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Pharmacochemical investigation of *Rosmarinus officinalis* L. cultivated by clonal micropropagation and hydroponic combined method

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ABSTRACT

Background: Rosemary - Rosmarinus (R.) officinalis L. is a versatile herb that plays an important role in functional foods and offers various health benefits through its bioactive compounds (BAC). Rosemary is an evergreen shrub native to the Mediterranean region and has been a popular aromatic, medicinal, and culinary herb since ancient times. Extracts and essential oils of R. officinalis are popular for their exceptional antioxidants and antibacterial and anti-inflammatory properties. R. officinalis tops the list of known natural antioxidants in the food industry. BACs from rosemary are used in various foods: edible oils, shelf-stable beverages, and meat products. Rosemary's phenolic compounds have immunostimulant and strong antioxidant properties. In addition, rosemary extract in combination with ascorbic acid has a synergistic effect with excellent antioxidant properties.

Recently, the development of in vitro tissue culture and hydroponic technologies has contributed to introducing and producing healthy planting materials of exotic, aromatic, and pharmacologically valuable plant species in many countries worldwide. In vitro micropropagation has several distinct advantages, including producing large numbers of seedlings in a relatively short time due to the usually high propagation rates. Micropropagation can be carried out at any time of the

year. The plants produced in vitro are generally free from microbial diseases and valuable genotypes can be freed from plant viruses. The method is based on utilizing the unique totipotency of plants, where tissues under the exogenous influence of physical and chemical factors initiate the formation of a completely new plant. Hydroponic cultivation of medicinal and aromatic plants is an innovative agrobiotechnology for growing plants without soil and is becoming increasingly popular. It offers many advantages that can increase the quality and sustainability of rosemary as a functional food.

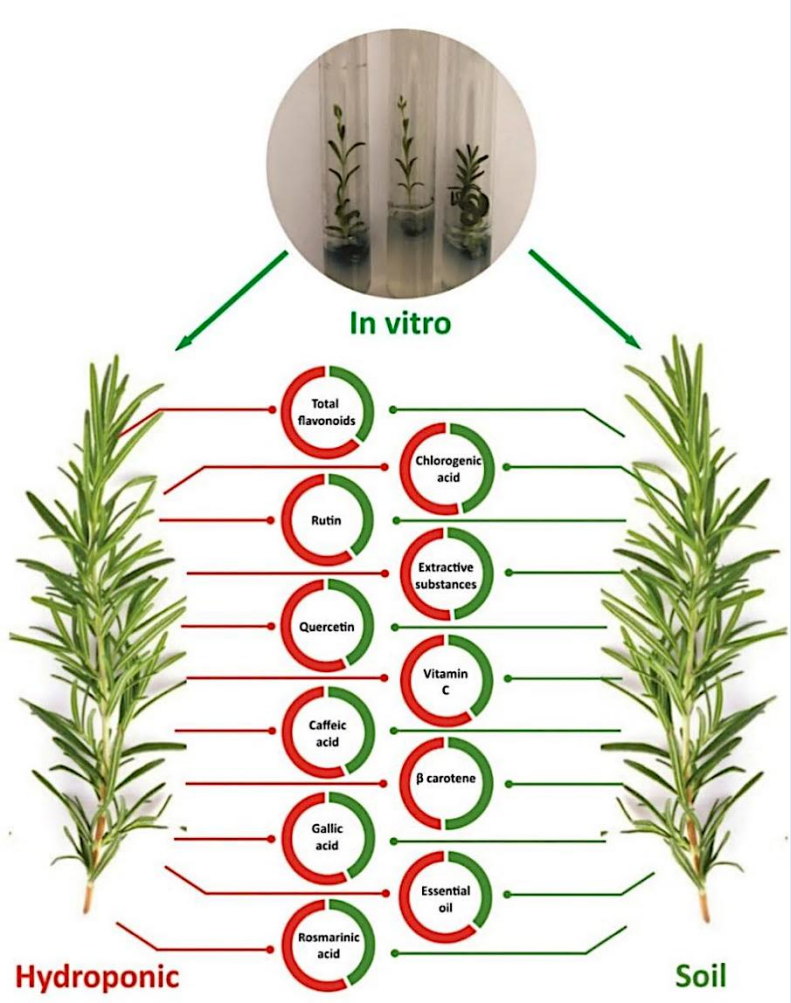
Objective: To implement a combined method of in vitro clonal micropropagation and hydroponics to obtain R. officinalis plant material with improved properties.

