Influence of Medium pH, Seasonal Variation and Subculture on *In Vitro* Production of Rosmarinic Acid in Four Lamiaceae Members

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ABSTRACT

Medicinal herbs are important sources of natural antioxidants. Conditions favoring oxidative stress such as drought, temperature, pH or light stresses cause the formation of high oxidized diterpenes. Rosmarinic acid (RA) is one of the most commonly occurring diterpenes and it is widely spread in family Lamiaceae. Since the influence of pH on the antioxidant activity is seldom taken into account, the main objective of this study was to investigate the influence of four different pH levels on RA accumulation in callus culture of four lamiaceae members through three successive subcultures, also the effect of seasonal variation on RA accumulation *in vivo* and *in vitro*. It was found that the highest RA accumulation in leaf tissues and in callus culture occurred during the summer and the second subculture in all four species, also it was found that the pH has a significant influence on RA accumulation in callus culture of the four species, the best pH was 6.8 in all species except in sage callus culture where the RA concentration increased significantly at pH 5.8. The highest species in RA concentration in field production was the sage followed by the rosemary, thyme and oregano and was (0.048, 0.045, 0.035 and 0.026 mg RA/gm fw) respectively, while *in vitro* culture the highest RA accumulation occurred during the second subculture in the summer at pH 6.8 in Rosemary, Thyme and Oregano (0.086, 0.073, 0.069 mg RA/gm fw) respectively and the least accumulation occurred in sage in the second subculture during the summer at pH 5.8 (0.062 mg RA/gm fw).

Key words: Rosmarinic acid, pH, callus culture, seasonal variation, Lamiaceae.

INTRODUCTION

Herbs and aromatic plants are important sources of natural antioxidants (Minnunni *et al.*, 1992). The diterpenes (rosmarinic acid and carnosic acid) are very strong antioxidants produced in the chloroplast *via* a non-mevalonate isopentenyl diphosphate pathway, used to protect plant tissues against free radicals and lipid peroxidation caused by stress (McGarvey and Croteau, 1995).

The most important diterpenes are carnosic acid, carnosol, 12-O-methylcarnosic acid, rosmarinic acid, iosrosmanol and 11, 12-di-O-methylisorosmanol. (Munne-Bosch *et al.*, 1999).

The conditions favoring the oxidative stress such as drought, temperature, pH or light stresses causes the formation of high oxidized diterpenes such as rosmarinic acid, isorosmanol and 11,12-di-O-methylisorosmanol, which are enhanced in the leaves through the Shikimate pathway. (Andarwulan and Shetty, 1999).

Rosmarinic acid (RA) is one of the most commonly occurring caffeic acid esters in the plant kingdom, it is widely spread in family Lamiaceae. The molecular structure of RA is α-O-caffeoyl-3,4-dihydroxyphenyllactic acid, it has an antimicrobial, antiviral, anti-inflammatory effect and an efficient natural antioxidant, which makes it a very valuable product for the pharmaceutical, cosmetic and food industries. (Munne-Bosch and Alegre, 2001).

Chloroplasts, where RA is functioned in, are the most organelles exposed to oxidative damage in plant cells since they function under high oxygen and light tension. As a result of de-regulation of photosynthetic activity due to stress, more free may cause disruption of membranes and the death of the cells if the plants are not protected by antioxidant defenses. (Osmond *et al.*, 1997 and Asada, 1999).

Some studies have investigated the effect of pH on the antioxidant properties of polyphenols (Tyrakowska *et al.*, 1999; Lemanska *et al.*, 2001) and have reported that the pH-dependent behavior is related to hydroxyl de-protonation, a considerable increasing in antioxidant activity was observed with an increase in the pH level.

The pH causes the formation of hydroxyl OH group this reactive molecules are highly destructive to lipids, nucleic acid, and protein, the plants scavenge and dispose of these reactive molecules by use of antioxidant defense system. (Buchanan *et al.*, 2002).

pH can affect ion uptake and affect existing ionic competitions. The competition among protons and other cations and other anions is of fundamental importance for the mineral nutrition of plants. Many studies have shown that low pH levels are associated with inhibited cation uptake, whereas anion uptake may not be influenced at all. (Pasqua *et al.*, 2002).

On the other hand *in vitro* growth and organogenesis are pH-dependent. The slightly acidic pH value, ranging from 6.3 to 5.7, is optimal especially for root formation of most species of *Maranta* (Mohsen and Ibrahim, 2002).

