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EXOGENOUS CYTOKININS APPLICATION INDUCES CHANGES IN STOMATAL AND GLANDULAR TRICHOMES PARAMETERS IN ROSEMARY PLANTS REGENERATED *IN VITRO*

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ABSTRACT

Rosemary (*Rosmarinus officinalis* L.) is a native Mediterranean herb, used to typically prevent oxidation of fats and oils in foods and cosmetics. Rosemary represents an effective source of natural antioxidants with analgesic, diuretic, digestive and anti-inflammatory effects. This study is carried out to properly evaluate if exogenous cytokinin applications typically led to hyperhydration symptoms or any histological variability in stomatal density, stomatal distribution, glandular trichomes density and size of leaf epidermis. The colloidal technique was used for observation and measurement of leaf epidermis characters. The plantlets during *in vitro* culture were developed on MS media and two different PGRs ratios (BAP) / auxin (NAA) were tested: (i) 1 mg l^{-1} / 0.1 mg l^{-1} (ii) 2 mg l^{-1} / 0.1 mg l^{-1}. *In vitro* leaves of both categories were taken for analyses 4 weeks after the first subculture and were compared with *in vivo* ones. The study showed that *in vivo* plantlets have hypostomatic leaves, meanwhile, *in vitro* plantlets of both categories consist of amphistomatic leaves. In all cases, there are observed anisocytic stomata. *In vitro* leaves have a higher stomatal density, value which is increased in higher concentrations of BAP and thus transpiration rates are affected. *In vitro* specimens show about 3 times higher trichomes density in comparison with *in vivo* ones, but their diameter is about 2 times lower. Glandular trichomes size and density are affected by BAP concentration in nutrient media. These results convincingly show that *in vitro* culture can be used successfully to improve secondary metabolites production by intentionally altering the growing physical and chemical conditions.

Keywords: rosemary, in vitro propagation, cytokinins, glandular trichomes

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a native Mediterranean herb, used to prevent oxidation of fats and oils in foods and cosmetics. Rosemary represents an effective source of natural antioxidants with analgesic, diuretic, digestive and anti-inflammatory effects **(Vallverdú-Queralt *et al.*, 2014)**. Presently, the increasing demand for secondary metabolites has intensified the practical application of *in vitro* culture methods to increase the amount of these products in micropropagated plants under controlled environmental conditions. With great interest is finding a suitable micropropagation protocol, especially in the ratio of growth regulators, to enable high productivity. In such cases, there would be variations between *in vivo* and *in vitro* plantlets about the density of trichomes and secondary metabolic production. In this case, these might be constructive variations as they help to get the most productive plant lines **(Jain, 2001; Gaspar *et al.*, 2002)**.
Various culture conditions as the use of sealed vessels to prevent microbial infection and of the artificial nutrient media, the reduced intensity of light, etc. are factors that lead to the formation of plantlets with altered morphology, physiology and anatomy in comparison with *in vivo* plants. The differences refer to the shape of the leaf, cuticle structures, density and morphology of trichomes, distribution and function of the foliage, formation of the calcium oxalate crystals, etc. **(Hazarika, 2006; Roberts *et al.*, 1994; Soukup *et al.*, 2004)**. Many of these changes may be responsive to stress caused by *in vitro* culture conditions as a result of which the first symptoms of hyperhydration occur. Such changes are considered as adaptations to the different external conditions during *in vitro* propagation **(Kevers *et al.*, 2004)**.
The aim of this research is to develop an *in vitro* propagation protocol to improve secondary metabolites production by modifying PGRs ratio in the nutrient media. Epidermal characters (stomata and glandular trichomes) of *in vivo* and *in vitro* plantlets of *Rosemary* L are evaluated and compared to test if exogenous cytokinin concentrations led to any morphological and histological variability, especially in glandular trichomes density and diameter.

