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# Rosmarinic Acid Production and Expression of Tyrosine Aminotransferase Gene in *Melissa officinalis* Seedlings in Response to Yeast Extract

M. A. Nasiri-Bezenjani[1], A. Riahi-Madvar[2*], A. Baghizadeh[2], and A. R. Ahmadi[3]

## ABSTRACT

**Effects of yeast extract (YE) (0, 0.05, 0.1, 0.2, 0.5 and 1%) were investigated on rosmarinic acid (RA) accumulation and tyrosine aminotransferase (TAT) gene expression in *Melissa officinalis* seedlings at different time intervals (4 and 17 hours). Based on the results, only YE concentrations of 0.05%, 0.1%, and 0.2%, for the 17-hour treatment, significantly stimulated RA biosynthesis pathway. At this elicitation time, flavonoid content and *TAT* gene expression significantly increased by the increase in YE concentration up to 0.2% as compared to the control. The maximum amount for both of them was seen at 0.1% YE treatment, where the RA accumulation was drastically elevated. Furthermore, the aerial parts were improved when root lengths were decreased; this was observed in seedlings that were treated by YE for 30 days. Overall, these observations can be attributed to the oxidative stress induced by YE, as a consequence of its uptake by the plant, as revealed by increasing activities in superoxide dismutase and catalase.**

**Keywords:** Antioxidant enzyme activity, Flavonoids, Elicitor, Morphological properties.

## INTRODUCTION

Lemon balm (*Melissa officinalis*) is a member of the Lamiaceae family, which spreads extensively from western part of Europe (Cosge *et al*., 2009) to western and central parts of Iran. Leaves of this plant are thin and fluff; the flowers are white or pink with small panicles, which are formed during the summer (Ali-Madad, 1996). Today, the extract of Lemon balm is used extensively in various fields, such as medicine, food industries, as well as perfume and cosmetics productions (Adinee, *et al*., 2008). Extracts of Lemon balm have various pharmaceutical properties including antiviral, anti-inflammation (Bagdat and Cosge, 2006; Abu-shanab *et al*., 2006) and antioxidant effects (Herodez *et al.,* 2003). It is known that most of these effects are related to its active ingredient, rosmarinic acid (Park *et al*., 2008).

Rosmarinic acid is an ester of caffeic acid known by the chemical name of 3, 4 di-hydroxyl phenyl lactic acid and has the chemical formula of $C_{18}H_{16}O_8$ (Petersen *et al*., 1993). This compound has been reported in most species and genus of Boraginaceae, Lamiaceae, sub-family of Nepetoidue and also some genus of ferns (family Blechnaceae) and Araceae plants

[1] Department of Biotechnology, Faculty of Science and Modern Technology, Graduate University of Advanced Technology, Kerman, Islamic Republic of Iran.
[2] Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Islamic Republic of Iran.
[*] Corresponding author; e-mail: ariahi@icst.ac.ir
[3] Department of Civil and Survey Engineering, Graduate University of Advanced Technology, Kerman, Islamic Republic of Iran.





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such as *Arum italicum* (Petresen *et al.,* 2009). This phytochemical has antioxidant properties (Herodez *et al*., 2003) and anticancer activities (Scheckel *et al.,* 2008) as well as broad industrial applications.

In plants, RA biosynthesis takes place through the flavonoids biosynthesis pathway. Flavonoids are classified as antioxidant compounds that play a role in creating the color in plants; they protect the plant against attacks from pathogens and exposure to UV radiation and also act as an indicator on the conditions due to tension and scavenging of free radicals (Hamilton *et al.*, 2003). Biosynthesis of these metabolites starts via two separate pathways that use two amino acids, namely, L-phenylalanine and L-tyrosine, as substrate (Petersen and Simmonds, 2003). Tyrosine aminotransferase (TAT) is one of the first enzymes in entrance of this pathway, which uses tyrosine as a substrate to produce hydroxy phenyl pyruvate. cDNA of this enzyme has been sequenced in some plants including arabidopsis (Lopukhina *et al*., 2001), *Medicago truncatula, Solenostemon scutellarioides*, *Salvia miltiorrhiza*, *Glycine max,* and *Coleus blumei* (GenBank Accession No. of DQ006809; AJ458993.1; DQ334606.1; DQ003328.1, and AJ458993, respectively). Multiple sequence alignment of different TAT protein sequences has shown that the enzyme has a conserved amino acids sequence in various plant species (Lopukhina *et al*., 2001).

