ISSN 2321-807X

Correlation between PAL, medicarpin, phenol and flavonoid content in *Medicago sativa* L. at different growth stages

*Zarinkamar Fatemeh, Asadi nastaran, Saeideh Soleimanpour

Faculty of biological Sciences, Tarbiat Modares University, Tehran, Iran

ABSTRACT

Alfalfa (*Medicago sativa* L.) is from Fabaceae family that has several flavonoid compound in roots and shoots. in alfalfa the major phytoalexin is medicarpin. In this study, total phenolic and flavonoid compounds and phenylalanin ammonia lyase **(**PAL) activity in different stages of development were measured. In this research the concentration of medicarpin by HPLC were studied in different stages of development. The lowest and highest level of the concentration of medicapin were in seedling stage and budding stage of growth, respectively. The results indicated that contents of total phenolic compounds and total flavonoid and PAL activity increase with developmental stage ,but decrease in flowering stage of growth.

Key words: *Medicago sativa* L; PAL activity; total phenol and total flavonoid content; Medicarpin; Stages of development.

Faculty of biological Sciences, Tarbiat Modares University. Tehran, Iran.

*E-mail: zarinkamar@modares.ac.ir

Council for Innovative Research

Peer Review Research Publishing System

Journal: Journal of Advances in Chemistry

Vol. 10, No. 7

editorjaconline@gmail.com

www.cirjac.com

ISSN 2321-807X

INTRODUCTION

Higer plants synthesized a wide variety of phenolic compounds during normal growth and development (8). Leguminous plants are a rich source of flavonoids that accumulate a wide range of phenolic secondary compound, including isoflavonoid conjugates (5). There has been intensive studies of the mammalian health promoting effects of isoflavonoids, such as prevention of several cancers, reduction of serum cholesterol, protection against lipoproteins oxidation, and prevention of bones loss (1) Isoflavonoids are particularly prevalent in the Papilonoideae subfamily of the Leguminosae (10). *Medicago sativ*a L. from Fabaceae family is a perennial herbaceous plant. It has several phenolic and flavonoid compounds in root and shoot (3). Flavonoids accumulate constitutively usually in the forms of glucosides and malonyl glucosides primarially in plant roots and then in shoots.

In alfalfa (*Medicago sativa* L.) the major phytoalexin is medicarpin (7,11). Medicarpin generally accumulate at relatively high concentration in the cell vacuole and are chemical associated with sugars. It is synthesized via the isoflavonoid branch of phenylpropanoid metabolism phenylalanin ammonia lyase (PAL) is the enzyme which introduces phenylalanine to the phenylpropanoid pathway is involved in the first step committed to falvonoid biosynthesis (10, 12). In the present study variation of total phenols and total flavonoids and relation between the changes of PAL activity, as a key enzyme in phenol production and phenylpropanoid production were investigated in different stages of growth. The aim was to determinate of the was a link PAL activiy and concentration medicarpin and development stages.

MATERIAL AND METHODS

Plant material

Medicago sativa L. cv. Hamedani was grown in plastic pots containing a mixture of soil: sand (1:1), under natural light condition at greenhouse. The plants were irrigated every day and maintained at day/night temperature of 27-30°C and18-20°C, respectively. Roots and shoots and flowers were harvested by hand at the seedling stage(4days), for the budding stage (20days) shoots and roots selected. At the flowering stage(140 days), shoots and roots with opend flowers were harvested. Their shoots and roots and flowers were separated and dried for medicarpin analysis, and some of them were frozen in liquid nitrogen and placed on -80°C freezer for biochemical analysis.

Total flavonoid assay

For measurement of total falvonoid content, 0.1gr root and shoot were ground in 3ml of acidified ethanol (99:1, ethanol: HCl) then samples were centrifuged at 12000 rpm for 20 min and the supernatant of each sample was gently boiled for 10 minutes in a water bath at 80°C. The absorbance was measured at 270, 300, 330 nm and the flavonid content was calculated using an extinction coefficient of 33000 M^{-1} cm^{-1} (4).

Total soluble phenolic compounds assay

The Folin-Ciocalteau reagent was used to estimate total phenolic content. The extract (60-300 µl) was diluted with deionized water to 4.8 ml, and 300 µl of Folin-Ciocalteau reagent was added and shaken. After 8 min 900 µl of 20% sodium carbonate solution was added with mixing. The solution was left at 4.0°C for 30 min before reading the absorbance at 765nm. Gallic acid (0-50 µg) was used as standard, and the result were reported as mg gallic asid equivalent per gram of fresh weight (6).

