



## Investigation of Nitrogen and Phosphate Effect on Growth and Rosmarinic Acid Production in Transgenic *Mentha aquatica* Hairy Root Induction

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### Abstract

*Mentha aquatica* L. as an important medicinal plant is known for its phenolic acids with pharmaceutical properties, especially rosmarinic acid. In order to obtain high amounts of secondary metabolites, the establishment of hairy root cultures through *Agrobacterium rhizogenes* infection is considered as a suitable alternative. In order to compare the abilities of *A. rhizogenes* strains to induce hairy roots, the leaf and shoot explants of *M. aquatica* were used for hairy root induction by five different bacterial strains (ATCC 15834, A13, R318, A4, 9534). Furthermore, the effect of phosphate, nitrogen, and reducing environmental acidity on growth and rosmarinic acid content of hairy roots were studied. The results indicated that the percentage of rooting in stem explants (50%) was higher than the leaves (42.6%). Among the bacterial strains, the strain A4 showed the highest rooting efficiency. It was also demonstrated that phosphate deficiency and increased phosphate levels significantly increased the rosmarinic acid content of hairy roots. Various effects on hairy root biomass were observed in response to different phosphate levels. However, increasing the amount of nitrogen did not affect the studied factors. It was concluded that different types of explants have different potentials of hairy root formation in interaction with bacterial strains.

**Keywords:** Hairy Roots, *Mentha Aquatic*, Phosphate, Nitrogen, Rosmarinic Acid

### Introduction

Medicinal plants of the mint family (Lamiaceae), are known as valuable sources of diverse secondary metabolites. The genus *Mentha* is one of the most important members of this family in terms of secondary metabolites production. This aromatic plant with wide distribution and significant commercial importance is cultivated in all five continents of the world [1-4]. This genus contains numerous economically significant species, such as *M. aquatica* (water mint, common mint) which is also known as *M. hirsute* Huds., *M. acuta* Opiz, and *M. acutata* [5].

Polyphenolic compounds are an important group of secondary metabolites in the mint family. Phenolic acids, such as rosmarinic acid (RA) are the main phenolic compounds that are found in high quantities in this family, especially the genus *Mentha* [6]. The biosynthesis

of RA, as an ester of caffeic acid is accomplished through the phenylpropanoid pathway with L-phenylalanine and L-tyrosine as precursor molecules [7]. RA plays an important role in the plant's defense system against fungi and bacterial infections or predators [8]. Bioactivity of RA as an antioxidant, antibacterial, antiviral, and anti-inflammatory agent has been recognized. This compound is used commercially as a preservative in the food industry [6]. Free radicals are involved in causing several diseases such as cancer and atherosclerosis by damaging the cells. RA reduces the risk of such diseases due to protective effects against cell damage [9]. Furthermore, RA has been shown to reduce inflammation in the affected areas by preventing the accumulation of C<sub>3</sub>b, a protein involved in inflammatory activities [9].

Hairy root cultures are considered as a suitable alternative for producing large-scale secondary compounds such as rosmarinic acid. The phenotype of hairy roots induced by *A. rhizogenes* is identified through rapid and hormone-

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independent growth, lack of geotropism, lateral rooting and genetic stability. In addition to being able to grow *in vitro* under sterile conditions, hairy roots can synthesize the metabolites normally found in roots and other plant organs at higher levels compared to the mother plant [10-12].

Several factors affect the growth rate and secondary metabolites content of hairy roots, including plant species, different lines of hairy roots, environmental nutrients and root environmental conditions. Among the medium nutrients, carbon, nitrogen and phosphorus play an important role in plant growth and formation of secondary metabolites [13].

In this study, the potential of *M.aquatica* for hairy root induction and production of rosmarinic acid was investigated. Moreover, the rooting capacity of different explants induced by five *A. rhizogenes* strains, the effect of pH, nitrogen and phosphorus on the growth rate of hairy roots and rosmarinic acid content were evaluated.

## Material and Methods

### Plant Materials and Explants Preparation

The cuttings of *M. aquatica* L. were collected from Hamedan, Iran and after approval by voucher specimens they were propagated and grown in greenhouse conditions. Leaf and stem segments (approximately 2 cm in length) from seedlings that were grown to a desirable level were used as explants. Firstly, the explants were washed in water containing a small amount of detergent. The sterilization of explants was carried out using the following procedure: 70 % ethanol (2 min), rinse with sterile double distilled water, 1 % solution of sodium hypochlorite (NaClO) containing one drop of Tween 20 % (3 min), followed by 4 times rinse with sterile double distilled water.

The explants were finally placed on sterile filter paper to remove excess water.

