynthetic capacity for secondary metabolites production (Banerjee et al., 1998; Kittipongpatana et al., 1998) and genetic stability in contrast with low yield levels of useful secondary metabolites synthesized by plant cell culture technology (Rokem and Goldberg, 1985; Charlwood and Charlwood, 1991). Accordingly, Rahnama et al. (2008) have described an efficient protocol for development of the hairy root culture of *S. marianum* and production of silymarin, by using *A. rhizogenes.* Since the tricyclic phenylbenzopyran structure of flavonoids biosynthetically derived from phenylalanine and malonyl-CoA in the phenyl-propanoid pathway (Matkowski, 2008), the precursor feeding strategy has been therefore found to effectively stimulate compound accumulation in hairy root cultures (Morgan and Shanks, 2000; Bohm and Mack, 2004). Although, the combination of substrate with the elicitation or stress treatment (permeabilization) was found an efficient strategy for compound production in *Taxus chinensis* and *Panax notoginseng* cell cultures and *Datura innoxia* Mill. hairy roots (Qian et. al. 2005; Wang et al. 2005; Boitel-Conti et al., 2000). However, flavonolignan production in response to precursor feeding has not been still investigated in hairy root culture of *S. mariaunm*. In this study, we aimed to report the silymarin production in hairy root cultures of S. *marianum* achieved through the PHE feeding.

Materials and methods

Hairy root culture

Hairy root culture of *S. marianum* used in this study was transformed by *Agrobacterium rhizogenes* (AR15834), and the genetic transformation of these hairy roots was confirmed by polymerase chain reaction (PCR) according to the method

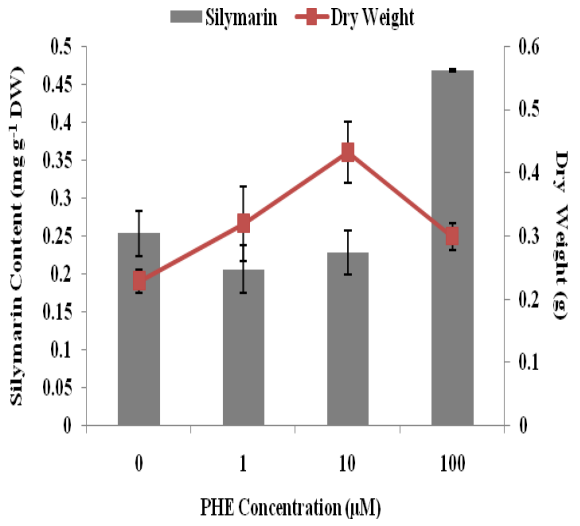

Fig 1. Effect of PHE concentrations (0, 1, 10 and 100 µM) on biomass and silymarin production in hairy root cultures of *S. marianum* 72h after treatment. The flavonolignans were analyzed with HPLC. Values are means of triplicate results and error bars show standard deviations.

described by Rahnama et al. (2008). All experiments were carried out in shake-flask cultures with 100-ml Erlenmeyer flasks on orbital shaker set at 150 rpm and incubated at 25º C in the dark. Each flask contained 50 ml liquid Murashige and Skoog (MS) medium supplemented with 30 g l^{-1} sucrose and 0.1 mg l^{-1} NAA. The medium was adjusted to pH 5.7 before autoclaving and inoculated with six 1 cm pieces of roots from 30-day old cultures (Murashige and Skoog, 1962).

Precursor preparation

A total of 165 mg of PHE was dissolved in distilled water and stock solution (10 ml) was prepared and sterilized (0.22 µM filter) for feeding. Different concentrations were made with culture media. Precursor, PHE was added to the media at 1, 10 and 100 µM concentration on 30th day and the hairy roots from flasks were harvested after 0, 24, 48, 72, 96, 120h of addition. Control cultures received equal amount of medium instead of precursor and harvested at the same time as experimental flasks. All experiments were replicated three times.

Extraction and determination of silymarin and naringenin

Hairy roots harvested from the shake-flasks and dried by tissue paper were measured in terms of fresh weight (FW). Lyophilized powdered hairy root samples were measured in terms of DW. The samples were defatted with petroleum ether. The flavonolignans were extracted from the dried residue with 10 ml of methanol at 40º C for 8h. The methanolic solution was concentrated to a dry residue. The extract was dissolved in 2 ml of methanol and kept at 4º C in darkness (Rahnama et al., 2008; Cacho et al., 1999). Silymarin were quantified by high performance liquid chromatography (HPLC) analysis as described by Hasanloo et al. (2005a, b).