At a pH of 4.0, 5.0 and 6.0, both flowers and vegetative buds were formed, whereas at a pH of 3.0, only few flowers were formed, and at a pH of 7.0, only vegetative buds were formed. (Pasqua *et al.*, 2002).

Since the influence of pH on the antioxidant activity is seldom taken into account, the main objective of this study was to investigate the influence of different pH values of cultivation medium and seasonal variation on RA accumulation in four lamiaceae members through three successive subcultures.

MATERIALS AND METHODS

Plant material

Rooted cuttings of four Lamiaceae members (*Rosmarinus officinalis*, *Origanum majoranum*, *Thymus vulgaris* and *Salvia officinalis*) with height 10-15 cm were obtained from Desert Research Center in Cairo and planted in the Faculty of Agriculture Experimental Station- Alexandria University, in the winter of 2009 and planted under field conditions.

Leaf segments (about 5 mm from the fourth node from the growing point) were disinfested for 10 minutes in 10% bleach plus 2-3 drops of Tween 20. Then leaf segments were rinsed with sterile distilled water and then placed on the medium.

Medium used for callus induction

Medium used was MS basal salt medium with vitamins from Sigma (M5519), 4.43 gm/liter medium + 3% sucrose (30 gm/L sucrose) + 1.5 mg/L TDZ + 0.5 mg/L IAA + 6 gm/L agar. (Tawfik *et al.*, 1992).

Four different pH levels were used (4.8, 5.8, 6.8 and 7.8), 1 N NaOH and 1 N HCl were used to adjust the different pH concentrations.

Medium was autoclaved for 20 minutes at 110 ^{0}C and 120 bar/cm. (Tawfik *et al.*, 1992). All tubes were placed under cool white florescent light at intensity of 66–52 µmol/m2/sec (depending on the bulb age) for 16 hrs and 8 hrs dark at a temperature of 25± 1 ^{0}C. After two months, the second callus subculture was performed and the callus tissues were placed on new a medium containing the same basal medium and the same pH levels (Figure 1).

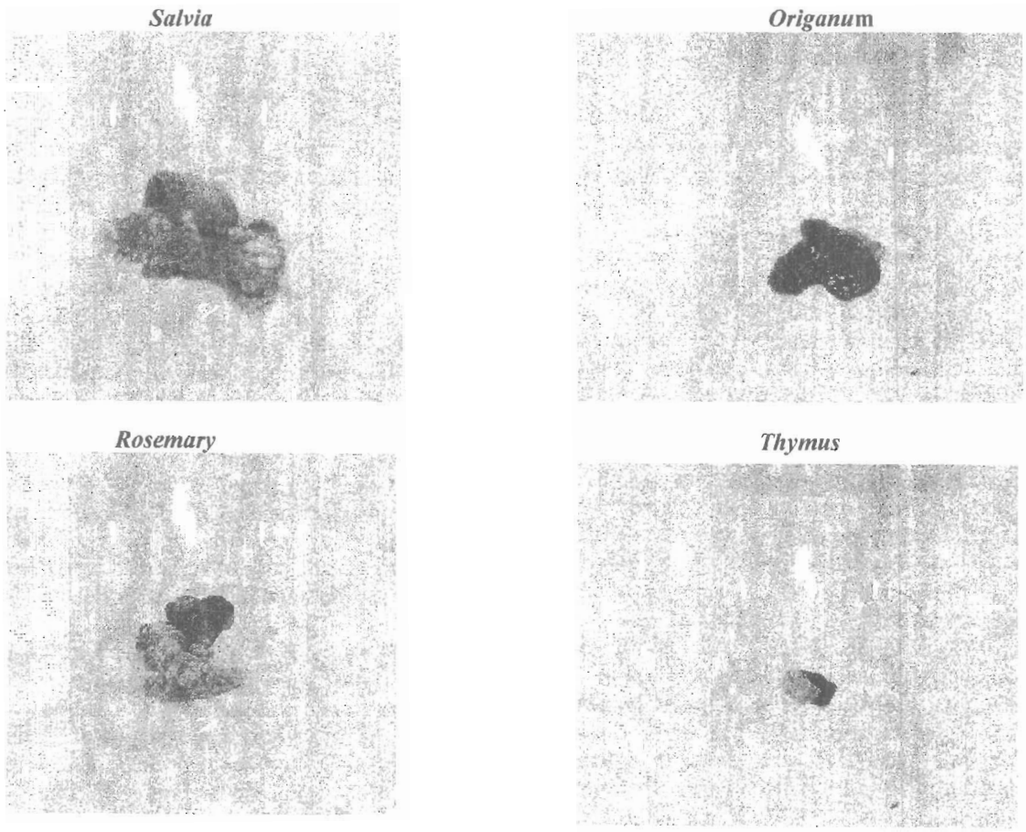

Figure 1: The first callus culture of the four Lamiaceae members after two months on the basal growing medium.