PAL activity assay

Phenylalanine ammonia-lyase (PAL) was extracted from 0.1 g of roots, shoots and flowers with 6.5 ml of 50 mM Tris-HCl buffer (pH=8.8) containing 15 mM of β- mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 50,000 g for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate of cinnamic acid production, according to Wang *et al*. (2006). Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of double distilled water and 0.1 ml of enzyme extract were incubated at 37°C for 1 h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl, and the product was extracted with 15 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 was quantified with the absorbance measured at 280 nm. One unit of enzyme activity was defined as the amount of PAL that produced 1 µmol of cinnamic acid within 1 min and was expressed as µmol of cinnamic acid mg/protein/min. Total protein was assayed with bovine serum albumin as the standard using the dye-binding method of Bradford (1976).

Preparation of samples solution

All samples (0.1gr of roots, shoots and flower) were accurately weighed and extracted with 5ml 80% methanol by refluxing at 80°C for 3 hours, samples were centrifuged at 4000 rpm for 10 min and the supernatants were filtered through nylon membrane filters (0.45µm) concentrated by evaporator. Then, each sample was added 3ml 80% methanol, then 20µl of sample were injected into the HPLC (High Performance liquid chromatography) column for analysis.

HPLC instrumentation

HPLC was conducted by a knauer system. A column of Lichrosorb RP 18, 5/0 µM 4.6×250 mm was used. Detection wavelength was set at UV 283 nm. Flow rate was set 1/0 ml min^{-1}.The mobile phase is the mixture of acetonitrile and

phosphoric acid used in the linear gradient elution are shown in Table 1. Isoflavonoid medicarpin quantification was based on a calibration curve, plotting peak area as monitored at 283nm against khown concentrations of medicarpin (10).

Table 1. Gradiant elution program using mobile phase A and B flow rate 1.0 ml/min

Time(min)	A%	B%
5	5	0
10	5	10
25	10	17
30	17	23
65	23	50
69	50	100

A: acetonitril

B: phosphoric acid

Satistical analysis

Three replicates of three growth stage samples were used for Statistical analysis Statistical analysis was carried out using execel. Data were subjected to analysis of variance (ANOVA), and then means were compared by Duncan's method in MSTATC program ver. 2.1. Differences at $P<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Changes in PAL activity and flavonoid levels and total phenols during development

Medicago sativa L .has phenolic and flavonoid compounds in roots and shoots . Total phenolic and flavonoid compounds and have been studied in different of stages of development in roots and shoots and flowers. Also in this study activity of PAL enzyme was investigated in *Medicago sativa* L. Total phenol contents of plant tissues varied with during development. The changes in activity of PAL in during development were compared with cganges in total flavonoids and total phenols. Phenol content in shoots of alfalfa increased in during development and reached the highest level at budding stage. The results for roots are also similar. Stem had higher phenol (51.3 mg/g FW) content when compared to other tissues. The lowest levels of phenols in root detected in the seedling stage whereas the highest content was detected during the budding stage. There was a correlation between PAL activity and total flavonoid concentration. Since PAL is also involve in the biosynthesis a range of other secondary products and only part of its activity may be available for flavonoid biosynthesis. Better correlation may have been observed between PAL activity and total phenol (1). PAL may be a key factor controlling the channeling of phenylalanine into phenolic synthesis and hence flavonoid biosynthesis. At all of development the level of PAL activity would have allowed the production of much higher levels of flavonoids than actually accumulation. Total flavonoid concentration in alfalfa aerial part was rather high as compared to other plant tissues (9). The observed decrease in PAL activity, with the concentration of flavonoids activity dropping. the variation of total phenolic and flavonoid compounds content in the alfalfa depend on many factors. It decrease in the stage of flowering. These differences can be attributed to the different stage of plant metabolism. Total flavonoid and phenolic compounds concentrated in the green parts and lower in the white parts. Analysis of PAL activity there was a wide variation in the level of PAL activity at different growth stages. PAL activity was highest in budding stage in root (0.31 µmol cinamic acid /mg/pr) dropped to low levels during seedling stage (0.09 µmol cinamic acid /mg/pr). There was coordination induction of flavonoid biosynthetic their enzymes, including PAL, during developmental regulation of biosynthesis. The reults for total flavonoid contant and total phenolic and PAL actvity in studied samples are presented in Fig 1, Fig.2 and Fig3.