Extraction and assay of PAL

PAL was extracted from dried hairy roots (0.1 g DW) with 6.5 ml of 50 mM pH 8.8 Tris-HCl buffer containing 15 mM of β-Mercaptoethanol in an ice cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 15,000 rpm for 30 min, and the supernatant was collected for enzyme assay. PAL activity determined based on the rate of cinnamic acid production as described by Ochoa-Alejo and Gomez-Peralta (1993). Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM PHE, 0.4 ml of double distilled water and 0.1 ml of enzyme extract incubated at 37º C for 1h. The reaction was finished by addition of 0.5 ml of 6 M HCl, and the product was extracted with 10 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the cinnamic acid concentration wherein was quantified with the absorbance measured in 290 nm. One unit of PAL activity is equal to 1 µM of cinnamic acid produced per minute.

Chemicals

Standards of silymarin, SB and TXF were purchased from Sigma-Aldrich; SC and SD were obtained from Phytolab (Humburg, Germany); Phenylalanine was from Sigma-Aldrich.

Statistical analysis

The data are given as the mean of at least three replicates. Statistical analysis was performed with SAS software (Version 6.2) using ANOVA method with Duncan test set at $\alpha' \leq 0.05$.

Results

Effects of different concentrations of PHE on root biomass and silymarin accumulation

Hairy root cultures (30-day old) were treated with four different concentrations (0, 1, 10 and 100 µM) of PHE (Fig. 1).
As it is shown, in comparison with non-treated culture (0.23 g), the highest dry biomass (0.43 g) was recorded when 10 µM PHE was added to the medium and then followed by plummeting at 100 µM (0.30 g) 72h after treatment. Whereas, it was seen that PHE supplementation at 100 µM concentration led to drastic surge in silymarin accumulation from 0.25 in control to 0.47 mg g^{-1} DW in hairy roots 72h after treatment. The contents of SB, ISB, SC, SD and TXF in these samples were 0.03, 0.02, 0.06, 0.12 and 0.14 mg g^{-1} DW, respectively, while in non-treated hairy roots the corresponding values were 0.02, 0.02, 0.07, 0.09 and 0.03 mg g^{-1} DW, respectively. However, the stimulatory effect of 100 µM PHE were noted where the TXF, SD and SB contents were greatly rose up to a 4.16, 2.43 and 1.58 fold, above control, after 72h, respectively (Table 1).

Effects of feeding time on root biomass and silymarin accumulation

The time-course of the effect of PHE on root growth and silymarin content are presented in Fig. 2. The dry weight of PHE-treated hairy roots was slightly risen after 24h (0.25 g)

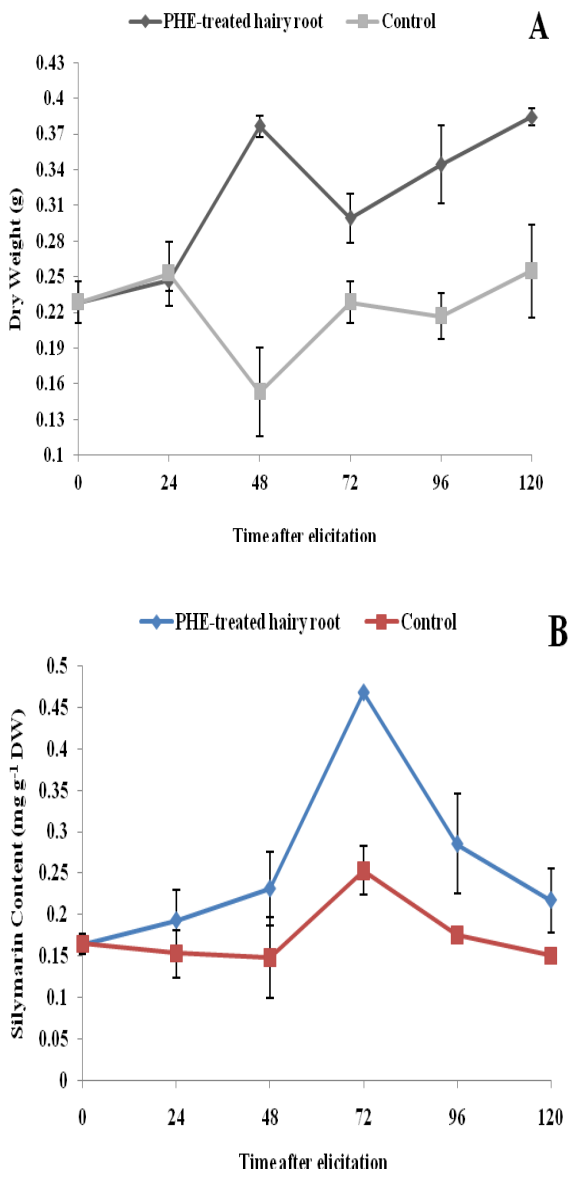

Fig. 2. Time courses of biomass (A) and silymarin production (B) in non-treated (control) and treated (100 µM PHE) hairy roots of *S. marianum*. The flavonolignans were analyzed with HPLC. Values are means of triplicate results and error bars show standard deviations