MATERIAL AND METHODS

Plant material and *in vitro* conditions

The mature Rosemary plants were carefully collected during March from Dajti Mountain, Tirana, Albania. For *in vitro* culture, after surface sterilization, the shoot tips were cultured on MS media **(Murashige and Skoog, 1962)**. Two different ratios of BAP / NAA were tested: (MS I) 1 mg l^{-1}/0.1 mg l^{-1} and (MS II) 2 mg l^{-1}/0.1 mg l^{-1}. At least 20 replicates were used for each treatment and both treatments were repeated 3 times. The media was supplemented with sucrose 3% and agar-agar 0.55%. pH value was adjusted at 5.5 before autoclaving. The cultures were maintained under 16 h light and 8 h darkness. The nutrient medium for each treatment contained the same hormonal combination during inoculation and subculture stage. *In vitro* leaves were taken for analyses 4 weeks after the first subculture.

Epidermal characters evaluation

For the comparative assessment between *in vivo* and *in vitro* plants, density of stomata and glandular trichomes on the upper leaf epidermis was evaluated. Regarding stomata's distribution, evaluation was made for both leaves surfaces. Samples were obtained via the colloidal technique which enables observation of stomata and glandular trichomes impressions in the slide utilizing an optical microscope. For each variant are analyzed at least 5 leaves with five microscopic fields each of them. For microscopic measurement was taken the third or the fourth leaf from the top. The measurements of stomata and glandular trichomes for *in vivo* samples were realized using a 10x magnification, meanwhile for *in vitro* ones a 40x magnification. Photomicrographs of the micrometer objective (10 µm) in 10x and 40x were used for the calibration of microscopic fields via Optika Vision 3.4 program. The calibration was realized by calculating the diameter of the microscopic field in 10x and in 40x, respectively 1867 µm and 450 µm. After the counting of stomata and glandular trichomes, their

density/mm^2 was calculated. Terminologies of stomatal complex types used in this research are those of **Dilcher (1974)** and **Wilkinson (1979)**.

Statistical analyses

Results were analyzed by Student's Test and Analyses of Variance ($P < 0.05$). All data, presented as means associated with the standard error, are carefully evaluated by computer using the statistical evaluation program JMP 7.0.

RESULTS AND DISCUSSION

Stomata distribution, type and density of *in vivo* plants and *in vitro* treatments

In vivo and *in vitro* leaves were examined for stomatal distribution on the two sides of leaf epidermis. From microscopic evaluation, it could be observed that *in vivo* plants are hypostomatic because stomatal complexes are present only in the lower leaf epidermis. *In vitro* leaves, in both treatments, result to be amphistomatic because stomatal complexes are present in the upper and the lower epidermis. As it can be seen from figure 1 a, b, the type of stomatal complex is anisocytic (with three accompanying cells surrounding the guard cells)

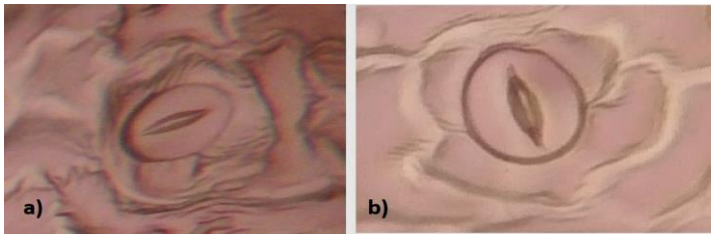

Figure 1 Anisocytic stomata in the upper epidermis of rosemary plantlets grown under *in vitro* conditions a) MS I b) MS II

Regarding stomatal distribution, the present data are supported by other reports in the literature **(Munné-Bosch *et al.*, 1999; Ullah *et al.*, 2013)**. Such distribution is considered as ecological adaption to prevent the significant loss of water from leaves during hot and dry days. However, depending on natural growth conditions, in many habitat types can be found rosemary plants which have amphistomatic leaves due to high air humidity **(Mbagwu *et al.*, 2008)**. Differences in stomata distribution relate not principally to the culture conditions but also to the nutrient content. Such results are reasonable given that *in vitro* plantlets grow in high air humidity conditions, while during *in vivo* growth conditions the rates of transpiration are high **(Kozai and Smith, 1995, Pospisilova *et al.*, 1999)**.