ISSN 2321-807X

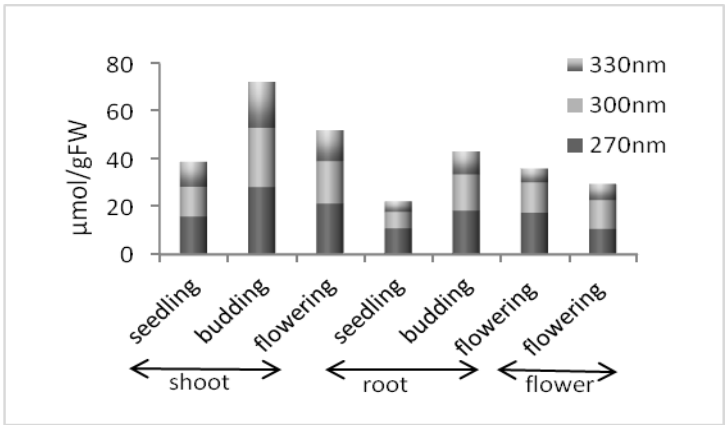

Fig .1: Total flavonoid levels at different developmental stages

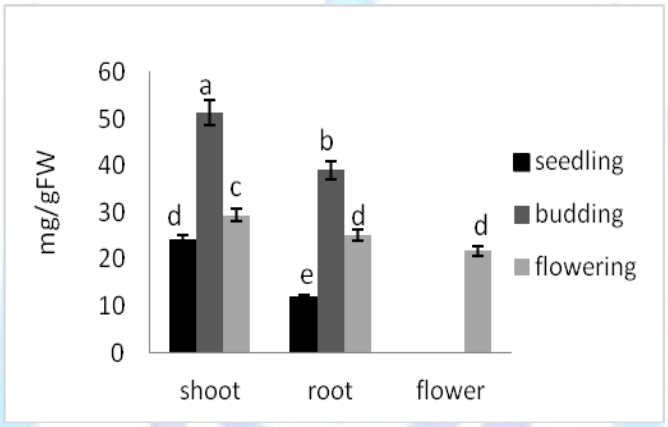

Fig.2: Total soluble phenolic compounds content at different developmental stages.

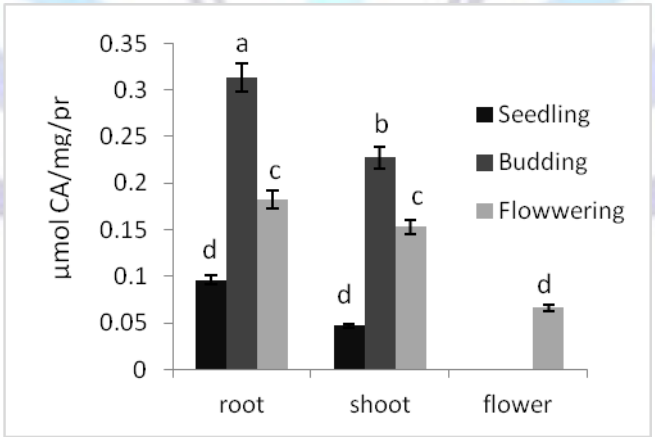

Fig .3: Phenylalanine ammonia lyase (PAL) activity determined in various stage of development.

ISSN 2321-807X

Separation of the medicarpin by HPLC

HPLC chromatogram of the ethanol extract in the alfalfa indicated the medicarpin standard were well separated, with retention time of 60 min. in order to determine the concentration of medicarpin, samples were separate into roots and shoots and the methanolic extracts from each part were analyzed (Fig.4).