### Bacterial Cultures and Infection of Explants with *A. rhizogenes*

To prepare the bacterial suspension for inoculation, five different strains of *A. rhizogenes* (ATCC 15834, A13, R318, A4, and 9534) were grown overnight in liquid Luria & Bertani (LB) medium [14] containing 50 mg/L rifampicin at 28 °C. After centrifugation at 4500 rpm for 15 min, the bacterial pellet was gently suspended in ½ MS medium containing 100 µM acetosyringone. The leaf explants were inoculated by immersion in bacterial suspensions [15]. Bacterial culture on solid medium was used for direct inoculation of stem explants [16].

### Genomic DNA Extraction from *M.aquatica* Hairy Roots and PCR Analysis

Extraction of genomic DNA was performed using the CTAB method [17]. In order to confirm the transgenic nature of hairy roots, the presence of the *rolB* gene was approved by PCR analysis using the following specific primers:

Forward: 5'-GCTCTTGCAGTGCTAGATTT-3'

Reverse: 5'-GAAGGTGCAAGCTACCTCTC-3'

PCR analysis using *virG* gene-specific primers (F: 5'-GGTCGCTATGCGGCATC-3' and R: 5'-CCTGAGATTAAGTGTCCAGTCAG-3') was also carried out to confirm any bacterial contamination.

### Effect of Environmental Acidity, Phosphate and Nitrogen on Hairy Roots Growth

For this purpose, emerged hairy roots were grown for one month in MS medium containing 15 mM nitrogen (+N), 2.5 (+P) and 0.6 mM (-P) phosphate and pH=5.5. Root biomass and rosmarinic acid content were measured after one month in comparison to control medium (10 mM nitrogen, 1.25 mM phosphate and pH= 5.8).

### Evaluation of Rosmarinic Acid Content in Hairy Root Cultures in Response to Different Treatments Using HPLC Analysis

50 mg of oven-dried hairy roots (50 °C) was finely ground and extracted with 2 mL of 70% ethanol, followed by centrifugation at 10000 rpm for 10 min. The supernatant was passed through a 0.45 µm filter (Orange scientific) and stored as a pure extract for chromatographic analysis at 4 °C. Chromatography analysis was performed by a Knauer high-performance liquid chromatography (HPLC) system (Germany) with a C<sub>18</sub> column (250 mm × 4.6 mm, 5 µm particle size, Eurospher 100-5, Germany) in the central laboratory of National Institute of Genetic Engineering and Biotechnology (NIGEB). The mobile phase consisted of water containing 0.1% trifluoroacetic acid (solution A) and acetonitrile containing 0.1% trifluoroacetic acid (solution B). The absorbance of standard solutions and samples were measured at 210, 254, 280, 330 and 360 nm, while the flow rate was 1 mL/min. The following gradient elution program was set in 20 minutes: In the first 15 min 90% solution A, 15-17 min 90% solution B and 17-20 min 90% solution A. A stock solution (1000 ppm) was prepared by dissolving 1 mg of standard rosmarinic acid (Sigma-Aldrich, USA) in 1 mL deionized water. Moreover, in order to obtain the linear amplitude of the standard curve, the stock solution was diluted in appropriate concentrations.

### Data Analysis

The experiments were conducted based on a completely randomized factorial design with three replications

including 20 explants per replicate. The percentage of transformation was calculated as follows: (the number of explants inducing hairy root / the number of infected explants)  $\times 100$

The correlation coefficients of the calibration curves were calculated using SAS v9.1.3 software. In addition, the significant differences between the treatments were analyzed by Tukey test.

## Results

### Effect of Bacterial Strain and Explants Type on Hairy Root Induction

In this study, hairy root induction ability of different strains of *A.rhizogenes* (ATCC 15834, A13, R318, A4, and 9534) in leaf and stem explants of *M.aquatica* was determined. The results demonstrated that percentage of hairy root induction in the leaves and stems were 42.6 % and 50 %, respectively. Also, the highest rate of hairy root induction was observed in stem explants inoculated with *A.rhizogenes* strain A4. Hairy roots emerged from leaves were far weaker in quantity and quality than stem explants and after a while, they turned brown and necrosed. In comparison, induced hairy roots on stem explants were ticker and grew faster in MS medium (Fig. 1). An analysis of variance (ANOVA) (Table 1) showed that the interaction between the effects of different bacterial strains and explants types on hairy root formation was statistically significant ( $p$ -value  $< 0.01$ ). As shown in Figure 2, the comparison of means using Tukey's method indicated that the highest rate of hairy root induction was obtained in stem explants infected by strain A4. The hairy root formation rate of the strain A4 was significantly different from the other strains ( $p < 0.05$ ).

