Plant Archives Vol. 19 No. 2, 2019 pp. 4149-4154 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF PHENYLALNINE CONCENTRATION ON ROSMARINIC AND SALICYLIC ACID IN THE CALLUS CULTURE OF *BORAGO OFFICINALIS* L.

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Abstract

This study performed to produce *in vitro* Rosmarinic (RA) and Salicylic acid (SA) via callus induction from explant petiole of *Borago officinalis* L., callus induction was carried out on MS medium supplement with different concentration of Naphthallen acetic acid (NAA) with 2.0 mg. L^{-1} of Benzyl adenine (BA). The results showed the highest fresh weight reached was 32.4 g at 2.0 mg. L^{-1} of NAA with 2.0 mg. L^{-1} of BA and that the MS medium supplement with 0.5 mg. L^{-1} of Thidiazuron (TDZ) and 1.0 mg.L^{1} of NAA, was the best medium to callus maintenance. The effects of phenylalanine (phe) at the concentrations 20, 40 and 60 mg.L^{-1} subsequently on the accumulation of RA and SA was investigated. The chemistry identification of RA using HPLC technique showed that the natural rate of RA in the leaves at the flowering stage was 63.21% with the concentration 77.12 µg. ml the rate of accumulation of RA reached was 66.23% with the concentration of 249.98 µg. ml at 20 mg.L^{-1} of Phe. The chemistry identification for SA using HPLC that the natural rate of SA in the leaves at the flowering stage was 36.67% with the concentration 44.88 µg. ml. the rate of SA reached 46.19% with the concentration of 132.04 µg.mL^{-1} at 60 mg.L^{-1} of Phe.

Key words : Salicylic acid, *Borago officinalis,* Rosmarinic

Introduction

Borago officinalis is annual herbaceous plants belong to Boraginaceae family, its cultivated for several medicinal uses because of his contain of secondary metabolites such mucilages, alkaloids, tanins, saponins, essential oil, calcium, potassium (Gupta & Singh, 2010), might been the most importantfaced the cultivated of borage is difficult germination of their seeds and low resistance to environment (sajirani *et al.,* 2011).

Rosmarinic acid (RA) is on of important natural antioxidant compounds that spread in Braginaceae and Laminaceae, it get attentions because its biological activities include anti-microbial, anti-viral, anti-allergic, anti-inflammtory (Xu *et al.,* 2008).

Salicylic acid (SA) its phenolic compound that act on regulation growth and development process in plant, and have activity against pathogens, it forms mean of defense for plant, also effect on stomatamovement, ethylene biosynthesis and photosynthesis by effecting on leaves, stracture of chloroplaste and another photo pigment, also it used as elicitor in plant tissue culture (Babel *et al.,* 2014).

Plant tissue culture have been used to produced secondary metabolites which produce accendently from metabolic pathways such as carbohydrates, amio acids and fats, they don't have specific function, these compound found in plant which it consider the main source for these medicinal compounds and to study the undiscovered compound until know to development pharmaceutical compounds (Chattopadhyay *et al.,* 2002 & Vanisree *et al*., 2004 & Sarin, 2005). Some studies show theaddition of precursors such Phenylalanine can accumulation secondary metabolites (Elshennawy *et al.,* 2017), Phenylalanie stimulate the production of phenolic acids and flavonoids (Masoumian *et al.,* 2011).

Study of Masoumian *et al.,* (2011) refers to was 3mg. mL^{-1} of phenylalanine enhance the production offlavonoids from *in vitro* cultured of *Hydrocotylebonariensi*s, while refers Goswami & Mathur (2012) in their study that MS

medium supplement with 75 mg. mL^{-1} gave the highest stimulation for the flavonoids, study of Balvanyos *et al.,* (2002) showed the Con. 66 $mg.L^{-1}$ of pheinduced the increasing of lobelin alkaloid in the culture tissue of *Lobelia inflate* L.

