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# Effects of Different Plant Hormones on *Salvia officinalis* Cultivated *in vitro*

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**Abstract:** Nodal explants of *Salvia officinalis* were grown on Murashige and Skoog (MS) media supplemented with various concentrations of naphthyl acetic acid (NAA), indole-3-butyric acid (IBA), benzylamino purine (BAP) and kinetine to induce adventitious shoots regeneration and micropropagation. The highest rate of growth was achieved on MS media supplemented with BAP (2.22 μM) (B05) and with kinetine (4.65 μM) and NAA (2.68 μM) (KN). The rooting process could be initiated only on KN medium. Histo-anatomical investigations revealed no significant structural modification on these media, especially on KN medium. At the same time, the non-rizogenetic action of NAA in *Salvia officinalis* was demonstrated.

**Key words:** Anatomy, *in vitro* cultures, nodal explant, micropropagation

## INTRODUCTION

*Salvia officinalis*, from Lamiaceae family, is a semi-woody shrub that gets up to 60 cm tall, originates from the Mediterranean region of North Africa, Spain and the Balkans. It has been grown as a medicinal and culinary herb for thousands of years and can now be found in gardens everywhere. Modern research has confirmed antiseptic, estrogenic, anti-inflammatory and anti-microbial properties in sage extracts. Very high variability was detected among individual plants, especially in the case of essential oil composition (D'Antuono *et al*., 2002).

*In vitro* cultures of different species of *Salvia* genus in general and of *Salvia officinalis* in particular have been investigated by many authors. Kintzios *et al*. (1999) underlined the effect of explant age, plant growth regulators and culture conditions on somatic embryogenesis and rosmarinic acid production from leaf explants of *Salvia officinalis* and *Salvia fruticosa*. Higher concentrations of the cytokinin benzyladenine (BA) or the cytokinin-like compound can induce direct adventitious shoot (Tawfik and Mohamed, 2007). Makunga and van Staden (2008) developed for *S. africana-lutea* an *in vitro* cultivation protocol. Adventitious shoot induction was most successful using hypocotyls as explants for propagation on Murashige and Skoog medium supplemented with 4.4 μM BA only or 2.9 μM IAA and 9.3 μM kinetin, respectively. Misic *et al*. (2006) develop a protocol for *in vitro* propagation of Balkan endemic plant *Salvia brachyodon*. BAP was effective in axillary buds promotion and all tested auxins (IAA, IBA and NAA) stimulated the rooting of *S. brachyodon* shoots.

The histo-anatomical investigations regarding *in vitro* cultivated plants in general are quite rare in the literature. Through micropropagation, it is possible to obtain a large number of plants from one single genitor (with valuable characteristics). The problems that could be occurring during the acclimatization process are caused by structural changes. The reduction of the cuticle, the presence of the aeriferous cavities and weakly developed vascular tissues were already reported by Picoli *et al*. (2001), which investigated stem anatomy in non-hyperhydric and hyperhydric eggplant. Donnelly *et al*. (1985) found size reduction of palisade parenchyma, compared with the spongy parenchyma in the case of plantlet's leaves obtained from *in vitro* cultures. Despite the richness of the literature concerning the chemical compounds from *Salvia officinalis* (cultivated *in vivo* or *in vitro*) (Santos-Gomes *et al*., 2002; D'Antuono *et al*., 2002) the histological data about the plantlets regenerated *in vitro* are missing so far.

In this study, both the morphological and histological aspects were considered in order to establish the most appropriate culture media for micropropagation in *Salvia officinalis*.

In a complementary study we have analyzed the seedling structure of *Salvia officinalis* grown *in vivo* (Gostin and Toma, 2001), the data obtained being further used in the present study to spotlight the anatomical differences between the *in vivo* and *in vitro* grown plantlets.

## MATERIALS AND METHODS

**Plant material and culture conditions:** The donor plants used for initiating the *Salvia officinalis* culture were supplied by the Stejarul Research Center of Piatra Neamt.

Axillary buds were excised and used as explants. They were sterilized with 0.1% mercury chloride ($HgCl_2$) solution for 12 min. After rinsing with sterile water, the explants were inoculated in 100 mL Erlenmayer flasks, containing 15 mL culture medium. The basal medium was composed according to Murashige and Skoog (1962). Nodal explants from sterile plantlets were used for testing the morphogenetical reaction in media with different combinations of growth regulators. All media contained 30 g $L^{-1}$ sucrose as carbon source; the pH of the media was adjusted to 5.7, prior to addition of 0.7% agar and autoclaving (1.2 atm, 121°C, 30 min). The cultures were maintained at 25±2°C, with 16 h daylight during a period of 6 weeks.