However, several studies have shown the involvement of tyrosine aminotransferase and phenylalanine ammonia-lyase on accumulation of RA under stimulation by different elicitors. Various elicitors have been used for inducing RA biosynthesis in cell culture of Boraginaceae and Lamiaceae species (Yan *et al.,* 2006; Mizukam *et al.,* 1992; Szabo *et al.,* 1999). To our knowledge, no report has been published regarding the effect of any elicitor on RA production and relationship between *TAT* gene expression and the RA accumulation in *M. officinalis* seedling.

Yeast extract (YE) obtained from the *Saccharomyces cerevisiae* (Nur Cahyanto *et al*., 2011) is recognized as a biotic elicitor; which has been extensively used for induction of RA in various plant species (Mizukam *et al.,* 1992; Szabo *et al.,* 1999; Yan *et al.,* 2006).

YE is a complex of various compounds such as peptides, polysaccharides, vitamins, and inorganic compound such as Zn, Co, and Ca ions as well as some unknown component (Mizukam *et al*., 1992). The exact member of this elicitor that stimulates biosynthesis of secondary metabolites in plants is not clearly understood. It has been suggested that stimuli effect of YE on biosynthesis of secondary metabolite may be due to certain cations such as Zn and Co or other components that have not been identified yet (Sandra *et al*., 2000).

In the present study, effects of different concentrations of yeast extract have been investigated on the active ingredient content of *M. officinalis*; as well as *TAT* gene expression profile. Additionally, the effect of this elicitor has also been investigated on some morphological properties of 30-days-old seedlings.

## MATERIALS AND METHODS

### Preparation of Culture Media

Seeds of *M. officinalis* were obtained from the Pakan Bazr Co. Esfahan, Iran. The seeds were sterilized using ethanol 70% and hypochlorite sodium 2% for one and 10 minutes, respectively. Thirty seeds with about 5 mm spacing, were placed in Petri dishes. The plates were then transferred to an incubator under controlled temperature of 28±2 $^{o}$C and relative humidity of 56±3 percent. Solidified basal media of MS (Murashige and Skoog, 1962) containing 0.8 agar was used as a medium for cultivation;





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here, two series of media were prepared. In series 1, the sterilized seeds were placed on the surface of the medium; the plantlets was used to analyze the YE (Sigma) effect, on flavonoid pathway including RA production, *TAT* gene expression, and flavonoid content. In series 2, which was used to measure the YE effect on morphological properties, the sterilized seeds were placed on the surface of the medium containing yeast extract.

### Effects of Yeast Extract on Flavonoid Biosynthesis Pathway

The 30-days-old seedlings grown on the medium without YE (series 1) were separated from their medium and washed thoroughly using distilled water. Different concentrations of YE [0 (as a control), 0.05, 0.1, 0.2, 0.5, and 1%] solution were prepared by dissolving appropriate amounts of YE in distilled water without dilution. The seedlings (about 50) were transferred into 50 mL of YE solution in 150 mL flaks. The flaks were shaken at 100 rpm on an orbital shaker for 4 and 17 hours.

The treated seedlings were then washed to eliminate the surface elicitor. The seedlings were divided into two groups. The first group was dried in the shade (for three days) and was used to measure the RA concentration. The second group, which was frozen in liquid nitrogen and kept at -80°C, was used for measuring the *TAT* gene expression and flavonoid content.

### Measurement of Rosmarinic Acid Concentration

In order to analyze the effect of YE on rosmarinic acid content, extraction and measurement of its concentration were performed in accordance to Wang *et al.* (2004) method with minor modifications. In brief, 100 mg of the dry tissue was grinded and suspended in 10 mL of 30% ethanol, and was sonicated for 15 minutes; then, the solution was centrifuged for 20 minutes at 4,000 rpm and 25°C. The distilled water was added to the supernatant until the volume of 20 mL was reached. The solution was filtered (0.2 µM of syringe-headed filter) prior to being injected to column ($C_{18}$, 250×4.6 nm) of High Performance Liquid Chromatography (HPLC) (Agilent 1100 series) in order to separate the RA. The RA was detected at 330 nm and authentic standard (Sigma− Aldrich St. Louis, MO, USA, Code No. 536954) solution was used to determine elution time for RA peak and its concentration (mg $g^{-1}$).