Methods: The experiments were carried out using tissue culture and hydroponic methods. In tissue culture, explants were propagated on Murashige and Skoog (MS) medium (pH 5.8) supplemented with various concentrations and combinations of cytokinins and auxins, carbohydrates (15-30 g/l), and agar-agar (0.6 %). Various concentrations of growth regulators: 6-Benzylaminourine (BAP), indole-3-butyric acid (IBA), and α-Naphthaleneacetic Acid (α-NAA), were used for callus induction and shoot multiplication. The plants in hydroponics were fed twice daily with Davtian nutrient solution. The content of phenolic compounds and β-carotene was determined spectrophotometrically (Agilent Cary 60 UV-Vis spectrophotometer) and the vitamin C content was determined by titrimetric method. The essential oil of R. officinalis was isolated by water distillation from fresh plant material during the flowering period.

Results: The results showed that concentrations of 0.5 mg/L α-NAA and 1.0 mg/L BAP in MS medium stimulated callogenesis, followed by organogenesis with 5-6 adventitious shoots. Clonal micropropagation on MS medium with half macro- and micronutrient concentrations of 0.2 mg/L and 0.3 mg/L IBA stimulated 80 and 95% root formation, respectively. The survival rate during the acclimatization of rosemary microplants in outdoor hydroponics and soil conditions was 89 and 70%, respectively. In hydroponic plants, the content of total flavonoids, rutin and quercetin exceeded that of soil-grown plants by 1.5, 1.4 and 1.2 times respectively. In hydroponics and soil rosemary, the phenolic acids formed the following decreasing series: chlorogenic acid > rosmarinic acid > gallic acid > caffeic acid, with chlorogenic acid exceeding rosmarinic, gallic and caffeic acids by 1.4 and 1.5 times; 2.4 and 2.5 times; 2.5 and 2.8 times, respectively. Vitamin C content was 1.3 times higher in hydroponic plants of R. officinalis than in soil-grown plants, while the content of β-carotene showed no significant differences. In the fresh leaves of hydroponically grown rosemary the essential oil content was 0.2 %, which is 1.7 times higher than in soil-grown ones.

Conclusion: The combined method of clonal micropropagation and hydroponics can produce healthy rosemary plant material, which can serve as a rich source of BAC and essential oil for use in antioxidant herbal teas, spices, food supplements, cosmetics, etc.

Keywords: in vitro culture, micro plants, BAC, flavonoids, phenolic acids, essential oil



Graphical Abstract: Pharmacochemical investigation of Rosmarinus officinalis L. cultivated by clonal micropropagation and hydroponic combined method.

INTRODUCTION

Rosemary (*Rosmarinus (R.) officinalis* L.) is a medicinal plant from the Lamiaceae family. Rosemary has long been valued for its culinary applications and remarkable health benefits, leading to its widespread inclusion in the functional food sector [1-3]. Rosemary is an aromatic, medicinal and culinary herb of universal importance and is rich in phytochemicals that contribute to its powerful antioxidant, anti-inflammatory and antimicrobial properties. Rosemary is a rich source of bioactive compounds (BAC), including rosmarinic, chlorogenic,and caffeic acids, flavonoids, tannins, vitamins (B2, B6, C), carotenes and essential oil which play a crucial role in promoting human health [4-5]. *R. officinalis* is a potential functional ingredient with beneficial properties in several chronic human diseases. Rosemary's BACs help fight