Another parameter monitored in the upper leaf epidermis for *in vitro* plantlets is stomatal density in both hormonal treatments during *in vitro* culture. From the results it could be noticed a considerable difference in this parameter between plants grown in MS I and the ones grown in MS II. Stomatal density increases considerably with the increase of BAP concentration in the nutrient media (Tab 1).

Table 1 Evaluation of stomata in the upper leaf epidermis for the three categories under study

	Distribution of stomata	Stomatal density/microscopic field (40x) S = 0.159 mm^2	Stomatal density /mm^2
In vivo	Hypostomatic	-	-
***In vitro* MS I**	Amphistomatic	1.44 ± 0.57	9.06
***In vitro*MS II**	Amphistomatic	6.64 ± 1.62	42.26

Note: *The values not connected by the same letter are very different between them*

Plants regulate the intensity of transpiration depending on the physical-biological changes by modifying many physiological and molecular mechanisms. The rate of transpiration is mainly regulated by the guard cells, through changes of the stomatal size and density **(Osakabe *et al.*, 2014; Nir *et al.*, 2014)**. Increased concentration of cytokinins in culture medium increases the values of transpiration. This is justified because cytokinins stimulate cytokinesis of plant cells and increase stomatal density **(Miller *et al.*, 1956; Schaller *et al.*, 2014)**. According to **Farber *et al.*, (2016)** it is proposed that under dry conditions the levels of endogenous cytokinins decrease. In these conditions growth and stomatal density are reduced. Consequently, the values of transpiration are also reduced, which improves survival of plants under dry conditions.

Trichomes density and diameter of *in vivo* plants and *in vitro* treatments

In adult plants and *in vitro* regenerated ones are observed glandular and non-glandular trichomes in the upper leaf epidermis. The glandular trichomes are categorized as peltate and capitate. Peltate trichomes consist in a four-celled head and up to 8 basal cells. Capitate trichomes are of different types. They might consist in a single-cell head structure or in e bicellular one, and in both cases with a mono or bicellular stalk, as observed in figure 2. Characteristic is the presence of oxalate crystals in the upper leaf epidermis in the three categories under study.

For all types of glandular trichomes (peltate and capitate) was evaluated their density and diameter in *in vivo* and *in vitro* treatments. From data processing results that in terms of glandular density, *in vitro* plantlets in both treatments represent significant statistical differences compared to the plants grown *in vivo*. The density in both *in vitro* treatments is about 3 times higher than in *in vivo* grown plantlets (28 glands/mm^2). By comparing *in vitro* treatments, results that MS II medium with more excessive concentrations of cytokinins induces a higher density of glandular trichomes than MS I (79.75 and 74.47 glands/mm^2 respectively).

Regarding the diameter of glandular trichomes, the differences between *in vivo* and *in vitro* leaves samples are significant, but there is an opposite relationship in comparison to the density. In *in vivo* plantlets, is observed a two times higher glandular trichomes diameter (28.62 µm), compared to plant leaves grown in MS I and MS II (13.30 and 14.07 µm respectively). Regarding *in vitro* treatments, MS II plantlets show a moderately higher diameter than those cultivated in MS I. Statistical calculations show a profoundly significant variation between in vivo and in vitro MS I and MS II plantlets for both parameters under study (Tab 2, figure 3).