**Growth regulators treatments:** The effects of different auxins (NAA naphthyl acetic acid, IBA indole-3 -butyric acid) and cytokinins (BAP benzylamino purine, Kinetine) were tested. Six combinations of growth regulators were used in this experiment (Table 1). For each variant 10 Erlenmayer flasks were used. In each flask 4-6 nodal explants were inoculated. The biometric analysis was made after 6 weeks of culture. For each variant, the number of leaves/plantlet, the length of the shoot and the length of the leaves (including petiole) were considered. Each data set was analyzed using ANOVA and the Least Significant Difference (LSD) test at the 95% probability level (with SPSS 1.6 EV software).

**Histo-anatomical analysis:** Culture explants were removed after 6 weeks of culture (when the shoots are completely developed). For the anatomical analysis, callus and shoots fragments were fixed in FEA (formol, 70% ethanol and acetic acid 1:19:1) and dehydrated with series of increasing ethanol concentrations. The material was passed through gradated ethanol/xylene mixtures (100% ethanol, 3:1, 1:1, 1:3 and 100% xylene) and embedded in paraplast x-tra (Sigma). Transversal sections (~12 μm thick) were made with a rotary microtome (Euromex-Holland). The tissues were stained with ruthenium-red and methyl-blue and mounted in Canada Balm. The photos were made with a Minolta photo camera using a trinocular Novex (K-range) microscope.

Table 1: Variants and concentrations of growth regulators used in this experiment

| | Growth regulators (μM $L^{-1}$) | | | |
|---|---|---|---|---|
| Variants | BAP | K | IBA | NAA |
| B02 | 0.89 | - | - | - |
| B05 | 2.22 | - | - | - |
| B1 | 4.44 | - | - | - |
| IBA | - | - | 4.92 | - |
| N2 | - | - | - | 10.74 |
| KN | - | 4.65 | - | 2.68 |

## RESULTS

**Morphogenetical reports:** From nodal explants on B02 medium, after 6 weeks of culture shoots with a relatively good growth rate were obtained (4.47 cm). Their leaves were thin and atypical (Fig. 1a).

On B1 medium the shoots growth was similar with that of the B02 medium; evidence of hyperhydricity was observed for that (Fig. 1b).

The concentration of 2.22 μM BAP provided shoots for 100% of explants associated with a high number of leaves per explants (8.15 mean leaves per explant) with 3.63 relative length of the leaves (Table 2); their aspect was quite normal, without signs of vitrification. The regenerated shoots have no callus at their basis. The length of the multiplied shoots varied between 4.03 and 4.59 cm in all analyzed medium (which contain BAP in various concentrations) (Fig. 1c).

From the explants on N2 medium resulted the most untypical plantlets. Their leaves are thin, without a developed lamina, with pale green callus at their basis (without organogenetic capacity) (Fig. 1d).

On IBA medium the rizogenesis process is absent; a small callus could be observed at the basis of the plantlets (Fig. 1e).

The combination between kinetin and NAA (KN medium) was the most effective, providing the most intense growth rate; the plantlets have normal aspects and the number of leaves/plantlet was significantly high as related to the previous variants (9.79 mean leaves per explant) (Fig. 1f).

**Histo-anatomical reports:** Under the influence of the NAA (10.74 μM) present in the N2 medium, a generalized proliferation of the explant's basal portion occurs. The cortical and the medullar parenchyma were the most active zones and a mass of callus results from their activity. Initially, such divisions occur in all directions (Fig. 2a). The callus is compact, having a high histogenic potential. In callus, vascular formations with a circular shape, made mostly of tracheids from the generative zone are

Table 2: The effect of various combinations of growth regulators on morphological parameters of regenerated plantlets of *Salvia officinalis* after 6 weeks of culture

| Variants | No. of leaves/plantlet | Leaves length | Shoot height |
|---|---|---|---|
| B02 | [y]6.46±1.33a | 1.91±0.25a | 4.40±0.49a |
| B05 | 7.84±1.86a | 3.39±0.29b | 4.52±0.45a |
| B1 | 7.90±1.44b | 3.28±0.43bc | 4.08±0.38b |
| IBA | 4.38±1.12c | 3.05±0.30c | 3.85±0.37b |
| N2 | 3.76±0.92c | 2.10±0.21a | 3.13±0.34c |
| KN | 9.84±1.40d | 3.64±0.23d | 4.55±0.64a |