Materials and Methods

Plant part sterilization and callus induction

Petiole of plant leaves has been used in the callus induction experipment, explant sterilization with wash solution for 5 min, then left under running water for 15 min, after this washing with ethyl alcohol 70% for one min then washing with sterile water to remove alcohole effect from the plant parts, after that soaked in sodium hypochlorite solution 10% for 20 min, after that washing with sterile water for three times each one take 5 min.

The plant part was transferred to the MS medium supplement with different concentrations 0.5, 1.0, 1.5, 2.0 $mg.ml^{-1}$ of Naphthalen acetic acid (NAA) wih 2.0 $mg.ml^{-1}$ of Benzyl adenine (BA). The culture incubated in the growth chamber and temperature conditions were 25 ± 2ºC with a continuous light system of 16 hours of light/8 hours of darkness, After 30 days of culture plant parts, recoerded callus characteristics,

Test the effect of concentrations of phenylalinine on the callus content of rosmarinic acid and salicylic acid

To study the effect of different concentrations of phenylaline 20, 40 and 60 $mg.L^{-1}$ on the callus content of rosmarinic acid and salicylic acid, pieces of callus about 1g in weight transferred to MS medium supplement by 0.5 $mg.L^{-1}$ of TDZ with 1.0 of NAA, the culture kept in growth chamber for 30 days, and then the callus collected and drying, kept in plastics tube in drying place until process extraction.

Sample extraction

Aqueos extract of leaves were prepared by dissolving 2g of leaves and 0.5g of callus powder in 20 ml HPLC methanol, the sample shaking and agitated in ultrasonic bath for 10min, then concentrated by evaporating the solvent with a stream of liquid N_2 until reach nearly 0.5 ml, then add some of mobile phase (deionized water acidified with 0.1% phosphoric acid, methanol, 2-propanol) to reach 1ml. The mixture were passed through 2.5 µm then 20µl were injected on HPLC column. The concentration for each compound were quantitatively determined by comparison the peak area of the standard with that of the sample. The analysis (Table 1) of the rosmarinic and salicylic acid of the studied samples was carried out based on the standard models by dissolving 25 µg of rosemarycin and salicylic acid in 1 mL of methanol.

Calculation

Concentration of Sample (µg/ml) =

$$\frac{area\ of\ sample \quad conc.of\ standard \quad dilution\ factor}{area\ of\ standard}$$

Table 1: Condition of separated by HPLC

The condition	Rosmarinic and Salicylic aid
Mobile phase	deionized water acidified with 0.1% phosphoric acid methanol, 2-propanol
Flow rate	1.2 ml per minute
Volum of injected sample	20 µ L
Separated temperture	30 Cº
Type of detection	Ultra violate at 230 nm
Reference	Zhang *et al.* 2012

Induction and maintenance of callus

The results (Table 2) for the callus induction from the petiole from *B. officinalis* showed significant differences, in the callus response to the MS medium, the fresh weight of the callus increased by increasing the concentration of NAA, the highest weight was 32.4 gat2 mg. L^{-1} of NAA + 2 mg of BA, While it weight were 12.58, 22.92 and 25.86 gon a medium supported by 0.5, 1.0 and 1.5 mg. L^{-1}. of NAA + 2 mg. L^{-1} from BA respectively, Compared with the control treatment, which