oxidative stress and inflammation, promote overall wellness and reduce the risk of chronic diseases such as heart disease, diabetes, and cancer. In addition, rosemary is thought to enhance cognitive function, potentially improving memory and concentration, which is particularly beneficial in today's fast-paced world [6-9]. Rosemary essential oil has hepatoprotective potential [10]. According to the literature [11], the combination of curcumin and rosemary essential oil enhanced the hepatoprotective effect, indicating the synergism of these two components. The therapeutic properties of this plant are also used in treating Alzheimer's disease [12]. Rosemary is one of the most powerful natural antioxidants in medicine and the food industry. [13-14]. Its extracts and essential oils are widely used in various foods: edible oils, shelf-stable beverages and meat products [15-16]. Rosemary extracts and essential oils can be useful to replace or even reduce synthetic preservatives in food. The phenolic compounds of rosemary have immunostimulant and strong anti-cancer properties. [17]. Rosemary is also used medicinally for the treatment and prevention of dyspepsia, mild cramps, migraines, depression, respiratory disorders, mild peripheral circulatory disorders, and muscle and joint pain [18].

Significant advances and developments in science, agriculture and agrobiotechnology in many countries around the world have promoted the use of plant tissue culture and hydroponic technologies [19-21]. The combination of these technologies facilitates the introduction of valuable, agriculturally and pharmacologically important species as well as the propagation of virus-free plant material and the increase of plant productivity [22-23].

The aim of this work is to use combined methods of *in vitro* micropropagation and hydroponics to obtain healthy rosemary plants with improved pharmacochemical qualities. Therefore, the development of biotechnology for growing rosemary by this combined method in Armenia will make it possible to grow genetically identical healthy plants and obtain high-quality plant material with high BAC content, which is relevant for the fields of functional food and medicine.

MATERIALS AND METHODS

The experiments were carried out at the Experimental Station and in the Tissue Culture Laboratory of the Institute of Hydroponics Problems (IHP), in the Ararat Valley, using methods of culturing plant tissues (*in vitro*) and hydroponics. To introduce rosemary into aseptic *in vitro* culture conditions, explants (apical part of stems, meristem) were pre-sterilized with 96% ethanol for 1 minute and then with 10% hydrogen peroxide (H_2O_2) for 20 minutes. The sterilized explants were then washed 3 times with sterile distilled water for 15 minutes. Under *in vitro* conditions, explants were passaged on Murashige and Skoog (MS) [22] medium (pH 5.7 - 5.8) supplemented with different concentrations and combinations of growth regulators (6-Benzylaminopurine (BAP), Indole-3-Butyric Acid (IBA), ~~and~~ α-Naphthalene Acetic Acid (α-NAA)), carbohydrates (15-30 g/L) and agar-agar (0.6%) for callus induction and shoot proliferation [23]. Microplants and tissues were incubated in a phytotron chamber at 20 - 25 C° with a photoperiod of 16 / 8 h and an illumination of 3 000 – 5 000 lux (Fig. 1a).

For hydroponics, the EBB and Flow system with automatic irrigation was used (Fig.1b).