### Molecular Analysis

#### Extraction of Total RNA and cDNA Synthesis

Total RNA was extracted from fresh weight (frozen sample) using RNA extraction kit as described by the manufacturer, CinnaGene Inc. The quality and quantity of extracted RNA was examined using 1% agarose gel electrophoresis and spectroscopy, respectively.

The first strand of cDNA was synthesized at 42°C for 120 minutes in the presence of 200 U $\mu l^{-1}$ MMuLV reverse transcriptase (Fermentas, EP0441, USA), 20 U RNase inhibitor, dNTP (final concentration each at 1 mM) and oligo $(dT)_{20}$ as a primer (Fermentas, SO131, USA).

#### PCR Amplification and Analysis of *TAT* Gene Expression

Comparison of gene expression in the presence of different concentrations of elicitor was conducted using semi-quantitative RT-PCR technique. cDNA amplification was carried out using *Taq* DNA polymerase in the presence of specific primers. Two primers were used for this purpose: forward primer 5´-*ATG GAG TTG CAG AAT TCA GCG*-3´and reverse primer 5´- *GGA GTG CCG TTC ACA GAA AG*-3´ were designed using Gene Runner (version 3.05) software based on tyrosine aminotransferase gene of *Salvia miltiorrhiza* and *Coleus blumei*, (Gene Bank Accession





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No. DQ334606 and AJ458993 respectively). Amplification of the *TAT* gene was carried out under the following condition: initial denaturation at 94°C for 5 minutes, 30 cycle amplification (94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute) and a final extension for 5 minutes at 72°C. The amplification of *Tubulin* gene, (a house keeping gene) used as an internal control, was conducted in the presence of specific primers: Forward primer 5´-*GCT TTC AAC ACC TTC TTC AGT G*-3´and reverse primer 5´-*CTT TCT CAG CTG AGA TCA CTG G*-3´, which were designed using Gene Runner software based on the *Tubulin* gene of *Triticum aestivum* (Gene Bank Accession No. DQ435671.1 ) under the same conditions as described above. The PCR products were analyzed on 1% agarose gel electrophoresis for 60 minutes at 90V.

The analysis of the TAT band intensity was carried out after normalizing by Tubulin related band on gel electrophoresis using Gene Tools software.

In order to confirm nucleotide sequence of *TAT* gene, the fragment which was amplified by *Pfu* DNA polymerase (Fermentas, EP057, USA) was sequenced using an automatic sequencer (MWG, Germany) based on specific forward and reverse primers.

## Measurement of Flavonoid Content

Flavonoid content was measured using Krizek *et al.* (1998) method. Based on this method, 0.1 g of fresh weight (frozen sample) was abraded well in 10 mL acidic ethanol (including ethyl alcohol 95% and glacial acetic acid at a 99:1 v/v ratio). After being centrifuged at 4,000 rpm for 15 min, the supernatant was separated and then gently heated in water bath at 80°C. Absorption of the output extract was measured at 270, 300 and 330 nm using UV-Visible spectrophotometer (Varian cary 50, Australia), considering the extinction coefficient of 33,000 $M^{-1}$ $cm^{-1}$ and flavonoid contents were calculated cumulatively.

## Protein Extraction and Assay of Antioxidant Enzymes

In determining the activities of antioxidant enzyme, fresh seedling samples (0.5 g) were grinded in liquid nitrogen; and they were homogenized in 5 mL of 50 mM sodium phosphate buffer (pH 7.5) including 1 mM EDTA, 1% PVP and 1 mM PMSF. The homogenate was centrifuged at 20,000 rpm and 4°C for 15 minutes. The supernatant was then used for protein concentration and enzyme assays. Protein concentrations in seedling´s crude extracts were determined according to Bradford (1976) method.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed in a 3 mL reaction mixture containing 2.5 mL 50 mM sodium phosphate buffer (pH 7.8), 0.1 mL 13 mM methionine, 0.1 mL 2 µM riboflavin, and 0.1 mL 75 µM NBT and 0.2 mL enzyme extract. Absorbance of the solution was tested by measuring its capacity inhibit, the photochemical reduction of Nitro-Blue Tetrazolium (NBT) at 560 nm. One unit of SOD is defined as the enzyme activity that reduces the photo reduction of NBT to blue formazan by 50% (Constantive *et al.,* 1977). SOD activity was expressed as enzyme units per milligram of fresh weight (U/mg protein).