Rosmarinic acid analysis

RA analysis was done for the field plants and for the callus tissues for three successive subcultures. The method of RA extraction used through this study was reported by (Lopez-Arnaldos *et al.*, 1995 and Komali and Kalidas, 1998).

Two hundred mg of leaf tissues (leaves from the fourth or fifth node from the growing point) or 300 mg of callus tissues were used. Leaf and callus tissues were blended each in 10 ml of 50% methanol and placed in water bath at 55 ^{0}C for 2 hours, then centrifuged for 10 minutes at 3500 rpm. One ml of extract was diluted with 9 ml methanol (50%) and absorbance was measured at 333 ηm using spectrophotometer (UNICO 3200). The rosmarinic acid concentration was calculated from the equation: $A= \varepsilon bc$.

Where: (A) is the absorbance at 333 ηm, (c) is the concentration of rosmarinic acid, (ε) is the extinction coefficient ε= 19000 L mol-1 cm-1 and (b) is the width of cuvette b= 1 cm. The extraction of RA from the four species was done four times through the year (Winter 1/12/2009, Spring 1/4/2010, Summer 1/7/2010 and Fall 1/10/2010) to test the seasonal variation of RA conc., also the callus was produced from leaf segments taken at the same four times and three subcultures were done for each season, the first subculture takes two months for callus production then one month apart for the second and the third subculture.

Experimental design:

The statistical design of the experiment was a split plot design with two factors. The main factor was the seasonal variation and the sub-factor was the pH levels, the number of treatments were (4 seasons X 4 varieties X 4 pH levels X 6 tubes/trt = 384 tubes). Snedecor and Cochran, (1980).

RESULTS

Using the SAS program (2002) and Tukey's multiple comparisons method for least significant difference Tukey (1994), it was found that there were significant differences between RA concentrations in the leaf tissues in the four seasons at significant level 0.05.

It was found that the highest RA conc. in leaf tissues of the four species in field produced plants due to seasonal variation was during the summer followed by the spring then the fall while the lowest concentration occurred during the winter (Figure 2) and (Table 1).

Also it was found that there were significant differences between the RA production in leaves of the four species; the highest species in RA production was the *Salvia* and *Rosemary* and there were no significant difference between them while there was a significant difference between them and the *Origanum* and the *Thymus* at significant level 0.05 (Figure 2) and (Table 1).

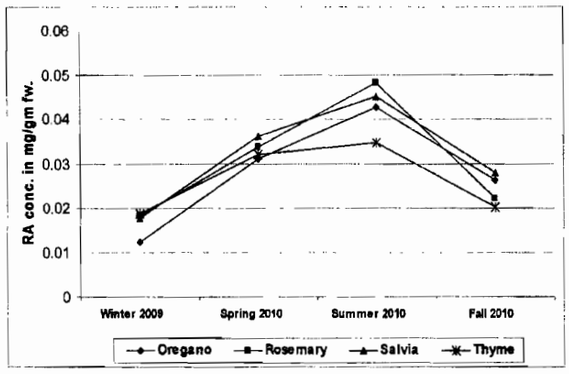

Figure 2: The effect of seasonal variation on RA production in leaves of field plants of the four lamiaceae members.

Table 1: The effect of seasonal variation on RA accumulation in leaves of four Lamiaceae members (*Rosmarinus officinalis*, *Origanum majoranum*, *Thymus vulgaris* and *Salvia officinalis*) in mg/gm fw.

Seasons	RA accumulation (mg/gm fw.)			
	Sage	Oregano	Rosemary	Thyme
Winter 2009	0.01774 c	0.01249 c	0.01816 d	0.01879 c
Spring 2010	0.03619 a	0.03115 b	0.03385 b	0.03197 b
Summer 2010	0.04514 a	0.04284 a	0.04824 a	0.03463 a
Fall 2010	0.02782 b	0.02618 b	0.02203 c	0.02012 c
Means	0.0317 a	0.0282 b	0.0306 a	0.0264 b

Same letters in the same column means there are no significant differences between treatments at P<0.05.