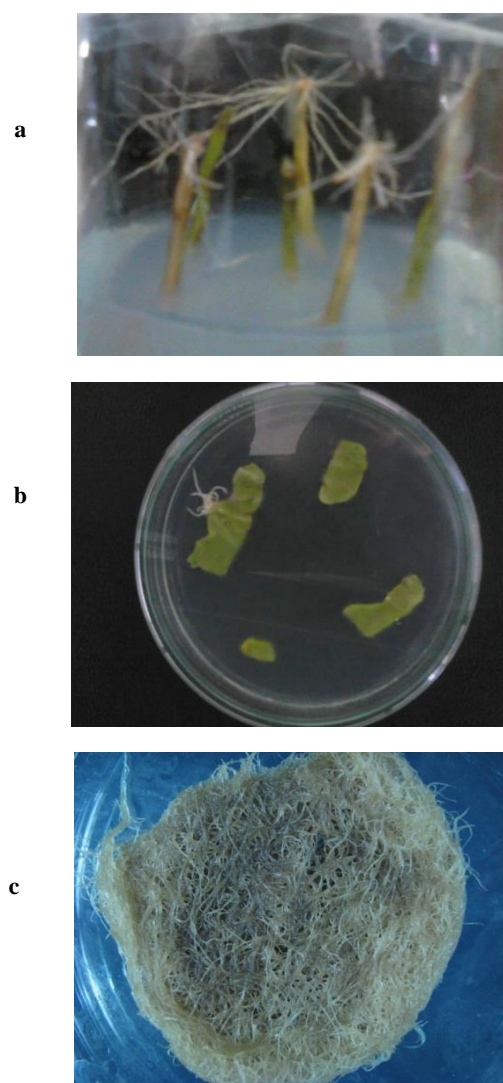
### Confirmation of Transgenic Status of *M.aquatica* Hairy Roots

Genomic DNA was extracted from hairy root samples by CTAB method to confirm the transgenic status of hairy roots. PCR analysis was performed with a specific primer pair designed to amplify a 423 bp fragment of the *rolB* gene. As shown in Figure 3, the presence of a 423 bp band indicated that the hairy roots were transgenic. PCR analysis with *virG* primers exhibited no contamination with *A.rhizogenes*.

### Effect of Acidity, nitrogen and Phosphate Concentrations in Medium on Hairy Roots Growth

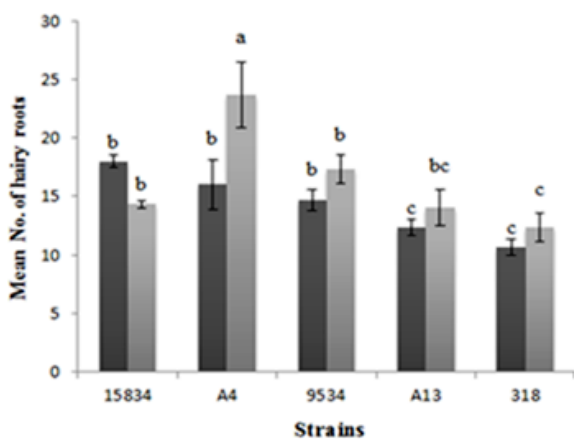
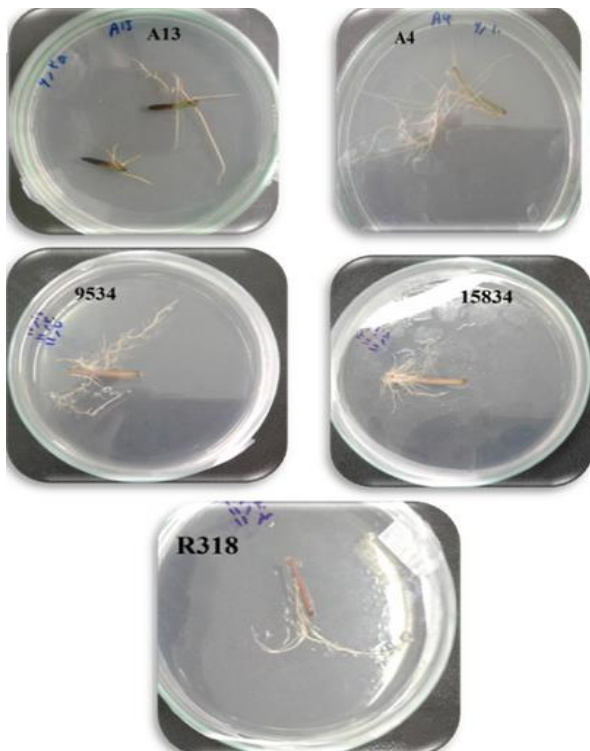
Hairy roots induced by *A.rhizogenes* strain A4 were grown in different media including liquid MS medium containing 15 mM nitrogen (an excessive amount of nitrogen), 2.5 mM and 0.6 mM phosphorous (phosphorous surplus and deficiency, respectively), and

medium with pH= 5.5. MS basal medium was considered as a control medium.