Catalase (CAT) (EC 1.11.1.6) activity was determined in a 3 mL reaction mixture containing 2.87 mL of 50 mM sodium phosphate buffer (pH 7.0), 30 µL $H_2O_2$ (15%), and 100 µL of enzyme extract. The rate of decrease in absorbance was measured at 240 nm. One unit of catalase is defined as the amount of enzyme that decomposes 1 µM $H_2O_2$ in 1 minute (Dhindsa *et al.,* 1981) and the activity was expressed in U $mg^{-1}$ protein.

## Measurement of Morphological Properties

Analysis of morphological properties was exclusively conducted on 30-day-old Lemon balm seedlings that were grown in the presence of effective concentrations of YE





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(0/05, 0.1 and 0.2%) for rosmarinic acid biosynthesis.

### Seed Germination

In measuring seed germination, each day, the germinated seeds (minimal of the radicals was about 1 mm) were counted and recorded. Seed germination was reported as the percentage of seeds germinated by the $15^{th}$ day.

### Measurement of Shoot and Root Length

Lengths of the roots and the shoots of 20 seedlings (30-day-old) randomly selected from each plate were measured using a ruler and were reported in millimeters.

### Statistical Analysis

Experiments were conducted with completely random designs in triplicate for each treatment. All experiments were repeated at least three times. The results were expressed as mean values ± standard deviation (SD). Duncan's multiple range tests were used to compare mean of the treatments at $P< 0.05$ using SAS 9.1.3 (service pack 4, version 6.1.7601) (Evans, 1999) software. Significance of the difference between mean values was determined by one-way analysis of variance (ANOVA).

## RESULTS

### Rosmarinic Acid Content

As shown in Figure 1, the elution time for RA occurred at 12 minutes after injection. Rosmarinic acid production was only affected in the presence of 0.05, 0.1, and 0.2% YE concentrations at 17 hours treatment; but not for higher YE concentrations (Figure 2). As indicated in Figure 2, in all treatments, the RA concentration was significantly increased compared to that of the control; and the highest accumulation was seen for the 0.1% treatment. The elicitor at 4 hours treatment did not show any significant effect on rosmarinic acid production for all concentrations (data not shown). It should be noted that all other experiments were performed on the seedlings that were exposed to YE concentration of 0.05, 0.1, and 0.2% for 17 hours, where the RA content was drastically elevated as compared