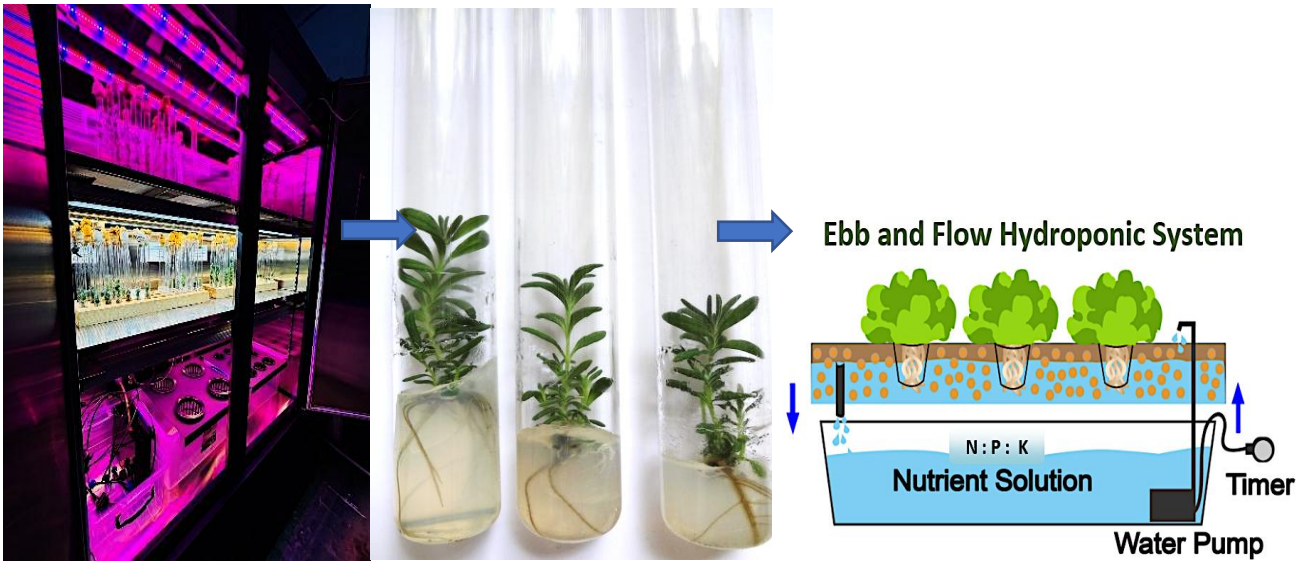


Figure 1: Rosemary micro-plants in phytotron chamber (a) and EBB and Flow hydroponic system (b)

Microplants (Fig. 1a) were used as a planting material in hydroponics and soil. Acclimatized microplants were planted in hydroponics and soil at a density of four plants per square meter. Black volcanic slag was used as the hydroponic substrate. In hydroponics, plants were fed twice daily with Davtyan nutrient solution (N 200 mg/L, P 65 mg/L, K 350 mg/L; pH 5.7-6.3; EC 1.0-1.1 mS/cm) [24, 26]. Artesian water was used as the nutrient solution. The aerial parts (20 cm from the top) of *R. officinalis* (Fig. 2a, b) were harvested from June to September.

Figure 2: Hydroponic (a) and soil (b) cultures of *R. officinalis*.

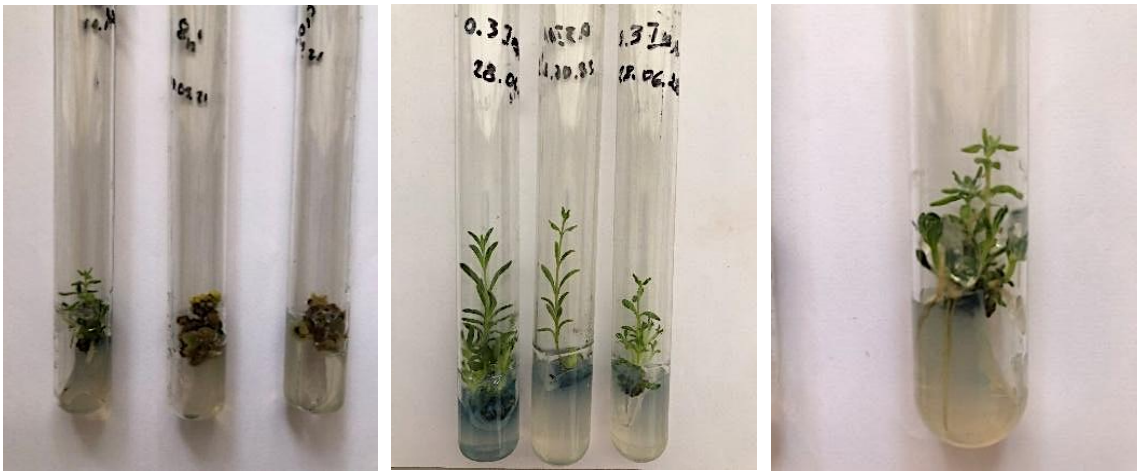
Pharmacochemical, Phytochemical Studies: The content of total flavonoids, rutin, quercetin, and phenolic acids in 70% ethanol extract of air-dried plant material of *R. officinalis* was determined by spectrophotometric method [24-25].