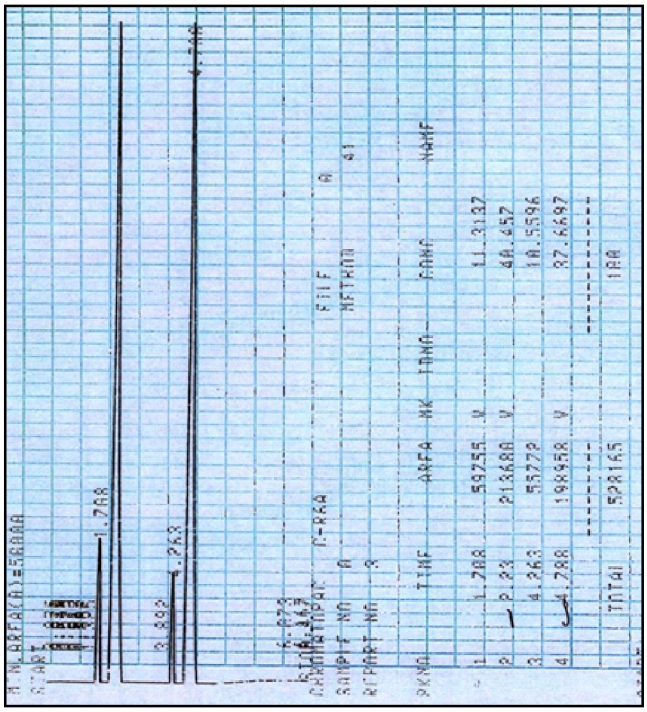

Fig. 1: Rosmarinic acid and salicylic acid curvesof the standard sample by HPLC

showed no response to the callus induction, the callus is characterizes by its green color and compact.

Table 2: Effect of different concentrations of NAA with 2.0 mg. L^{-1} of BA in the induction of the callus of the petiole of the leaves of *Borago officinalis*.

Concentrations of NAA mg. L^{-1} + 2.0 mg. L^{-1} BA	Induction%	Rate of fresh weight
0.0	0.0%	0.0 d
0.5	100%	12.58 c
1.0	100%	22.92 b
1.5	100%	25.86 b
2.0	100%	32.4 a

Identification and quantitative estimation of rosmarinic and salicylic acid by using HPLCE

The results showed (Table 3) that the extract of the field leaves samples contains rosmarinic acid 63.21% with a Conc. of 77.12 µg. ml and salicylic acid with 36.67% and 44.88 µg conc.µ.ml (Fig. 2) by comparison the retention time of rosmrinic acid 4.82 compared with the retention time of the standard sample 4.78 and retention time of salicylic acid 2.25 compared to the retention time of the standard sample 2.23.

Effect of phenylalanine onrosmarinic and salicylic acid

Results (Table 3) showed a gradual decrease in the percentage of rosmarinic acid by increasing phenylalin in concentrations, the highest ratio of rosmarinic acid was 66.23% with concentration 249.98 µg.ml. at 20 mg. L^{-1} of the phenylalnine (Fig. 3). While it was 62.08% and 53.80% at 160.81 and 153.78 µg. ml at 40 and 60 mg. L^{-1} of phenylalnin (Fig. 4 and 5).

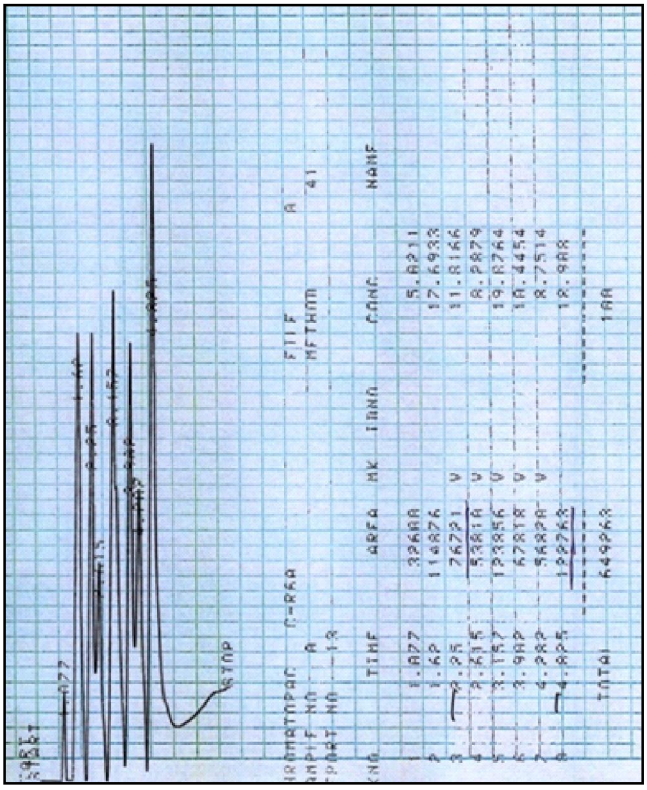

Fig. 2. Rosemarync acid and salicylic acid extracted from the field leaves *B. officinalis* by HPLC