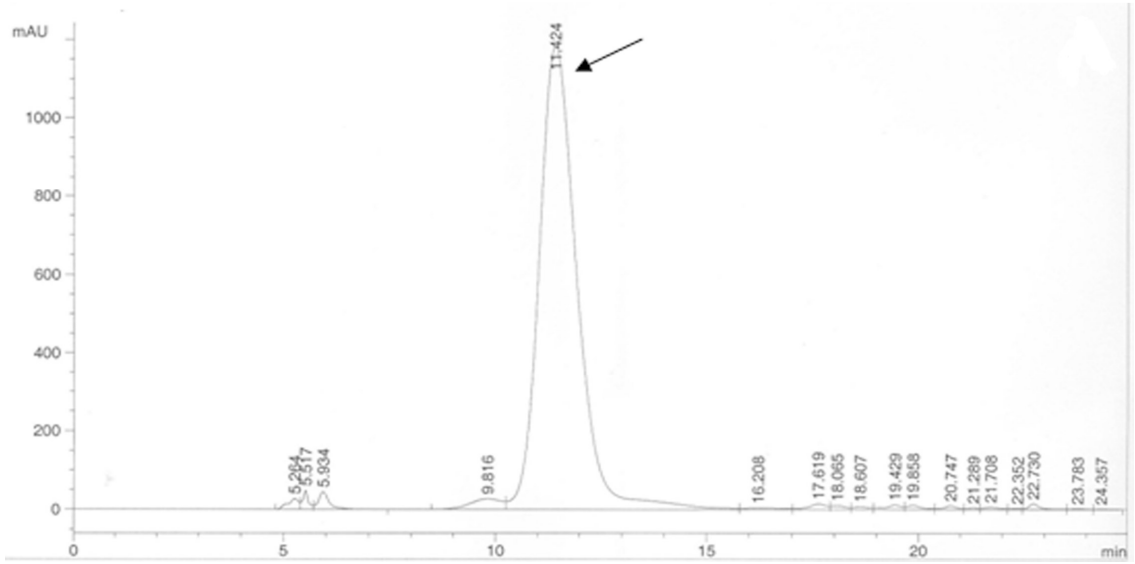


**Figure 1.** Chromatogram of rosmarinic acid. Elution time of standard RA occurred at 12 minutes after injection.





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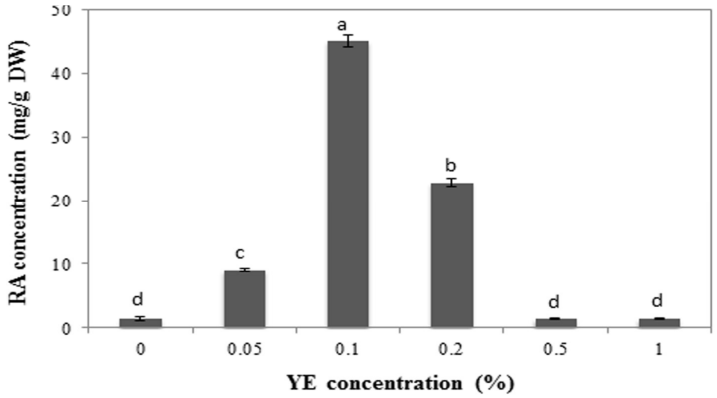


**Figure 2.** RA accumulation in seedlings that were treated for 17 h with different concentrations of YE. Bars with different letters are significantly different at $P \leq 0.05$, according to Duncan's multiple range tests.

to that of the control.

### Analysis of Tyrosine Aminotransferase Gene Expression

The cDNA library was utilized to amplify the *TAT* and *Tubulin* genes in the presence of specific primers. The PCR products analyzed on 1% agarose gel electrophoresis (for 60 minutes at 90V) were about 1,450 and 750 bp for *TAT* and *Tubulin* gene, respectively (Figure 3). The nucleotide sequence of amplified TAT fragment and deducing amino acid sequence were confirmed after blasting. The partial cDNA of the gene was registered in Gene Bank under accession number of JN863949.

As shown in Figure 4, the gene expression was significantly elevated in the treated seedlings compared to the control sample, and it was drastically enhanced at 0.1% treatment.

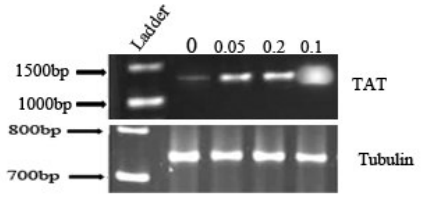


**Figure 3**. Expression of TAT and Tubulin in *M. officinalis*. The seedlings were treated with various concentrations of YE. The expression of gene was determined by a semi-quantitative RT-PCR method using Tubulin as an internal control.

### Flavonoid Content

As reported in Table 1, flavonoid content in YE-treated seedlings significantly increased with the increase in YE concentration in the medium.

### Enzymatic Antioxidant System

The activity of SOD and CAT are summarized in Table 1. As indicated by the data, the activity of enzymes was increased by the increase in YE concentration.

### Effect of Yeast Extract on Seed Germination

Percentage of seed germination was calculated at the end of the 15$^{th}$ day; which was more than 70% for the control and all treatments. As shown in Table 2, percentage of seed germination increased significantly for 0.05 and 0.1% treatments as compared to the control. The highest value for seed germination was obtained at 0.05% YE concentration, which was decreased by the increase in elicitor concentration.