There were significant differences in the RA conc. between the four seasons in *Salvia*, *Origanum*, *Thymus* and *Rosemary* and the highest RA conc. was found in the callus produced from leaf explant taken during the summer in all four species, and the peak was for *Rosemary* callus culture which reached 0.077 mg RA/gm fw (Figure 3) and (Table 2).

A significant increase in RA conc. was found in the second subculture in all four species and then RA conc. declined at the third subculture, while there were no significant differences in RA conc. between the second and the third subcultures in *Thymus* and *Origanum* (Figure 3) and (Table 3).

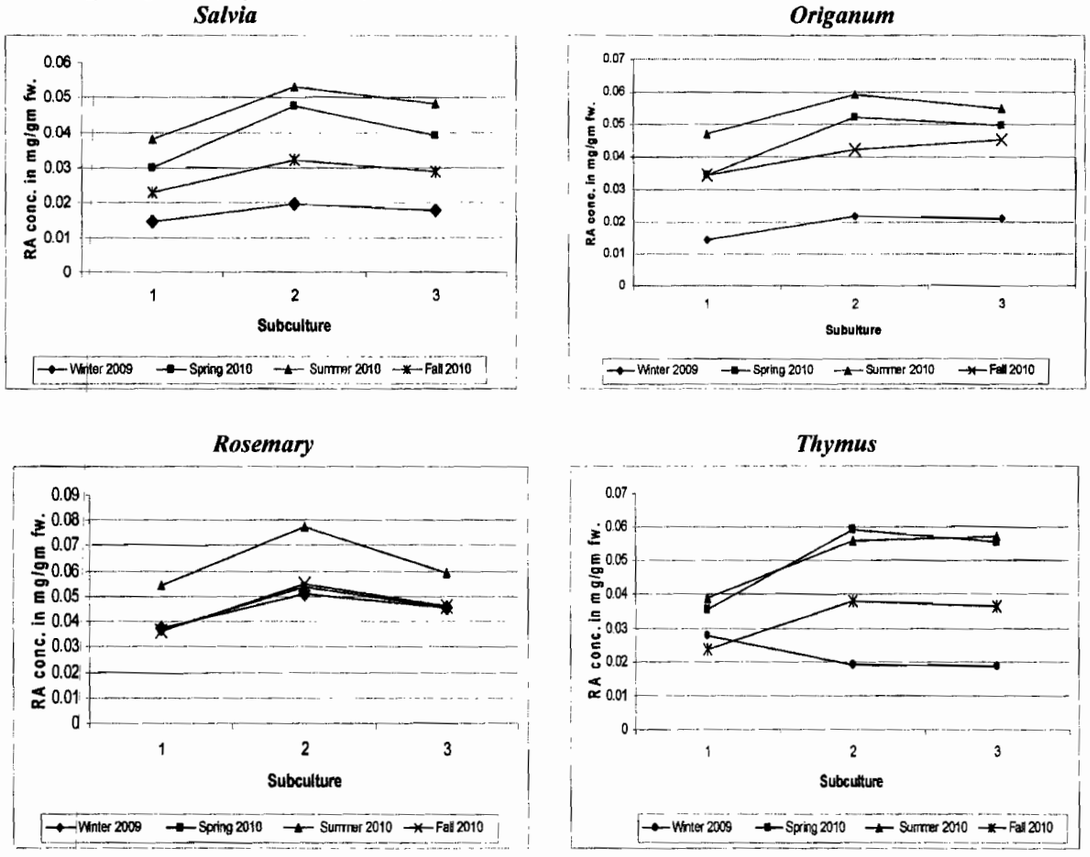

Figure 3: The effect of successive subcultures and seasonal variation on RA production in the callus of four Lamiaceae members.

Table 2: The effect of seasonal variation on RA accumulation in callus tissue of the four Lamiaceae members in mg/gm fw.

Seasons	RA accumulation (mg/gm fw.)			
	Sage	Oregano	Rosemary	Thyme
Winter 2009	0.017 d	0.019 d	0.0447 b	0.022 c
Spring 2010	0.039 b	0.045 b	0.046 b	0.05 a
Summer 2010	0.046 a	0.054 a	0.064 a	0.051 a
Fall 2010	0.028 c	0.041 c	0.0454 b	0.033 b

Same letters in the same column means there are no significant differences between treatments at P<0.05.