Vitamin C and β-carotene were determined in fresh leaves [26]. Essential oil of *R. officinalis* was isolated by hydro-distillation from fresh plants of *R. officinalis* during the flowering period [27-28]. Analyses were performed in three replicates.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 8 software (t-test). Experimental results are presented as mean ± standard deviation (SD) in at least three independent replicates.

RESULTS AND DISCUSSION

According to the results, concentrations of 0.5 mg/L α-NAA and 1.0 mg/L BAP in MS medium stimulated callus formation (Fig. 3 a) and organogenesis of up to 6 adventitious shoots (Fig.3b, c).



a. **b.** **c.**

Figure 3: Callus tissue (a), adventitious shoots (b), and rooted regenerated shoots (c) of *R. officinalis* in *in vitro* culture

Micropropagation on MS medium with half the reduced macro- and micronutrients and an IBA concentration of 0.2 mg/L and 0.3 mg/L stimulated root formation (Fig. 3c, Fig. 4a) by 80% and 95%, respectively. Survival rates for acclimatization of rosemary microplants under outdoor hydroponic conditions (Fig. 4 b) and soil (Fig. 2 b) were 89% and 70%, respectively.

a. **b.**

Figure 4: Rooted micro-plants of *R. officinalis* in *in vitro* culture (a) and transplanted in outdoor hydroponics (b).

The therapeutic efficacy of medicinal plants is determined by the BAC they contain. The study of the content of BAC in the plant materials of *R. officinalis* was carried out following the requirements of the State Pharmacopeial article [27].

The formation and accumulation of BAC in plants is a dynamic process that changes during the ontogeny of the plant and depends on numerous environmental factors. The natural climatic factors (seasonal variation) [29] and growing conditions [30] have a determining influence on the chemical composition of *R. officinalis* plants.