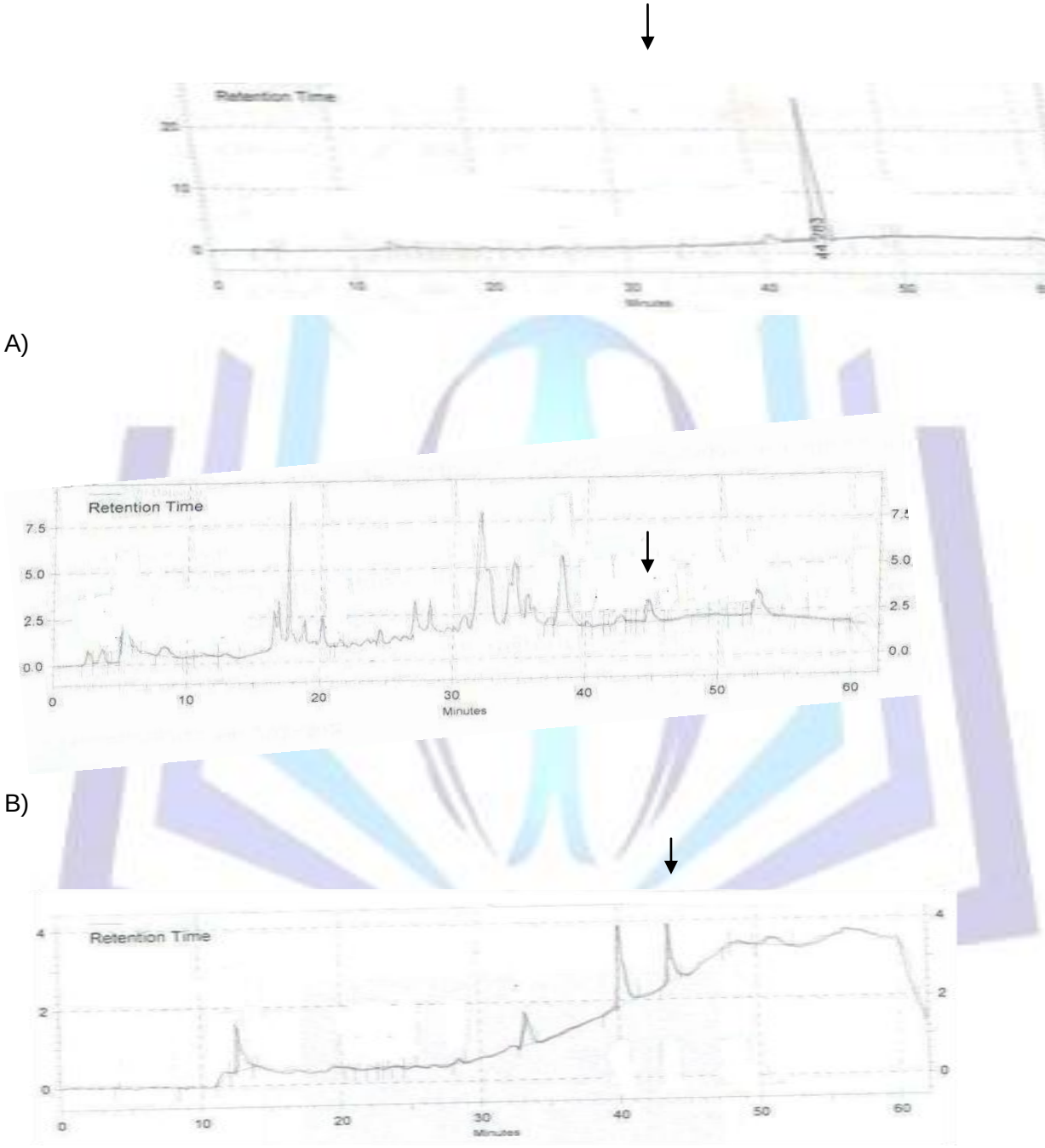

Fig .4: HPLC Chromatogram of extract from alfalfa A), extract from standard solution of medicarpin B), medicarpin in root C), medicarpin in shoot.

ISSN 2321-807X

Analysis of medicarpin

The isoflavonoid medicarpin in alfalfa have been studied to date in roots and shoots of 140 day old. In the present study we show that medicarpin is in roots and shoots in alfalfa, where it accumulates in a developmental manner. PAL which direct phe to the phenylpropanoid pathway, present in three in roots and shoots and flowers. PAL activity of roots increase stages of development in parallel to the increase in accumulation medicarpin, there was a significant correlation between PAL and medicarpin formation. The highest activities were observed in roots in budding stage (7.1 nmol g^{-1} FW) coinciding to the highest medicarpin accumulation. In shoots from 4-old-day seedling alfalfa medicarpin concentration increased during seedling growth, however its concentration was

considerably low (0.6 nmol g^{-1} FW) compared to that of the roots, while no accumulation of medicarpin detected in flowers. HPLC analysis of 20 day old samples showed the presence of medicarpin in shoots and roots, while amounts of medicarpin found in roots. Chromatography of the methanolic 140 day old plants showed the no medicarpin were present in flower, while shoots and roots had insignificant decrease in medicarpin concentration in stage of flowering at compare to medicarpin concentration in stage of budding. Results showed that significant difference in medicarpin concentration were observed among various plant tissues. Our observation showed that medicarpin accumulation was correlated with PAL activity. The result for PAL activity and medicarpin concentration in the studied samples presented in Table 2.