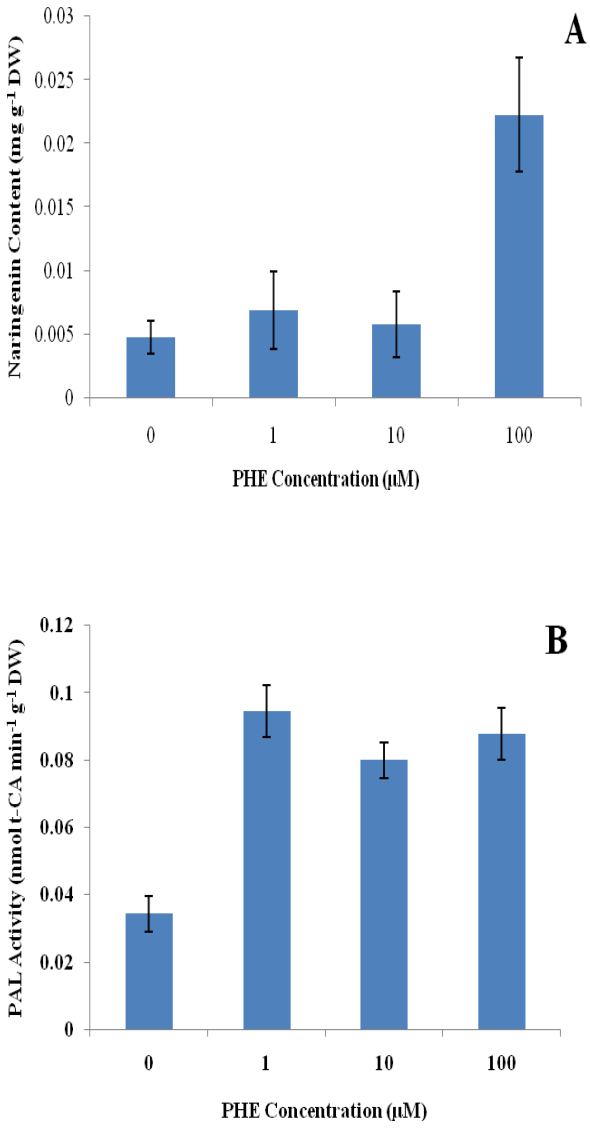

Fig. 3. Effect of PHE concentrations (0, 1, 10 and 100 µM) on naringenin content (mg g^{-1} DW) (A) and PAL activity (B) in hairy root cultures of *S. marianum* 72h after treatment. The naringenin were analyzed with HPLC. Values are means of triplicate results and error bars show standard deviations.

the same as control (0.25 g) and followed by shooting up to 0.38 g after 48h which was higher than that of the control (0.15 g) (Fig. 2). A significant drop in the dry weight of cultures was observed after 72h (0.30 g) and subsequently, enhanced after 96 and 120h (0.34 and 0.38 g, respectively). The DW of non-treated hairy roots was fairly steady after 72, 96h (0.23 and 0.22 g, respectively), before climbing after 120h (0.25 g), being below the treated hairy roots. Furthermore, there was a slight improvement in the production of silymarin after 24 and 48h (0.19 and 0.23 mg g^{-1} DW), 1.26 and 1.53 fold greater than that of the control at the same time (0.15 mg g^{-1} DW). The silymarin content in PHE-treated hairy roots was rocketing by 188% (0.47 mg g^{-1}DW) than that of the control (0.25 mg g^{-1}DW) after 72h and then plummeted to 0.29 and 0.22 mg g^{-1}DW 96 and 120h after treatments which were 1.61 and 1.47 fold greater than the corresponding controls (0.18 and 0.15 mg g^{-1}DW). The highest TXF, SD and SB accumulation were obtained after 72h (0.14, 0.12 and 0.03 mg g^{-1}DW) in PHE-treated media that proved 4.7, 1.3 and 1.5 fold increase than the untreated cultures (0.03, 0.09 and 0.02 mg g^{-1}DW, respectively; see Table 1). The gradual grow in the SCN and ISB content from 0.05 and 0.02 mg g^{-1}DW after 24h to 0.09 and 0.03 mg g^{-1} DW after 96h (1.5 and 3 fold higher than that of the control) was followed by a dramatic decline after 120h (0.05 and 0.02 mg g^{-1} DW, respectively).