Table 3: The effect of successive subcultures on RA accumulation in callus tissue of the four Lamiaceae members in mg/gm fw.

Subculture	RA accumulation (mg/gm fw.)			
	Sage	Oregano	Rosemary	Thyme
First	0.038 a	0.044 a	0.0592 a	0.043 a
Second	0.0336 b	0.043 a	0.0492 b	0.042 a
Third	0.0264 c	0.036 b	0.0412 c	0.0314 b

Same letters in the same column means there are no significant differences between treatments at P<0.05.

Results also showed that by increasing the pH in the callus growing medium from 4.8 to 6.8, the RA conc. increased significantly reaching its peak at pH 6.8 in all species except in *Salvia* callus culture where the RA conc. increased significantly at pH 5.8 during the summer culture (Figure 4), (Table 4).

The RA conc. declined significantly in all four species by increasing the pH to 7.8 (Figure 4).

Also it was found that applying the pH at high conc. (6.8 and 7.8) for successive subcultures caused a reduction in RA concentration from the first to the second to the third subculture, while the pH at low concentrations (4.8 and 5.8) led to an increase in RA accumulation in callus tissues with successive subcultures (Figure 5).

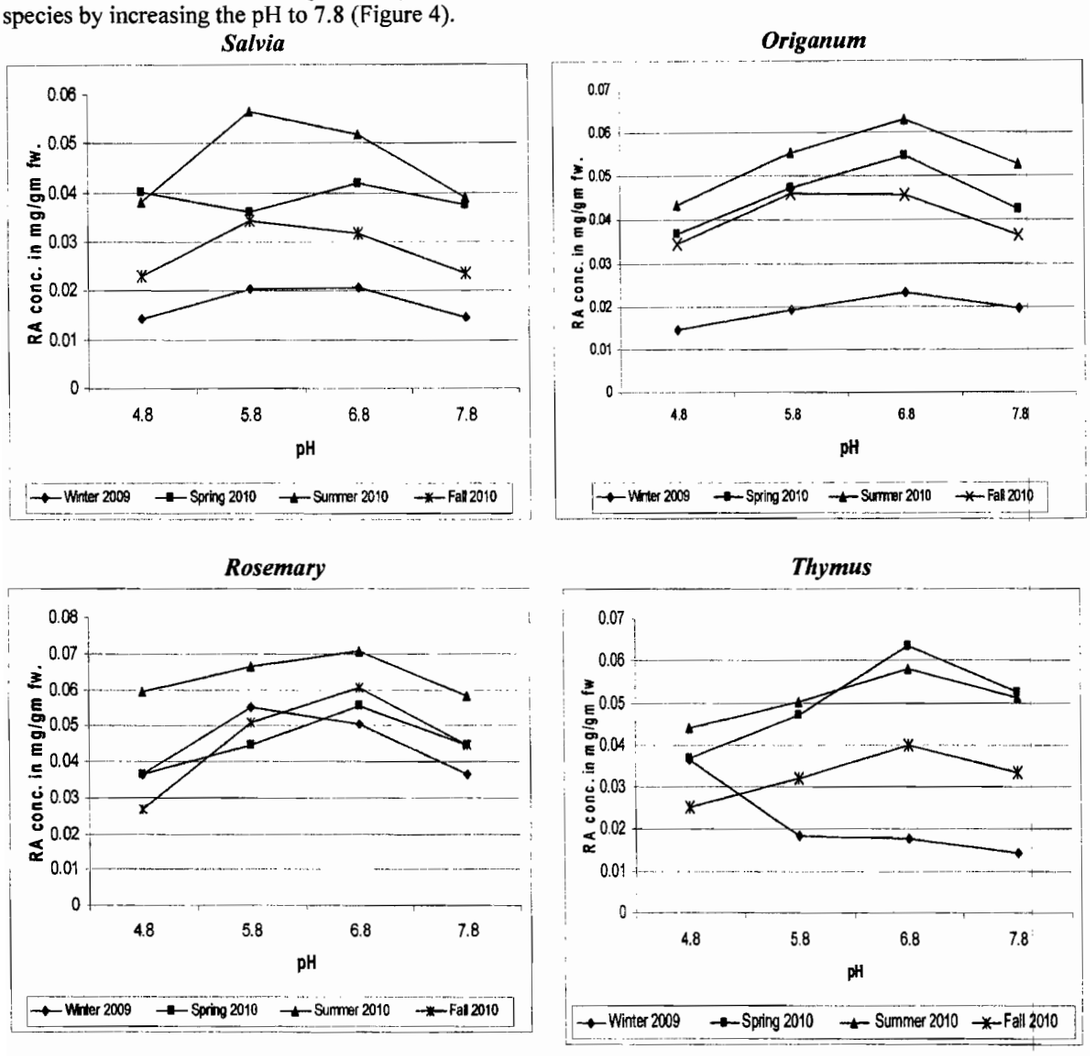

Figure 4: The effect of pH and seasonal variation on RA production in the callus of four Lamiaceae members.

Table 4: The effect of pH on RA accumulation in callus tissue of the four Lamiaceae members in mg/gm fw.

pH	RA accumulation (mg/gm fw.)			
	Sage	Oregano	Rosemary	Thyme
7.8	0.0287 b	0.038 c	0.046 b	0.038 ab
6.8	0.0365 a	0.0467 a	0.059 a	0.045 a
5.8	0.0368 a	0.0421 b	0.0543 a	0.0369 ab
4.8	0.0289 b	0.032 d	0.0398 c	0.0357 b

Same letters in the same column means there are no significant differences between treatments at $P<0.05$.

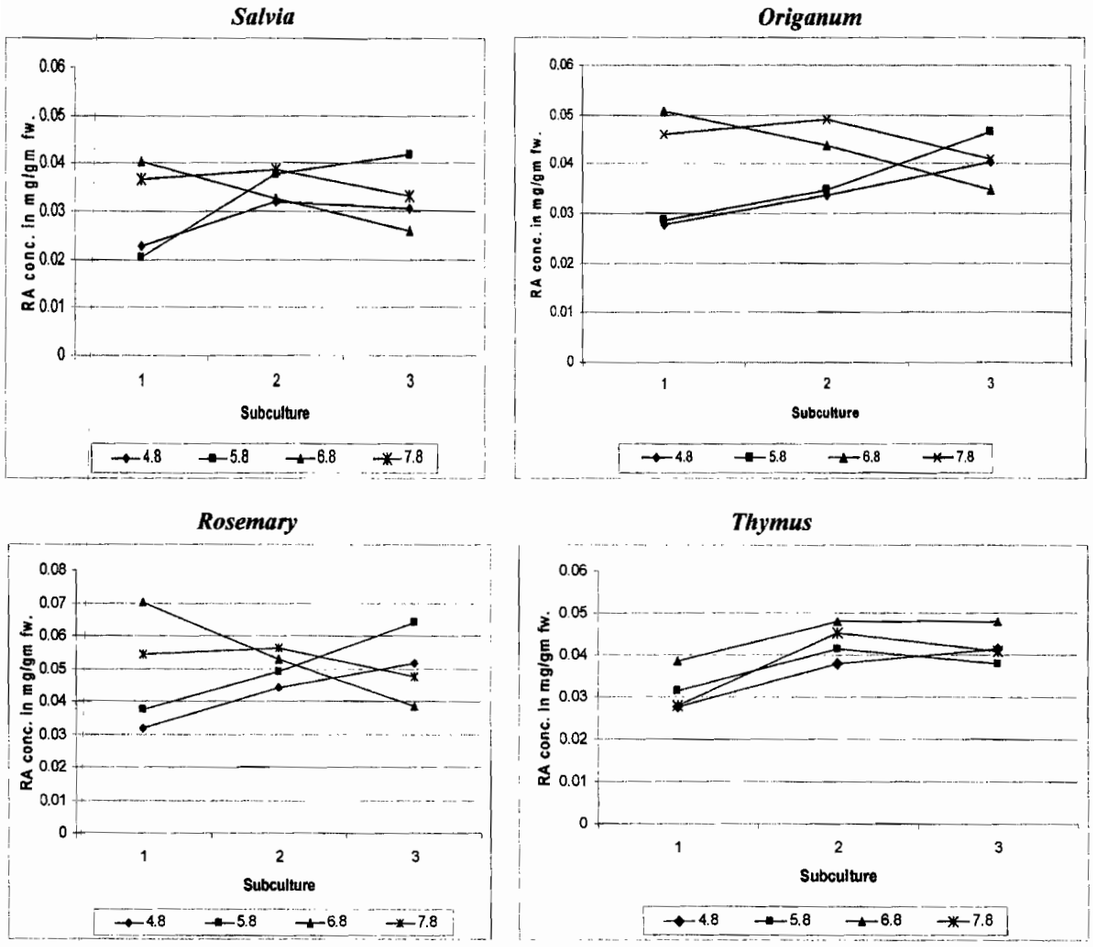

Figure 5: The effect of successive subcultures and pH on RA production in the callus of four lamiaceae members.