Table 2. PAL activity and medicapin concentration in *M. sativa* roots and shoots in stages of growth.

	PAL activity µmol CA mg^{-1} pr^{-1}	Medicarpin concentration nmol g^{-1} FW
Roots		
root- 4 days	0.09 ± 0^{d}	2.1 ± 0.3^{c}
root-20 days	0.31 ± 0.04^{a}	7.1 ± 4.0^{a}
root-140 days	0.18 ± 0.01^{c}	3.2 ± 2.0^{b}
Shoots		
shoot-7days	0.04 ± 0.09^{d}	0.1 ± 0^{e}
shoot-20days	0.22 ± 0.01^{b}	1.0 ± 0.3^{d}
Shoot-140days	0.15 ± 0.01^{c}	0.8 ± 0^{e}
Flower	0.066 ± 0.03^{d}	0 ± 0^{e}

Values are the mean± S.D. OF three experiments different letters signify significance (at least)

CONCLUSIONS

A positive relation between PAL and total phenol content. Maximum phenolic compounds was of the budding phase, while it decrease at flowering stage. The results obtained suggest that budding stage could be considered as the best stage for the harvesting of this plant. We found that the roots had the highest concentration of medicarpin in stsge of budding in compered with of all tissues plant tested. This isoflavonoid of alfalfa may have medicinal values for use in the future.

ISSN 2321-807X

References

1-Akoi, T., Akashi, T., Ayabe, Sh, 2000. Flavonoid of Leguminous Plant: Structure, biological activity and biosynthesis. Journal of Plant Research. 113:475-488.

2-Bradford, M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein dye binding. Analytical Biochemistry. 72: 248-254.

3- Dixon, RA., Choundhary, AD., Dalkin, K., Edwards, R., 1992. Molecular biology of stress induced phenylpropanoid and isoflavonoid biosynthesis in alfalfa. Phenolic Metabolism in Plants. 26: 91-138.

4-Ghanati, F; F. Khatami; Effect of UV irradation on cell viability, anthocyanin and falconoid contents of callus-cultured Malva neglecta analysis. Agrobios, New Dehli, India. Pp:438.

5-Gagnon, H; Tahara S.L; Ibrahim, R.K. 1995. Biosynthesis, accumulation and secration of isoflavonoids during germination and development of white lupn (Lupinus albus L.) Plant Phisiology. 106:195-202

6-Marinova, D., Ribarova, F., Atanassova, M; 2005. Total phenolic and total flavonoid in ulgrian fruits and vegetables. J. Univ. chemistry Technology Metal. 40:25-260.

7-Matsouka, I., Beri, D., Chinou, I., Haralampidis, K., Spyropoulos, C, 2011. Metals and selenium induce medicarpin accumulation and execration from the roots of fenugreek seedling: a potetial detoxification mechanism. Plant soil, Published online.

8-Stalikas, C; 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. Plant Biology Plant Pathology. 61: 89-101.

9-Stochmal, A; Oleszek, W. 2007. Seasonal and strucural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. Journal of food. Agriculture Environment. 5: 160-174.

10-Tsiri, D., Chinou I., Haralampidis, k., Halabalaki, M; Spyropoulos, C, 2009. The origin of copper induced medicarpin secretion from roots of young fenugreek seedling are regulated by copper concentration. Plant Sciense, 176:367-374.

11-U.Mackenbrock, W. Gunia, W. BARZ. 1993. Accumulation and metabolism of medicarpin and maackiain malonylglucosides in elicited chickpea (Cicer arietinum L.) cell suspension cultures. Plant Physiology. 142: 385-391.

12-Winkel-Shirely, B; 2001. Flavonoid Biosynthesis. A colorful model for Genetics, Cell Biology, and Biotechnology. Plant Physiology. 126: 485-493.

13 -Wang . J; Zheng L. P; Wu, J.Y and Tan, X. 2006. Involvment of nitric oxide in oxidative st Phenyalanin ammonia lyase activating and taxol prodaction induced by low-energy ultrasound in taxus yunnanensis cell suspension cultures. Nitric Oxid Biology and Chemistry. 15:351-358.