Effects of different concentrations of PHE on naringenin production and PAL activity

PHE at 100 µM concentration has stimulatory effect on naringenin production 72h after treatment which was 4.64

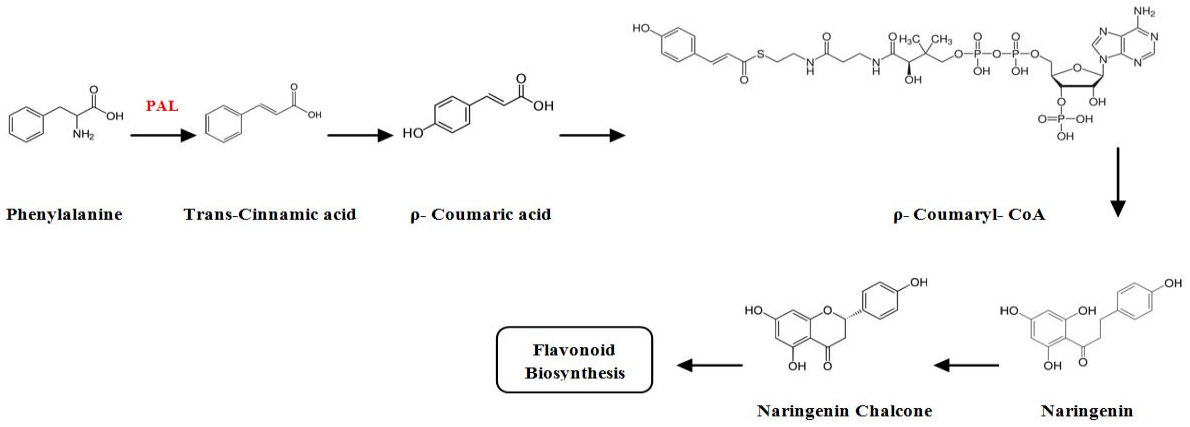

Fig. 4. Pathways of flavonoid formation in *S. marianum* hairy roots.

Table 1. Flavonolignans content (mg g^{-1} DW) in PHE-treated (100 µM) (A) and non-treated (control) hairy root cultures of *S. marianum* (B) for exposure time after feeding. The flavonolignans were analyzed with HPLC. Data shows means ± SD from triplicate experiments.

A

Exposure Time	Flavonolignan				
	TXF	SC	SDN	SB	ISB
0	0.034 ± 0.002	0.043 ± 0.002	0.048 ± 0.004	0.019 ± 0.002	0.021 ± 0.006
24	0.042 ± 0.004	0.049 ± 0.007	0.057 ± 0.006	0.018 ± 0.0005	0.016 ± 0.004
48	0.053 ± 0.006	0.058 ± 0.007	0.077 ± 0.007	0.022 ± 0.001	0.019 ± 0.003
72	0.143 ± 0.01	0.062 ± 0.003	0.117 ± 0.001	0.031 ± 0.002	0.021 ± 0.007
96	0.076 ± 0.016	0.089 ± 0.011	0.083 ± 0.016	0.027 ± 0.002	0.027 ± 0.006
120	0.039 ± 0.007	0.055 ± 0.002	0.079 ± 0.015	0.02 ± 0.003	0.015 ± 0.003

B

Exposure Time	Flavonolignan				
	TXF	SC	SDN	SB	ISB
0	0.034 ± 0.002	0.043 ± 0.002	0.048 ± 0.004	0.019 ± 0.002	0.021 ± 0.006
24	0.022 ± 0.001	0.044 ± 0.011	0.06 ± 0.015	0.014 ± 0.0007	0.014 ± 0.002
48	0.014 ± 0.003	0.041 ± 0.006	0.04 ± 0.0004	0.017 ± 0.006	0.012 ± 0.003
72	0.033 ± 0.008	0.075 ± 0.005	0.091 ± 0.005	0.02 ± 0.003	0.016 ± 0.004
96	0.024 ± 0.005	0.057 ± 0.007	0.069 ± 0.003	0.015 ± 0.002	0.012 ± 0.007
120	0.022 ± 0.003	0.048 ± 0.009	0.054 ± 0.008	0.015 ± 0.003	0.013 ± 0.002

fold greater than untreated cultures (0.005 mg g^{-1} DW). Naringenin content experienced two sharp rocketing by 741% and 464% more than that of the control (0.003 and 0.005 mg g^{-1} DW) after 24 and 72h, respectively (Fig. 3). The PAL activity was measured in different concentrations and harvesting times (Fig. 3) in order to determine whether this amino acid stimulated the first step of phenyl-propanoid metabolism (see Fig. 4). All PHE treatments at 1, 10 and 100 µM concentrations stimulated PAL activity after 72h, respectively. The PAL activity saw one peak on 72h (0.088 nmol t-CA min^{-1} g^{-1} DW) after adding 100 µM PHE while non-treated hairy roots of *S. marianum* were significantly plunged to 0.034 nmol t-CA min^{-1} g^{-1} DW.