Results (Table 4) showed increase of salicylic acid concentration with an increasing in phenylalanine concentrations, ratio of SA was 46.19% with a concentration 132.04 µg.ml at 60mg. L^{-1} of phenylalenine at (Fig. 5), While at the concentration 20 and 40 mg. L^{-1} of phenylalnine the ratio were 33.76% and 37.92% at 127.41 and 98.21µg. ml (Fig. 3 and 4), respectively.

Table 3: Retention time of isolate the rosmarinic acid and its percentage in the sample of leaves and the tissue culture of *B. officinalis* callus culture grown in different Con. Phe.

The sample		Retention time (Minute)	Curve area	Rosmrinic acid conc. (µg. ml)	Rosmrinic acid ratio
Leaves	4.82	122768	77.12	63.21%	
Phemg.	20	4.83	397888	249.98	66.23%
L^{-1}	40	4.82	255969	160.81	62.08%
	60	4.97	244769	153.78	53.80%

Table 4: Retention time of isolate the salicylic acid and its percentage in the sample of leaves and the tissue culture of *B. officinalis* callus culture grown in different Con. Phe.

The sample		Retention time (Minute)	Curve area	Rosmrinic acid conc. (µg. ml)	Rosmrinic acid ratio
Leaves		2.25	76721	44.88	36.67%
Phemg.	20	2.24	217811	127.41	33.76%
L^{-1}	40	2.25	167896	98.18	37.91%
	60	2.29	225788	132.04	46.19%

Discussion

The results of the callus induction from petiole of the leaves of the borage plant on a medium supported by concentrations of NAA overlapping with BA (Table 2), show the highest rate of callus weight recorded at the concentration of 2 mg.L^{-1} for both NAA and BA, The results of the experiment are consistent with the findings Mehrabani *et al.,* (2005) When callus Echiumamoenum was induced, the fresh weight of the callus was increased by increasing the concentrations of NAA, And agree with what has reached Almohammed Maher *et al.,* (2014) were

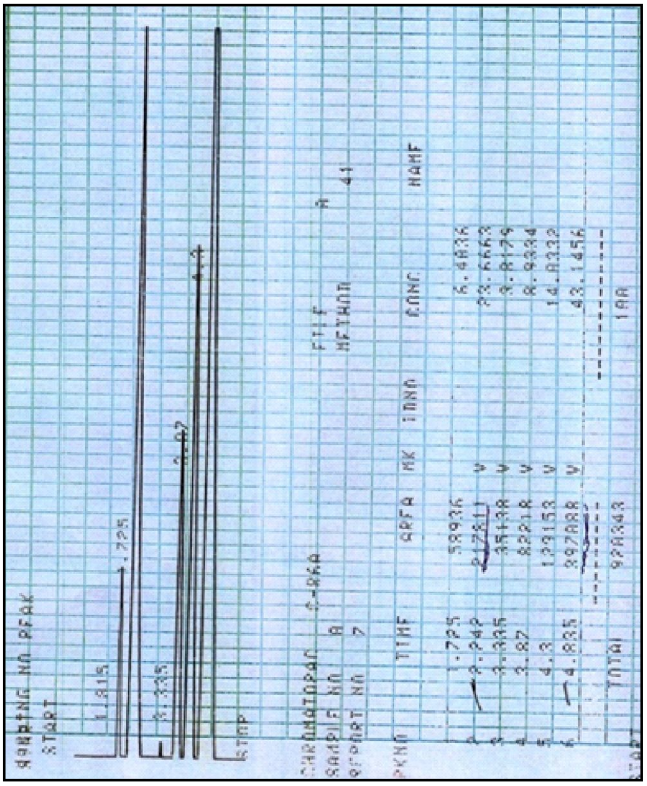

Fig. 3. Rosmarinic acid and Salicylic acid curve in the cullus culture of the leaves of*B. officinalis* treated with a concentration of 20 mg. L^{-1}phenylalenine by HPLC