**Fig. 1** Hairy root induction in (a) stem and (b) leaf explants of water mint using *A.rhizogenes*; (c) Hairy roots growth in liquid MS medium

The dry weight of hairy root samples grown in different nutritional and environmental conditions was measured in three independent replications. The experiments were conducted as a completely randomized design and Tukey's method was used to compare means. As can be seen in Figure 4, acidic treatment and phosphorous surplus in medium (2.5 mM) significantly increased the dry weight of hairy roots. Effect of acidity, nitrogen and phosphate concentrations in medium on rosmarinic acid content of hairy roots First, the purity and retention time of rosmarinic acid were investigated by the injection of rosmarinic acid standard solution into the HPLC system at different wavelengths of 210, 254, 280, 330, and 360 nm.



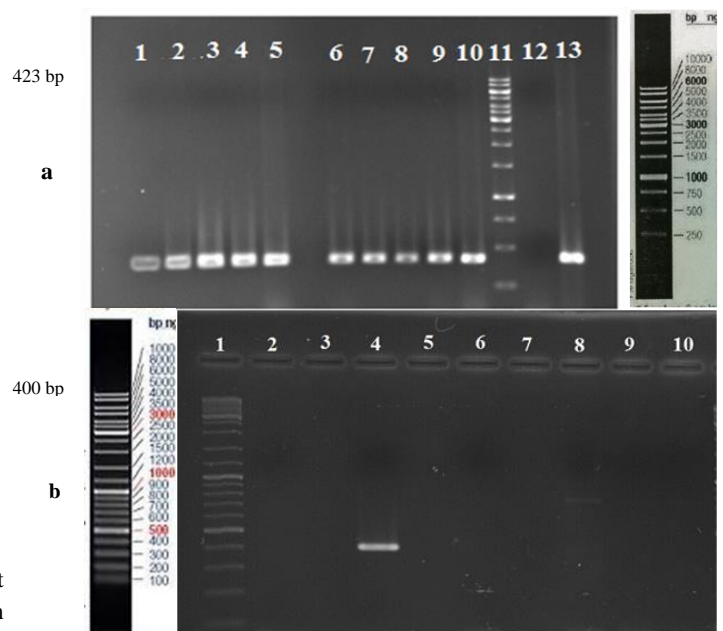
**Fig. 2** Effect of different bacterial strains on hairy root induction in leaf and stem explants of water mint. (a) Induction of hairy roots by different bacterial strains in stem explants; (b) Effect of explant types (leaf and stem) and bacterial strain on the average number of hairy roots in water mint. Each column represents the mean of three replicates. Dark gray and light gray columns represent the leaf and stem explants, respectively. Different letters above columns indicate significant differences ( $p < 0.05$ ) between treatments

The best peak shape of rosmarinic acid was obtained at 330 nm with a retention time of 24 min (Fig. 5A). The Standard curve was constructed by injecting three different volumes of rosmarinic acid standard solution in the range of 3, 5, and 7  $\mu$ l. The linearity of the curve was evaluated by linear regression analysis of the excel

software and the following regression equation was obtained:  $y = 2842.6x + 1667.5$  ( $R^2 = 0.9996$ ) (Fig. 5B)

The extract of hairy root samples, grown in different environmental conditions, was injected into the HPLC system (3 replicate injections) and the rosmarinic acid content of samples was quantified using the standard curve of rosmarinic acid. Tukey's method was used to compare the means (Table 2).

Quantification of rosmarinic acid content in hairy roots grown in different media indicated that the production level of this metabolite in medium with a phosphorous deficiency (24851.21 ng/mL) increased compared to the control medium (16543.69 ng/mL). Only a slight increase of rosmarinic acid accumulation (17498.16 ng/mL) was observed in medium with excess nitrogen however, this increase was not statistically significant. The results showed that rosmarinic acid production in media with excess phosphorous and acidic pH also increased up to 26647.02 ng/mL and 21224.13 ng/mL, respectively (Fig. 6).