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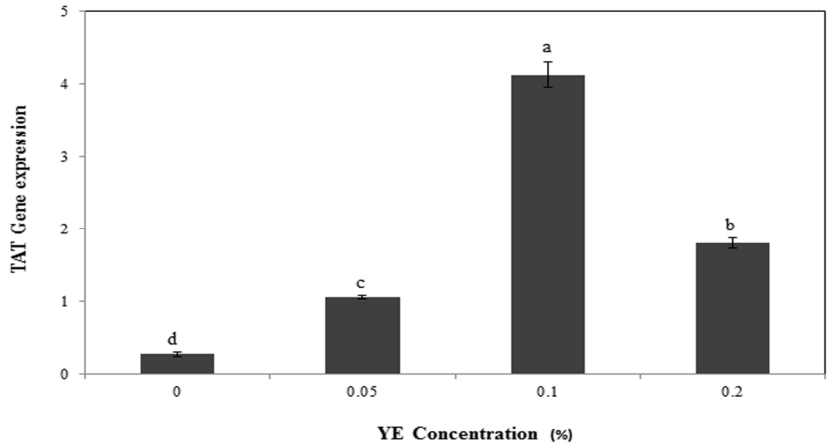


**Figure 4.** Comparison of *TAT* gene expression in seedlings that were treated by different concentrations of YE. Bars represent means±SD, $n$= 3. Signs with different letters are significantly different at $P\leq 0.05$ according to Duncan's multiple range tests. For further details see experimental procedures.

### Effect of Yeast Extract on Shoot and Root Length

The effect of YE on the root and shoot length are summarized in Table 2. Root length significantly decreased with the increase in elicitor concentration, while shoots length significantly increased as compared to the control; and the highest shoot length was obtained at 0.1% treatment.

## DISCUSSION

According to Figure 2, amongst the various concentrations that were tested, only 0.05, 0.1, and 0.2% concentration of this elicitor for the 17-hour treatment stimulated RA biosynthesis. None of the concentrations after 4-hour treatment, as well as 0.5 and 1% YE concentrations after 17-hour, had any

**Table 1**. Flavonoid content, CAT and SOD activity of the control and treated seedlings.[a]

| YE Concentration (%) | Flavonoid Content (μM $mg^{-1}$) | CAT Activity (U $mg^{-1}$ protein) | SOD Activity (U $mg^{-1}$ protein) |
|---|---|---|---|
| 0 | 124.51±1.73[b] | 0.129±0.005[d] | 3.510±0.342[c] |
| 0.05 | 126.47±2.27[b] | 0.187±0.006[c] | 6.270±0.190[b] |
| 0.1 | 140.77±0.35[a] | 0.324±0.003[b] | 8.043±0.071[a] |
| 0.2 | 139.37±1.11[a] | 0.399±0.002[a] | 8.037±0.508[a] |

[a] Data are triplicate and presented as the means±SD. Different letters in each group indicate significances at $P\leq 0.05$ according to Duncan's multiple range tests.

**Table 2.** Effect of different YE concentrations on seed germination and length of root and shoot.[a]

| YE Concentration (%) | Seed germination (%) | Root length (mm) | Shoot length (mm) |
|---|---|---|---|
| 0 | 72.95±2.41[c] | 44.66±0.00[a] | 9.16±0.06[c] |
| 0.05 | 88.33±0.88[a] | 34.37±1.15[b] | 15.65±0.33[a] |
| 0.1 | 81.35±1.26[b] | 31.73±0.53[c] | 15.83±0.19[a] |
| 0.2 | 70.67±0.58[d] | 30.17±0.12[c] | 14.58±0.37[b] |

[a] Data are triplicate and presented as the means±SD. Signs with different letters in each group indicate significances at $P\leq 0.05$ according to Duncan's multiple range tests.





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significant effect on the production of RA (data not shown).

The accumulation of RA significantly increased at YE concentrations up to 0.1% treatment, and decreased at higher elicitor concentrations. The positive effects of this elicitor on the biosynthesis of rosmarinic acid in *Coleus blumei* has been reported (Szabo *et al.,* 1999); as well as on the hairy root of *Salvia miltiorrhiza* (Yan *et al*., 2006).

On the other hand, the expression of *TAT* gene was also significantly influenced by YE concentrations and showed a similar pattern as observed for RA accumulation (Figure 4). The direct relationship between RA production and TAT activity has been reported by Yan *et al.* (2006). They found that increase in RA content in *Salvia miltiorrhiza* hairy root that was exposed to YE was accompanied with increase in activity of TAT.

As reported in Table 1, flavonoid content significantly increased with the increase in YE concentration and maximum production was obtained at 0.1% treatment, where the rosmarinic acid content was significantly elevated. Studies conducted by Naoumkina *et al.* (2007) established that this elicitor influences the entire pathway of flavonoid´s biosynthesis in plants.