DISCUSSION

From the results presented in this study it appears that, the concentration of RA in leaf tissues increased during the high light and temperature during spring and summer.

Munne-Bosch and Alegre (2001) and (Munne-Bosch *et al.*, 2000) found that the concentration of the diterpene isorosmanol in leaves of rosemary increased during spring and summer and then declined during the winter.

Same results were achieved by Salido *et al.* (2003) who reported that the highest level of rosemary oil was obtained during the summer from rosemary leaves also Luis and Johnson (2005) found that the concentration of RA increased during the summer and reduced during the winter in the *in vitro* rosemary production.

The increase in RA during the hot climate might be due to the closure of the stomata in order to reduce water loss during the day because of the increase in temperature and photoperiod during spring and summer, this leads to the reduction in carbon assimilation during the warm period of the day. The limitation of carbon uptake causes the chloroplast to be subjected to an excess of energy resulting in the down regulation of photosynthesis, this leads to the reduction of oxygen and to the formation of reactive oxygen species. To compensate the excess energy and the oxidation stress plants scavenge the reactive oxygen by antioxidants like tocopherols, carotenoids and diterpenes. This leads to the increase in RA concentration in leaf tissues during the spring and summer (Munne-Bosch and Alegre, 2001 and Ain-Lhout *et al.*, 2004).

RA concentration increased in *Coleus* cell culture with time and then decreased with the aging of the culture, the reduction of the RA concentration is due to the reduction in the PAL enzyme activity, which is an important enzyme in the biosynthetic pathway of the RA, and to the reduction in phenylalanine which is a main precursor in the RA biosynthetic pathway. (Razzaque and Ellis, 1977).

The concentration of RA in *Anchusa officinalis* declined with aging of callus. The reduction of RA was due to the "crowding of cells" and the

intracellular high concentration of the RA which leads to a feedback inhibition on further RA synthesis. (Wei Wen and Fei Lei, 1993).

The same results were reported by Lopez-Arnaldos *et al.* (1994). They attributed the decline of RA concentration with the aging of callus to the oxidation of caffeic acid by phenolases and peroxidases present in the cell culture and that the action of these enzymes may be responsible for the decomposition and disappearance of RA and to rosmarinic acid peroxidase, which is responsible for the oxidation of RA during aging of callus and causing the browning of the culture.

Chen Hui *et al.* (1997) mentioned that successive subculture of *Salvia miltiorrhiaz* caused a reduction in diterpenes found in the roots of the plant.

From the previous studies and the results presented in this study it appears that the successive subculture of aromatic and medicinal plants causes decrease in the production of the secondary metabolites, like RA, with the time (Figure 1).

In the presented results, it was found that the pH has a significant influence on RA accumulation in callus culture of the four species. These results are in consistent with those presented by (Pasqua *et al.*, 2002). Higher culture pH favored the production of extracellular bioactive antioxidants with scavenging free radical activities. (Chin-Hang and Ming-Yeou, 2008).

Jovanovic *et al.* (1994) and Sauerwald *et al.* (1998) mentioned that a considerable increase in antioxidant activity was observed with an increase in the pH. An explanation for this could be that at physiological pH, the antioxidant value of the poly-hydroxy-flavones is a combination of the activity of the neutral as well as of the deprotonated form in different ratios, where as at pH 3.5 the neutral form of antioxidant is prevalent, while at higher pH the mechanism of antioxidant action, i.e. hydrogen atom or electron donation occur.

With increasing medium pH, the antioxidant activity of flavan-3-ols increases significantly on its turn. These molecules have the same antioxidant activity at pH 3.5, but higher antioxidant activities were observed at pH 7.4. At this pH level the mechanism for the antioxidant action can be either hydrogen atom and/or electron donation (Lemanska *et al.*, 2001).