**Fig. 3** (a) Confirmation of the transgenic status of water mint hairy roots by PCR reaction. 1-5: Hairy root samples cultured in different medium conditions first repeat, 6-10: Hairy roots samples cultured in different medium conditions second repeat, 11: 1 Kb molecular marker, 12: Negative control (non-transformed roots), 13: Positive control (*A.rhizogenes* plasmid). (b) PCR analysis with *virG* primers. 1: Molecular size marker, Ladder mix, 2: Negative control (non-transformed roots), 4: Positive control (*A.rhizogenes* plasmid), 5-10: Hairy roots samples.

**Table 1** Variance analysis of the interaction between the effects of different bacterial strains and explants types on hairy root formation in water mint.

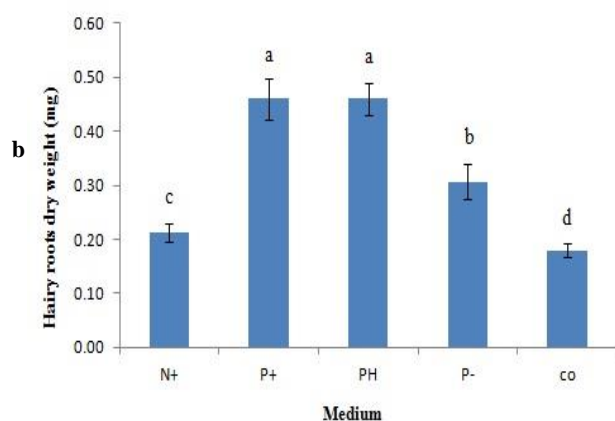
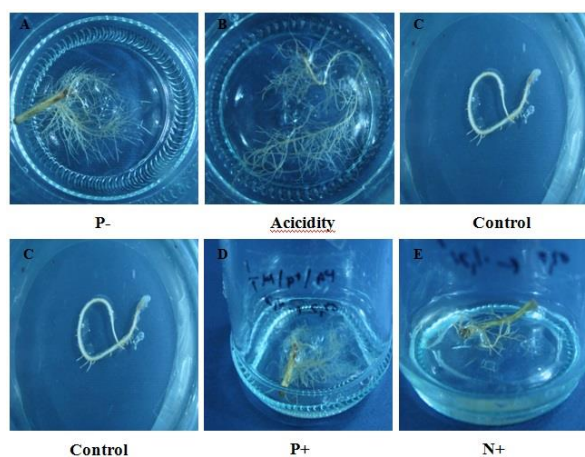
| Variability source               | Degrees of freedom (dF) | Average square      |
|----------------------------------|-------------------------|---------------------|
|                                  |                         | Rooting percentage  |
| Bacterial strain                 | 4                       | 61.16 <sup>**</sup> |
| Explants type                    | 1                       | 30.00 <sup>**</sup> |
| Bacterial strain × Explants type | 4                       | 24.33 <sup>**</sup> |
| Error                            | 20                      | 5.93                |
| Coefficient of variation (CV)    | -                       | 15.88               |

<sup>\*\*</sup> Significant at 0.01 level ( $p < 0.01$ )

**Table 2** Variance analysis of rosmarinic acid content of water mint hairy root samples grown in different media

| Variability source            | Degrees of freedom (dF) | Average square Rosmarinic acid |
|-------------------------------|-------------------------|--------------------------------|
| Treatment                     | 4                       | 39135035.2 <sup>**</sup>       |
| Error                         | 5                       | 0.1108129                      |
| Coefficient of variation (CV) | -                       | 4.92                           |

<sup>\*\*</sup> Significant at 0.01 level ( $p < 0.01$ )