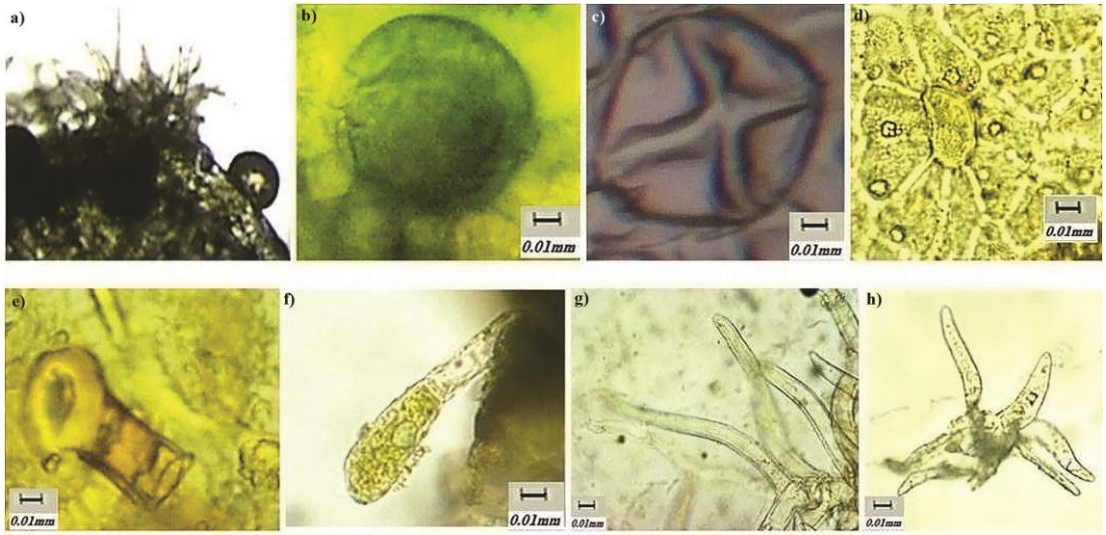

Figure 2 Types of trichomes observed in *Rosmarinus officinalis* L. plants under *in vivo* and *in vitro* conditions: a) stereomicroscopic view of peltate and non-glandular trichomes b) Peltate trichome c) peltate trichome with with four-celled head d) peltate trichome with 8 basal cells and presence of oxalate crystals e) capitate trichomes with two-celled head and bicellular stalk f) capitate trichomes with single-celled head and a bicellular stalk g, h) non-glandular furcate trichomes

Table 2 Evaluation of stomata and glandular trichomes in the upper leaf epidermis for the three categories under study

	Glandular trichomes density/microscopic field (10x)	Glandular trichomes density/mm^2	Glandular trichomes diameter (10x) (µm)
In vivo	77.90 ± 1.32 **A**	28	28.63 ± 0.21 **A**
***In vitro* MS I**	203.68 ± 8.69 **B**	74.47	13.31 ± 0.42 **B**
***In vitro*MS II**	218.08 ± 7.75 **B**	79.75	14.07 ± 0.35 **B**

Note: *The values not connected by the same letter are very different between them*

As noted by the results, the culture conditions have a considerable influence on both diameter and density of glandular trichomes. *In vitro* grown plantlets show a density of glandular trichomes three times higher and a diameter two times lower than *in vivo* plantlets. The obtained results suggest that a fundamental reason causing these changes is the presence of exogenous cytokinines in the nutrient medium during *in vitro* cultivation.

Presence and accumulation of essential oils are associated with the presence of specialized structures such as oil glands, secretory cavities, etc. According to **Gottlieb and Salatino (1987)** the production of essential oils and the formation of secretory structures are two processes closely related to one another. Consequently, secondary metabolites production is highly influenced by endogenous factors (stage of parent plant development or any specific organ, etc.), as well as by exogenous factors (biotic and abiotic) **(Sangwan *et al.*, 2001; Lima *et al.*, 2003; Gobbo-Neto and Lopes, 2007)**. These reports show that photosynthesis, photoperiod, light quality, climatic and seasonal changes, nutrition, humidity, salinity, temperature, growth regulators, and so on, are factors that affect the production of essential oils, both quantitatively and quantitatively.