The pH effect on the antioxidant activity could be attributed to different degree of dissociation of COOH and OH groups. A considerable number of dissociated carboxyl groups is present and ionization of OH groups is also favoured at pH 7.4, (Ordoudi and Tsimidou, 2006).

pH causes the formation of hydroxyl OH group (oxyradicals) this reactive molecules are highly destructive to lipids, nucleic acid, and protein, the plants scavenge and dispose of these reactive molecules by use of antioxidant defense system. (Buchanan *et al.*, 2002).

Di Majo *et al.* (2011) mentioned that the antioxidant properties of polyphenol compounds are strongly influenced by pH level. Increasing pH led to a considerable increase in observed antioxidant activity. The polyphenolic compounds are more sensitive to pH change than to the number and position of substitution groups.

It is important to clarify that the specific action measured *in vitro* does not necessarily reflect the *in vivo* antioxidant properties. In a biological system, in addition to chemical and environmental behaviors, there are many factors influencing antioxidant activity.

CONCLUSION

The highest RA accumulation in leaf tissues and in callus culture occurred when the explants were taken in the summer and the second subculture in all species, while the best pH was 6.8 in all species except in sage callus culture where the RA conc. increased significantly at pH 5.8, and it was found that the highest species in RA production in field production was the sage followed by the rosemary, thyme and oregano and was (0.048, 0.045, 0.035 and 0.026 mg RA/gm fw) respectively, while *in vitro* culture the highest RA accumulation occurred during the second subculture in the summer at pH 6.8 in rosemary, thyme and oregano (0.086, 0.073, 0.069 mg RA/gm fw) respectively and the least accumulation occurred in sage in the second subculture when the explants were taken during the summer at pH 5.8 (0.062 mg RA/gm fw).

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الملخص العربى

تأثير تركيز الأس الهيدروجينى السالب والتغيرات الموسمية وتكرار الزراعة على إنتاج حامض الروزمارنيك فى أربعة أصناف تابعة للعائلة الشفوية لبيئة زراعة الانسجة

هانى النجار، هدى المقدم، علا الشناوى

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النباتات الطبية هى المصدر الطبيعي لمضادات الأكسدة وظروف الإجهاد هى التي تحفز إنتاج مضادات الأكسدة مثل الجفاف، الحرارة، تركيز الأس الهيدروجيني السالب والضوء. كل هذا يؤدى الى تكوين تركيزات عالية من مضادات الأكسدة وخاصة التربينات الثنائية. حمض الروزمارينيك يعتبر من أشهر التربينات الثنائية وهو منتشر بكثرة في العائلة الشفوية. وحيث ان تأثير الأس الهيدروجيني السالب فى بيئة الزراعة على إنتاج مضادات الأكسدة فى زراعة الأنسجة لم يدرس بصورة واضحة، فان الهدف من هذا البحث هو دراسة تأثير الأس الهيدروجيني السالب على إنتاج حمض الروزمارينيك فى زراعة الكالس معمليا وذلك لأربعة أصناف من العائلة الشفوية(حصا لبان، المريمية، البردقوش، الزعتر) وكذلك دراسة تأثير التغيرات الموسمية وعدد تكرارات الزراعة على إنتاج حمض الروزمارينيك معملياً وحقلياً وذلك فى خلال الفترة من شتاء ٢٠٠٩ الى خريف٢٠١٠.

وقد وجد أن اعلي تركيز لحمض الروزمارينيك فى الكالس كان خلال النقل الثانى للكالس المنزرع فى فصل الصيف للأربعة أصناف موضع الدراسة.

ووجد أيضا أن للأس الهيدروجيني السالب تأثير كبير على تراكم حمض الروزمارينيك فى الكالس فى الأربعة أصناف وكان أفضل تركيز للأس الهيدروجيني السالب ٦,٨ فى كل الأصناف ماعدا المريمية حيث كان ٥,٨.

أيضا وجد ان اعلي تركيز لتراكم حمض الروزمارينيك حقلياً كان فى اوراق نبات المريمية ثم الحصا لبان ثم الزعتر ثم البردقوش على التوالى(٠,٠٤٨، ٠,٠٤٥، ٠,٠٣٥، ٠,٠٢٦ ملجم/جم وزن رطب). بينما كان اعلي تركيز لتراكم الحامض فى الكالس خلال ثاني زراعة للكالس للحصا لبان، ثم الزعتر ثم البردقوش ثم المريمية على التوالى.