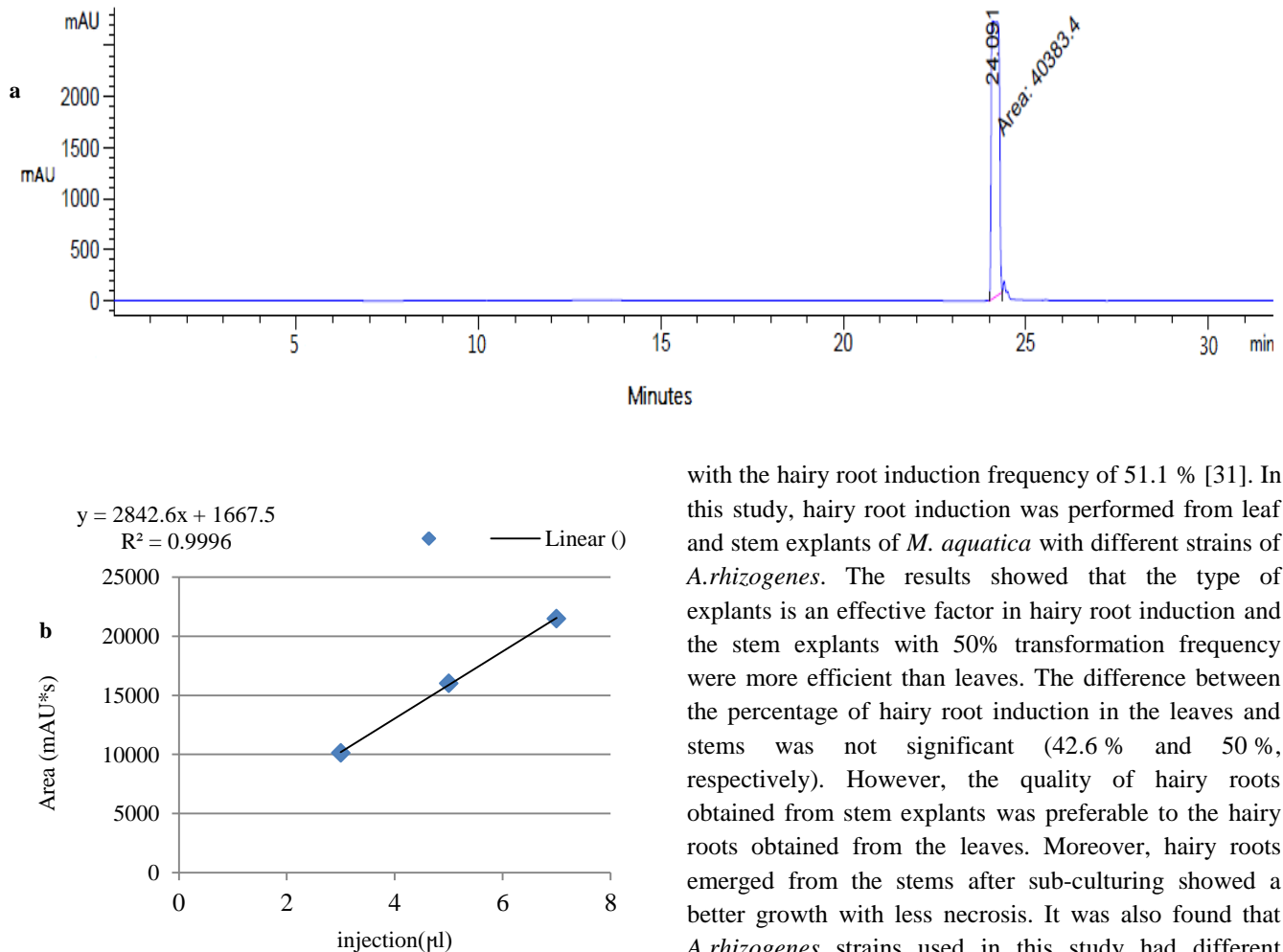


**Fig. 4** The effect of acidic condition, phosphate and nitrogen content on growth and dry weight of water mint hairy roots induced by *A.rhizogenes* strain A4 after one month of treatment. (a) Hairy root induction from stem explants of water mint in a different medium in comparison with the control condition (A- phosphate starvation, B- acidic condition, C- control, D- phosphate surplus, E- nitrogen surplus); (b) Comparison of hairy roots dry weight induced from stem explants of water mint in response to different treatments compared to control.

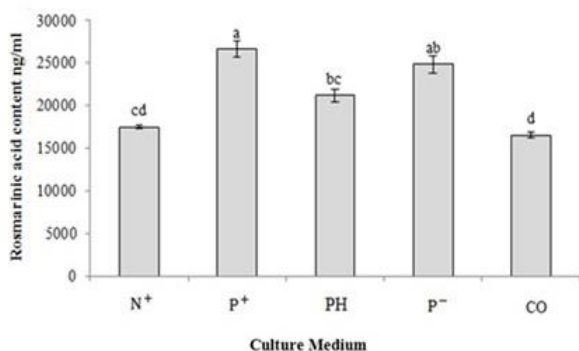
## Discussion

Plant secondary metabolites are of great interest due to their numerous medicinal benefits and properties [1]. The plants belong to the mint family have gained attention because of being a source of polyphenolic compounds such as rosmarinic acid and essential oils with various medicinal and therapeutic properties [18, 19]. The anti-cancer property of rosmarinic acid has been attracted interest from researchers in the last two decades. Among the members of the mint family, the highest content of rosmarinic acid has been observed in the genus *Mentha* [20].