Discussion

The application of PHE as the upstream biosynthetic precursor of phenyl-propanoid pathway could enhance secondary metabolite production in suspension cultures of strawberry and rudbeckia cells, *Taxus baccata*, *Taxus cuspidata*, *P. corylifolia* and *Ca. frutescens* (Edahiro et al., 2005; Luczkiewicz and Cisowski, 2001; Khosroushahi et al., 2006; Fett-Neto and DiCosmo, 1996; Shinde et al., 2009a; Lindsey and Yeoman, 1985). In this study, we investigated the effect of PHE feeding on hairy root growth and silymarin production in *S. marianum* cultures and therefore it was apparently expected this PHE supplementation accordingly has a beneficial effect on the level of target compound caused by increasing metabolic flux through phenyl-propanoid biosynthetic pathway. The addition of PHE led to improvement in rosmarinic acid yield in *Col. blumei* and *Salvia officinalis* suspension cell cultures as well (Ibrahim, 1987; Ellis and Towers, 1970). However, appropriate dosage of precursor and exposure time should be considered as the fundamental factors for successful feeding. As it was implied that PHE at 2 mM concentration increased the production of isoflavones in hairy root cultures of *Psoralea corylifolia* L. as compared with control while isoflavones levels greatly inhibited when 10 mM PHE was applied (Shinde et al., 2009b). Furthermore, in the same way, the addition of 1 mM PHE to the media resulted in an increase of paclitaxel content

and the greatest 10-DAB III accumulation in hairy root cultures of *Taxus x media* var. Hicksii, although, there were no taxanes in the hairy root cultures growing in the medium supplemented with 100 mM PHE (Syklowska-Baranek et al., 2009). Even though, various concentrations of PHE (1 mM for callus; 0.05, 0.1, and 0.2 mM for suspensions) in cell suspensions and callus culture of *Taxus cuspidata* produced the greatest increase in taxol accumulation (Fett-Neto et al., 1994). Moreover, Wielanek and Urbanek (2006) found that the biosynthesis of glucotropaeolin and gluconasturtiin relies on PHE in the process of stimulating glucotropaeolin production in *T. majus* hairy root cultures. Jie et al. (2005) reported that the production of phenylethanoid glycosides was enhanced by feeding precursors PHE at 0.2 mmol l^{-1} to cell culture of *Cistanche des er ticola* and the maximum dry biomass (6.2 g l^{-1}) was recorded when 1 mM of PHE was added to the medium. Results obtained here suggested that the cultures supplemented with 100 µM PHE after 72h was found optimal feeding condition for production of silymarin (1.84 fold higher than that of the control), without any significant influence on hairy root biomass, meanwhile, the highest dry biomass (0.43 g) was observed when 10 µM PHE was fed to the medium. However, the stimulatory effect of 100 µM PHE could be attributed to the TXF, SD and SB content were greatly rose up to 4.16, 2.43 and 1.58 fold greater than that of the untreated cultures after 72h, respectively. Furthermore, the naringenin content, flavonoid precursor indicated the similar pattern at 100 µM concentration 72h after treatment which was 4.64 fold greater than that of the untreated cultures (0.005 mg g^{-1} DW). In the other hand, all phenyl-propanoids originated from trans-cinnamic acid are synthesized from PHE by PAL. Kovacik et al. (2007) determined the stimulatory influence of L-PHE application in different concentrations on PAL activity (by 30, 76 and 90%, respectively) as well as accumulation of coumarin-related compounds in the chamomile (*Matricaria chamomilla*) leaf rosettes. The results obtained in this study indicated that all three concentrations of added PHE stimulated PAL activity and consequently supported the use of PHE as the substrate for the initial step of phenyl-propanoid metabolism which is in agreement with the affinity of PAL for PHE being known in dicotyledons (Jones and Hartley 1999; Cochrane et al. 2004; Ritter and Schulz 2004; Berner et al. 2006).

Conclusion

In this paper, silymarin and naringenin accumulation was strongly affected by PHE feeding. The greater PAL activity in various concentrations of PHE confirmed PAL affinity for the PHE as the upstream biosynthetic precursor of phenyl-propanoid pathway.

Acknowledgements

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