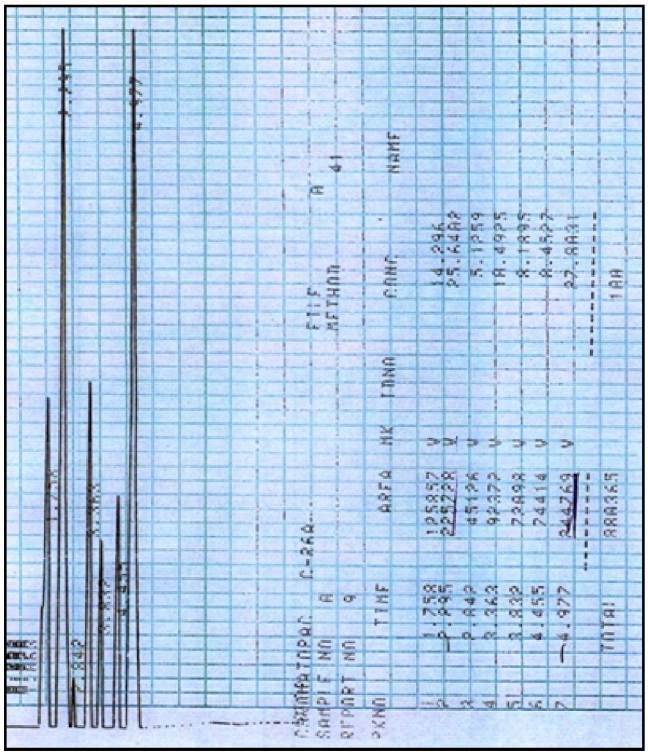

Fig. 4. Rosmarinic acid and Salicylic acid curve in the cullus culture of the leaves of*B. officinalis*treated with a concentration of 40 mg. L^{-1}phenylalenine by HPLC

a combination of (2mg/L) from each of BA and IAA

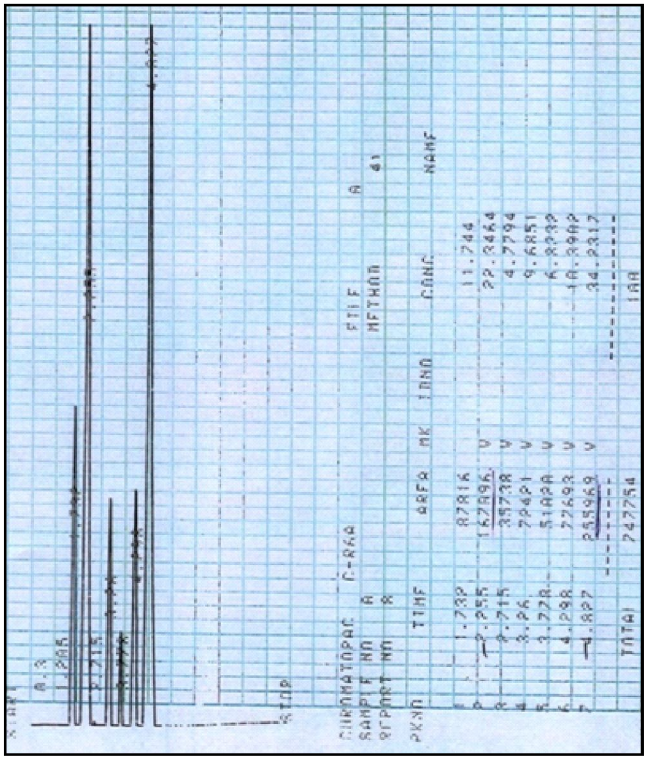

Fig. 5. Rosmarinic acid and Salicylic acid curve in the cullus culture of the leaves of*B. officinalis*treated with a concentration of 60 mg. L^{-1}phenylalenine by HPLC