Application of tissue culture techniques in medicinal plants has been introduced as an alternative for the production of valuable secondary metabolites. Recently, hairy root cultures have been suggested as a sustainable source for secondary compounds production [21,22]. *A. rhizogenes* transformation for hairy root induction have been carried out in several plant species, including many medicinal plants and the results have been indicated the high potential of hairy roots for the production of valuable pharmaceutical metabolites [16,23-28]. The aforementioned roots induced by *A.rhizogenes*, appear in different types of explants with different potentials. In other words, plant genotype and the type of explants affect hairy root generation and biomass. Moreover, different bacterial strains show different efficiencies in hairy root formation, biomass production, and metabolites accumulation [15,29,30]. Hairy roots induction by different strains of *A.rhizogenes* (13333, 15834, 9402, R1000, R1200, R1601 and A4) in leaf tissue explants of *Agastache rugosa* indicated that the strain A4 with the infection rate of 94.1 % had the best efficiency in hairy root formation [29].



**Fig. 5** (a) HPLC chromatogram of standard solution of rosmarinic acid at 330 nm; (b) Calibration curve of rosmarinic acid was constructed by three different injection volumes (3, 5, and 7 µl of the stock solution).



**Fig. 6** The effect of acidic condition, phosphate and nitrogen content on rosmarinic acid accumulation level of water mint hairy roots induced by *A.rhizogenes* strain A4 after one-month treatment. Each column represents the average of three repeats. Different letters above columns indicate significant differences ( $P < 0.05$ ) among treatments.

Nourozi *et al.* (2016) successfully induced hairy roots in *Agastache foeniculum* using A4 strain of *A.rhizogenes*

with the hairy root induction frequency of 51.1 % [31]. In this study, hairy root induction was performed from leaf and stem explants of *M. aquatica* with different strains of *A.rhizogenes*. The results showed that the type of explants is an effective factor in hairy root induction and the stem explants with 50% transformation frequency were more efficient than leaves. The difference between the percentage of hairy root induction in the leaves and stems was not significant (42.6 % and 50 %, respectively). However, the quality of hairy roots obtained from stem explants was preferable to the hairy roots obtained from the leaves. Moreover, hairy roots emerged from the stems after sub-culturing showed a better growth with less necrosis. It was also found that *A.rhizogenes* strains used in this study had different potentials in hairy root induction and the strain A4 with the highest transformation frequency (23.66 %), was selected as the most suitable strain for hairy root induction in *M.aquatica*. Various physicochemical agents have different effects on the potential of hairy root induction. Researchers have found that the components of culture medium and physical factors such as light and temperature greatly influence the production of secondary metabolites in plants [32,33].

Phosphorus plays an important role in plant growth and development. Only 20-50 % of the soil phosphorus is in the inorganic form and the rest is in the form of insoluble and inaccessible organic compounds which results in phosphate poverty in the world. Plants use different strategies to cope with phosphate deficiency. Increasing the number of secondary roots and the length of root hairs in order to enhance the phosphate uptake from the environment is an adaptation strategy that is very useful when plant roots are used as medicinal resources [34]. Metabolic changes are very critical for plant adaptation to phosphate deficiency. Primary metabolism remodeling is one of the adaptive mechanisms that have been well studied in plants in phosphate deficiency condition. The expression of genes and enzyme activities, involved in the process of carbohydrate and lipid metabolism bypasses, greatly enhance under phosphate deficiency