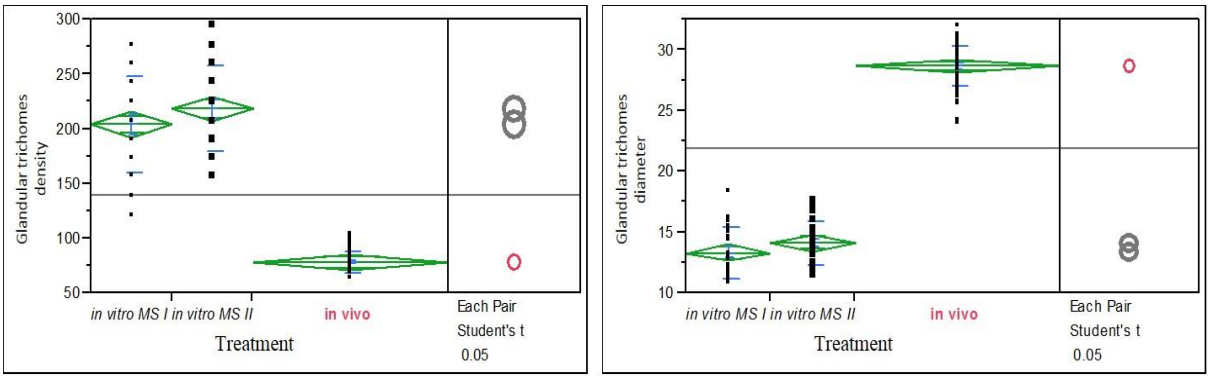

Figure 3 Oneway analysis for the three categories under study **a)** glandular trichomes density **b)** glandular trichomes diameter

Many studies have demonstrated differences in the density of trichomes or in the quantity of essential oils they contain by comparing *in vivo* plants with *in vitro* ones**. Farooqi and Shukla (1990)** point to the fact that growth regulators and/or plant hormones stimulate plant growth and biosynthesis of terpenes in a considerable number of aromatic-medicinal species, resulting in beneficial quality changes and quantity.

According to the reports of **Vantu and Gales (2009)** the density of the oily glands was greater both in the upper and lower epidermis of *Chrysanthemum morifolium* Ramat. Analysis of essential oils of *Curcuma longa* L. **(Kuanar *et al.*, 2009),** *Valeriana officinalis* L. **(Tousi *et al.*, 2010),** *Valeriana glechomifolia* Meyer **(Salles *et al.*, 2002)**, *Valeriana edulis* ssp. Procera **(Castillo *et al.*, 2002)**, *Salvia officinalis* L. **(Avato *et al.*, 2005)** propagated *in vitro* show an increase in their quantity and quality compared to those grown *in vivo*.

Somaclonal variations of a specific type can be extremely important in improving certain plant lines **(Jain, 2001)**. This trend is being used more and more today, especially in the use of tissue culture for the production of secondary metabolites. Thus, *in vitro* culture stress can cause constructive and destructive processes, and it is not only a selection factor but also a driving force to improve ecological adaptation processes **(Gaspar *et al.*, 2002)**.

CONCLUSION

Increased concentration of cytokinins in the nutrient medium induces morphological and physiological changes in leaf epidermis characters. The density of stomata increases significantly with the increase of BAP concentration in the nutrient media. *In vitro* grown plantlets show a density of glandular trichomes three times higher and a diameter two times lower than *in vivo* plantlets and these parameters increase in higher concentrations of BAP. It can be reasonably concluded that altering *in vitro* physical and/or chemical growth factors led to effective changes in epidermal characters responsible for secondary metabolites production. There are needed further studies to carefully analyse if fundamental changes in diameter and density of glandular trichomes affect positively or not the quality and/or quantity of essential oils.

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