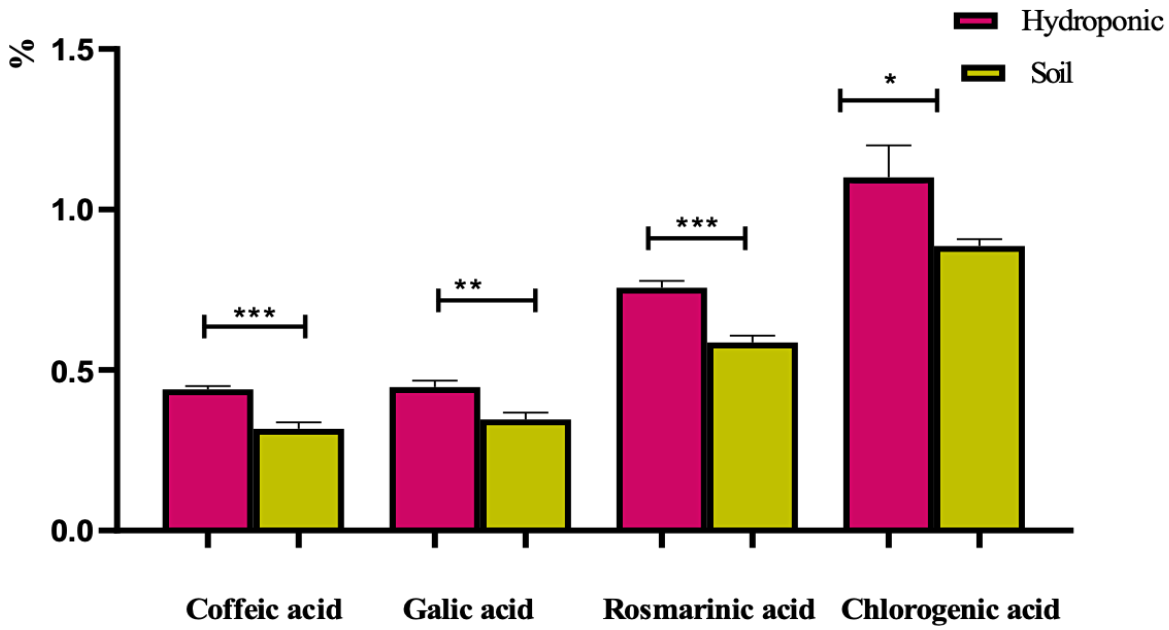


Figure 5: The content of phenolic acids in hydroponic and soil *R. officinalis*:
* P < 0.05, ** P < 0.01, *** P < 0.001

The pharmacochemical analysis of medicinal raw material, presented in Figure 5 shows, that the content of phenolic acids in *R. officinalis* did not depend on the growing conditions and formed an identical decreasing series: chlorogenic acid > rosmarinic acid > gallic acid > caffeic acid. The content of chlorogenic, rosmarinic, gallic, and caffeic acids in the studied soil and hydroponic medicinal raw materials ranged from 0.89 – 1.1%, 0.59 – 0.76%, 0.35 – 0.45%, and 0.32 – 0.43%, respectively. Thus, chlorogenic acid exceeded rosmarinic, gallic, and caffeic acids by 1.4; 2.4; 2 and 1.5; 2.5; 2.8 times respectively in hydroponic and soil plant raw materials. In addition, the content of chlorogenic, rosmarinic, gallic and caffeic acids was 1.3 times higher in hydroponic rosemary than in soil plants (Fig. 5). The seasonal variation has a significant influence on the accumulation of some polyphenols in *R. officinalis*, for example, according to the literature data, rosmarinic acid content was higher in the winter period (28.23 mg/g of dry extract (d.e.)), and significantly lower in summer (15.69 mg/g d.e.) [29]. In our experiments, the range of its content in June - September was 20.77 - 25.16 mg/g d.e..The extraction yield in the medicinal raw material grown in hydroponics (30.2%) was 1.1 times higher than in the plants grown in soil (Fig. 6 b). Interestingly, the data (11.2%) in the literature [31] were much lower than ours.

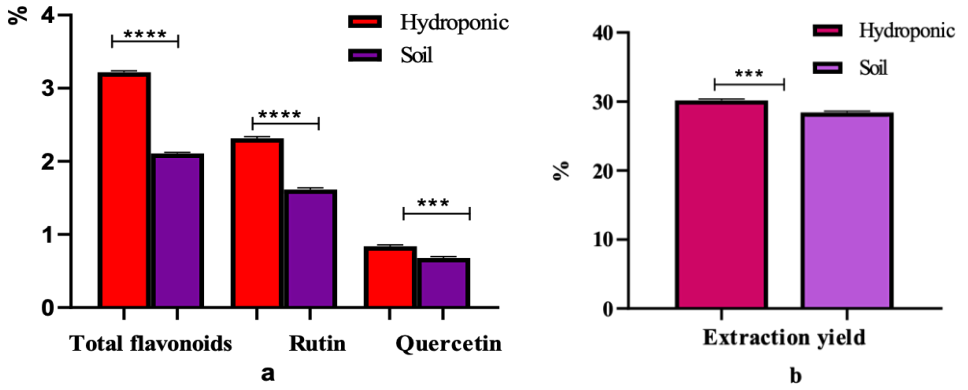


Figure 6: The content of flavonoids (a) and extraction yield (b) in hydroponic and soil *R. officinalis*: *** P < 0.001, **** P < 0.0001