condition which results in improved uptake and fixation of phosphorus and subsequently increase phosphorus utilization efficiency [34]. Secondary metabolism, which plays a key role in plant response to environmental conditions, is also induced under phosphate deficiency conditions. Phosphate starvation induced the accumulation of saikosaponin in *Bupleurum chinense* DC [35] and ginsenosides in *Panax ginseng* [36]. Induction of secondary metabolism depends on the adaptive capacity of plants under phosphate deficiency [34]. In research by Uozumi *et al.* (1995), phosphate reduction in *Ajuga hairy root culture* decreased the growth rate and increased the secondary metabolite content [37]. Liu *et al.* (2018) reported that phosphate deficiency leads to the accumulation of phenolic acids in *Salvia miltiorrhiza* hairy roots. They also showed that the hairy roots of *Salvia castanea* compared to *S.miltiorrhiza* were rapidly adapted to phosphate deficiency and their root biomass increased. The production of phenolic acids was affected by the application of different phosphate levels in hairy root cultures of *S.castanea* and *S.miltiorrhiza* [34]. Treatment of 1.24 mM phosphate in *S. miltiorrhiza* hairy roots increased the lithospermic acid B content, while the content of caffeic acid and rosmarinic acid were decreased. Reduction of phosphate level up to 0.6 mM did not have a significant effect on rosmarinic acid and lithospermic acid B content in both *Salvia* species, whereas the accumulation of caffeic acid was increased [34]. Different concentrations of phosphate (0.6 and 2.5 mM) used in the present study resulted in different effects on hairy root growth, biomass, and rosmarinic acid content. An increase in the number of secondary roots and biomass was observed in samples which were grown in lower levels of phosphate (0.6 mM) than the control medium (1.2 mM) (Fig. 4). However, the total biomass of hairy roots was decreased compared to medium with higher levels of phosphate (2.5 mM) (Fig. 4). Rosmarinic acid content was significantly increased in medium with 0.6 mM phosphate (Fig. 5). Our results indicated that increasing the phosphate content of the medium (2.5 mM), resulted in improved hairy roots biomass production. Despite the increase in biomass, the rosmarinic acid content was not significantly increased compared to medium with 0.6 mM phosphate. According to the results of this study, it can be said that water mint is able to regenerate primary metabolism and demonstrates an adaptive capacity to increase the content of secondary metabolites in phosphate deficient conditions. However, the plant response to the presence of additional phosphate in medium appears to be different from *Salvia*. Due to increased phosphate uptake under acidic conditions, the biomass of hairy roots in medium with pH=5.5 was similar to the phosphate surplus condition.

Nitrogen plays a critical role in plants homeostasis and affects crop yield, growth, and development. Plants are able to stabilize the mineral form of nitrogen as nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ). The natural growth of plants is depended on the ratio between these two ions. Soil acidity, oxygenation, and humidity affect this ratio. Nitrate ions after absorption by plant roots may be reduced to ammonium or incorporated into amino acids. Subsequently after reduction, nitrate ions are transferred to the leaves, accumulated in vacuoles or moved to the apoplast. Using the reduced form of nitrogen ( $\text{NH}_4^+$ ) by plants requires less energy than the  $\text{NO}_3^-$  ion, whereas the ammonium ion uptake results in more energy consumption, which is associated with the preparation of ketoacids from the aerial parts of plants. Moreover, ammonium uptake as a sole source of nitrogen is toxic in most crops and wild plants. Metabolite production and root growth are significantly affected by the nitrogen source [38]. Dlugosz *et al.* (2018) demonstrated that the source of nitrogen has an influence on hairy root growth, accumulation and secretion of saponin in hairy root cultures of *Calendula officinalis*. Their results indicated that nitrate was more suitable than ammonium as a source of nitrogen for hairy roots. However, the nitrate effect on accumulation and secretion of metabolites was line-dependent. [38]. The rosmarinic acid biosynthesis is also affected by the nitrogen source and the ratio of  $\text{NO}_3^-/\text{NH}_4^+$ . In a study by Ilieva and Pavlov (1999), using 0.09 gr/L ammonium ions (25 % of the standard medium) in the cell culture medium of *Lavandula vera* resulted in enhanced growth and rosmarinic acid content. A two-fold increase in rosmarinic acid accumulation was observed in the cell culture medium with a 1.2-fold concentration of  $\text{NO}_3^-$ . However, the  $\text{NH}_4^+$  reduction was more effective in rosmarinic acid induction [39]. In the present study, the effect of the increase in amount of nitrogen source ( $\text{NH}_4\text{NO}_3$ ) from 10 mM to 15 mM on biomass and rosmarinic acid content of *M.aquatica* hairy roots was investigated. The results indicated a significant increase in hairy roots biomass compared to control. However, the biomass of hairy roots was significantly decreased in comparison with other treatments. Furthermore, the rosmarinic acid content in hairy roots treated with 15 mM nitrogen showed a slight increase compared to the control medium (10 mM).

## Conclusion

*A. rhizogenes* induced hairy root culture has been suggested as a sustainable source for the production of various secondary metabolites. Hairy roots are able to grow rapidly and their growth rate is often faster than plant cell cultures. Moreover, in many cases, they have a high potential for the production of secondary metabolites more than the intact plant. The bacterial strains and

explants types, as well as different physicochemical factors affect the biomass and the type of metabolites accumulated in hairy roots. According to the results of this study, the type of explants was an effective factor in rooting rate of water mint and stem explants with 50% transformation frequency were more suitable compared to the leaf explants. Additionally, among the different strains of *A. rhizogenes*, the potential of the strain A4 was higher in hairy root induction in stem explants. Phosphate application had a positive effect on increasing biomass and rosmarinic acid content, either in deficient or surplus conditions.

### Declaration of Competing Interest

The authors declare that this article has no conflicts of interest.

### Acknowledgements

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