However, inducing oxidative stress in the treated plants with YE can be demonstrated by increases in activities of SOD, which plays an important role in catalysis of $O_2^{-\bullet}$ to $H_2O_2$ (Dong *et al*., 2002) and CAT, the key enzyme in scavenging of $H_2O_2$ (Ghanati *et al*., 2005) (Table 1). It has been known that $H_2O_2$, as a free radical, induces expression of many defense genes and secondary metabolites (Xu, *et al*., 2007). Based on the antioxidant properties of flavonoid component (Herodez *et al*., 2003; Hamilton *et al.,* 2003), it seems YE induced oxidative stress in the treated plant and the promotion of flavonoid biosynthesis pathway (including the increase of RA and flavonoid contents and also *TAT* gene expression) resulted in the response of the plant to reduce oxidative damage.

Additionally, some morphological properties including seed germination and roots and shoots elongation were also analyzed for the 30-day-old seedlings, which were grown in the presence of yeast extract. It is worthwhile to mention that the seed germination was affected by YE concentrations. While the seed germination started on the 5$^{th}$ day for the control, it started on the 2$^{nd}$ day in the presence of all YE treatments (data not shown). The percentage of seed germination significantly increased at 0.05 and 0.1% treatments as compared to the control. Whilst the highest percentage of seed germination was obtained at 0.05%, it decreased with the increase in YE concentration (Table 2).

Growth of the seedlings was also affected by the elicitor (Table 2). In contrast to decrease in root length, the shoot length was significantly improved in the presence of all YE concentrations. It may be proposed that the inhibition of the underground organs, and also over-development of the aerial organs, might be due to the various components of yeast extract including heavy metals (Mizukam *et al*., 1992).

In conclusion, this study has shown the positive effects of yeast extract on rosmarinic acid production in *M. officinalis* seedling, which was accompanied with more *TAT* gene expression under this condition. Yeast extract is a complex of various components that, within a short period (17 h) treatment, affected the biosynthesis pathway of the secondary metabolites and their gene expression; while in the long time treatment i.e. 30 days, different morphological properties appeared on the seedlings. It seems that these observations are related to the oxidative stress induced by this elicitor. However, more complementary studies are needed to determine all effects of the yeast extract on plants.

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## تولید رزمارینیک اسید و بیان ژن تیروزین آمینوترانسفراز در گیاهچه‌های بادرنجبویه در پاسخ به عصاره مخمر

**م. ا. نصیری بزنجانی، ع. ریاحی مدوار، ع. باقی زاده، و ع. ر. احمدی**

## چکیده

در این تحقیق، اثرات عصاره مخمر [۰، ۰/۰۵، ۰/۱، ۰/۲، ۰/۵ و ۱ درصد] بر تجمع رزمارینیک اسید و بیان ژن تیروزین آمینوترانسفراز (TAT) در دوره‌های زمانی مختلف (۴ و ۱۷ ساعت) در گیاهچه‌های بادرنجبویه بررسی شد. بر اساس نتایج، تنها غلظت‌های ۰/۰۵، ۰/۱ و ۰/۲ درصد عصاره مخمر بمدت ۱۷ ساعت تیمار، مسیر بیوسنتزی رزمارینیک اسید را بطور معنی داری تحریک کردند. در این زمان، با افزایش غلظت عصاره مخمر تا ۰/۲ درصد، محتوی فلاونوئید و بیان ژن تیروزین آمینوترانسفراز بطور معنی داری در مقایسه با شاهد افزایش یافتند و بیشترین مقدار هر دوی آن‌ها در غلظت ۰/۱ درصد عصاره مخمر مشاهده شد ، جائیکه تجمع رزمارینیک اسید نیز شدیداً افزایش یافته بود. علاوه بر این، با مشاهده بهبود رشد اندام‌های هوایی در گیاهچه-های تیمار شده با عصاره مخمر بمدت ۳۰ روز، کاهش طول ریشه مشاهده گردید. در مجموع، می‌توان نتیجه گیری نمود که این مشاهدات به القاء تنش‌های اکسیداتیو توسط عصاره مخمر پس از جذب آن توسط گیاه مربوط است، که با افزایش فعالیت سوپر اکسید دسموتاز و کاتالاز مشخص می‌شود.





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