gave high callus fresh weight for shoot tips, cotyledon leaves and hypocotyls, succeeded Abdollahi *et al.,* (2017) in the induction of callus from the pollen of the borage plant as it was the highest response to the callus at the concentration of 2mg.L^{-1} of Picloram interfered with 2mg.L^{-1} of 2, 4-D, the results indicated that the fresh weight of the induced callus was increased with the increase of NAA concentrations interfering with BA this is attributed to what referred Aghaei *et al.,* (2013) The low concentrations of NAA and BA have a low susceptibility to callus induction Compared to the high concentrations of NAA and BA which give high growth of calcium and also works to regulate the internal growth hormones of oxytin and other cytokines.

Results of the study indicated that medium 0.5mg.L^{-1} of TDZ overlapped with 1.0mg.L^{-1} of NAA is the most suitable medium for sustaining callus and maintaining the growth of plantations such as the borage plant This response is due to the fact that TDZ is classified as a synthetic cytokinein because of its similarity with natural cytokines in terms of its function which stimulates a number of biological processes in the plant and differs from it in its potential to stimulate the internal oxy-toxin and increase its effectiveness, especially in the dicotyle, as it works to make the necessary changes in the cell wall to prepare for the process of division as well as

increased absorption of nutrients and the process of metabolites when added to the medium of the plant in the process in vitro, its activity in this area is due to its low concentrations and weak absorption of callus, as well as positively affect the metabolism of purines and thus improves the storage of cytokines in their effective form and also reduces the process of catabolism (Guo *et al.,* 2011). Also study of Mok & Mok, (1985) when tracking the tagged molecules of the TDZ in Callus Phaseoluslunatus these molecules do not enter the metabolism only after 48 hours of presence in the medium and after the production of glucose residue, in other words, the TDZ avoids the enzyme cytokinein oxidase peroxidase of cytokines, also this enzyme is inhibited by Diphenyllurea compound which found in the structure of TDZ, uxin plant tissues also helps to improve the response of these tissues and increases cell division when exposed to TDZ, also TDZ increases the efficiency of second-carrier molecules such as the AMP cycle and the GTP cycle and increases cell division of treated Callus with it (Galuzka *et al.,* 2000, Jones *et al.,* 2007 (TDZ is a cytokinin that has the potential to support the persistence of the callus growth as well as its differentiation for plants that exhibit difficulty in growth and differentiation especially dicotyle (Lakshmanan & Taji, 2000).

The results of the study showed (Table 3) the low level of rosemarynic acid in the plantations of callus borage plant with increased concentrations of amino acid phenylalanine, this is what confirmed Hakkim *et al.,* (2011) the content of rosmarinic decreased in the cell suspension of the *Ocimum sanctum* after 18 days of culture, the maximum concentration was reached at the lowest concentration of phenylalanine 0.25g.L^{-1}, the treatment with phenylenin increased the concentration of salicylic acid (Table 4) the presence of phenylalnine with high concentrations increases the effectiveness of Phenylalanine Amino Lyase PAL, which is a key to activating the Shikimic path and the Phenylpropanoid pathway which produce phenolic compounds, PAL stimulates in plant tissues to treat wounds and defend against pests, as well as withstand against biotic and abiotic stress (Yan *et al.,* 2006), this explains the high concentration of salicylic acid in callus culture treated with concentrations of phenylalnine, whereas previous research has confirmed that the presence of amino acid triggers stimulates the building of secondary metabolites, these may be the mediatory or primary molecules of building secondary metabolites) Roy & Mukhopadhyay, 2012).

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