It was found that the cultivation method (hydroponic and soil) significantly affected the content of total flavonoids, rutin, and quercetin, which ranged from 2.11 - 3.22%, 1.62 - 2.32% and 0.68 - 0.84%, respectively (Fig. 6 a). The content of total flavonoids, rutin and quercetin in the hydroponic *R. officinalis* is 1.5, 1.4 and 1.2 times higher than in the soil variant, respectively. The rutin content was 2.8 times higher than quercetin in the hydroponic plants and 2.4 times higher in the soil plants (Fig. 6). Studies by some authors [32-36] showed that the chemical composition of propagated lines of *R. officinalis* varied dramatically depending on the place of growth: some were characterized by a high content of rutin (34.62 mg/100g d.e.) and quercetin (11.56 mg/100g d.e.) [37], others by a low content of total flavonoids (6.5 mg/g d.e.) and phenolic compounds, but a high content of triterpenoids (71.7 mg/g d.e.) and rosmarinic acid (46.3 mg/g d.e.) [31]. However, it is unclear whether this is related to the age of the plant or the stress, environmental, or genetic factors [31, 38].

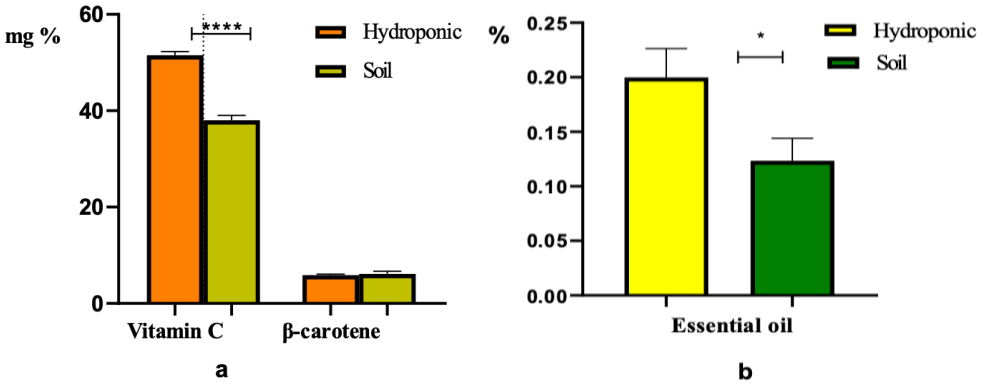


Figure 7: Vitamin C, β-carotene (a) and essential oil (b) content in the fresh leaves of *R. officinalis* in hydroponics and soil: * P < 0.05, **** P < 0.0001

Phytochemical analysis of *R. officinalis* showed that hydroponic plants (51.5 mg%) had 1.3 times higher vitamin C content than soil plants, and they did not differ significantly in β-carotene content (Fig. 7 a). The flowering period of *R. officinalis* lasts from August to March under hydroponic conditions in the Ararat Valley (Armenia). It was found that the content of essential oil in fresh hydroponic plant material (0.2%) collected in August-September was 1.7 times higher than in soil plants (Fig. 7 b). Rosemary extract in combination with

vitamin C has a synergistic effect with high antioxidant activity [39], and phenolic compounds can enhance the antimicrobial properties of the essential oil, making *R. officinalis* an effective preservative in the food industry [14-16, 30]. In addition, these compounds may work together to provide protection against pathogens and promote general human health [32, 35].

CONCLUSION

The development of biotechnology of a combined method of *in vitro* cultivation and hydroponics allows obtaining both healthy and genetically homogeneous plant material of *R. officinalis* with a high content of BAC. The results can serve as a practical basis for introducing the combined method of *in vitro* culture and hydroponics into production. Furthermore, the *R. officinalis* plant material can be utilized simultaneously for culinary, functional food, and medicinal purposes, maximizing its versatility and value.

Abbreviations: BAC - biologically active compounds; BAP - 6-Benzylaminopurine; d.e. - dry extract; IBA - Indole-3-Butyric Acid; IHP - Institute of Hydroponics Problems; MS - Murashige and Skoog; α-NAA - α-Naphthaleneacetic Acid; *R. officinalis* - *Rosmarinus officinalis* L.

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Authors' Contribution: All authors contributed to this study.

Competing interests: The authors declare no conflict of interest.

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