Data represented as a Mean±Standard error; y = Means within columns having different letters are significantly different according to the Least Significant Difference (LSD) at 0.05 level of probability

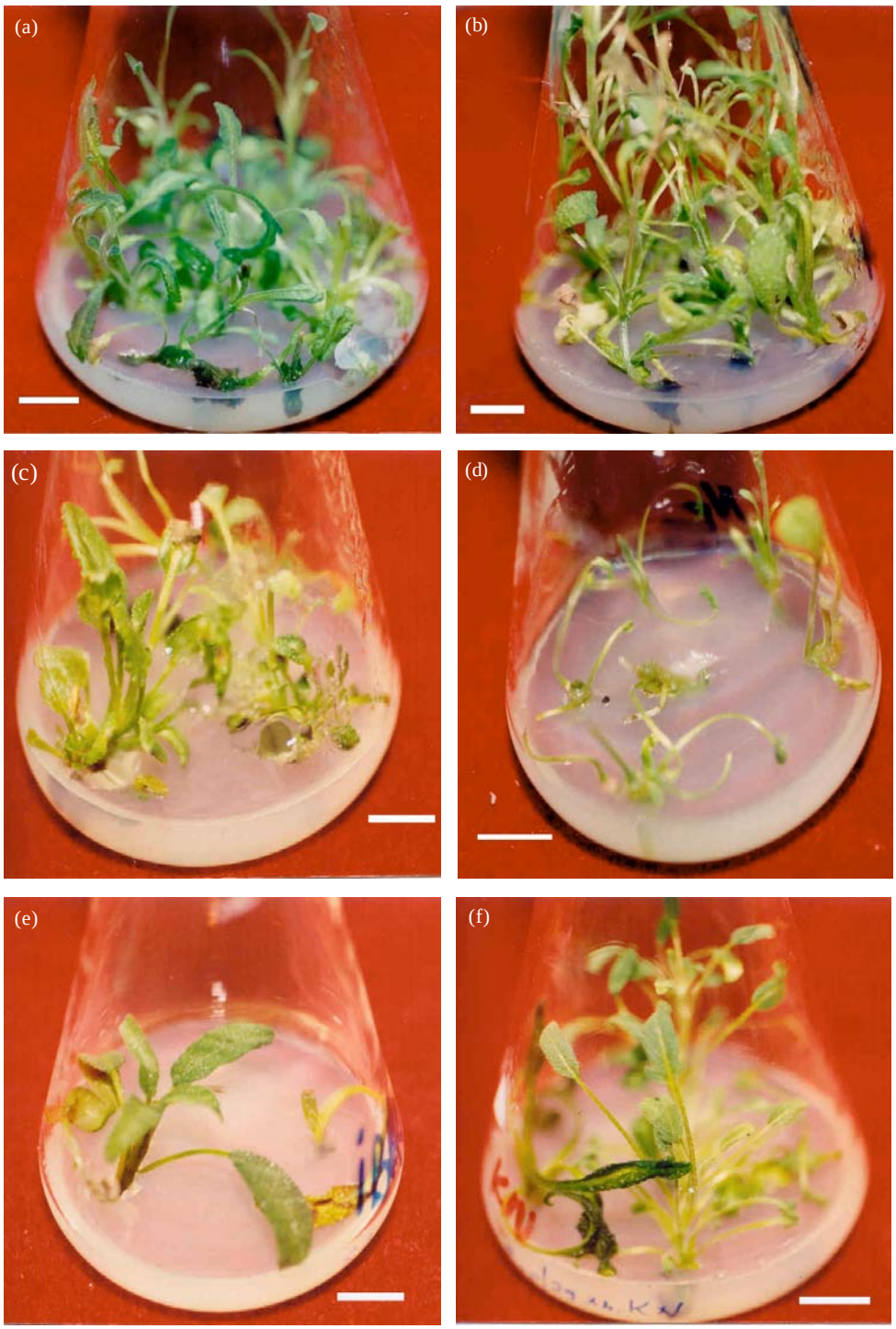


Fig. 1: Regenerated plantlets from nodal explants: (a) -On B02 medium supplemented with BAP (0.89 μM), (b) -B05 medium supplemented with BAP (2.22 μM); (c) -B1 medium supplemented with BAP (4.44 μM); (d) -N2 medium supplemented with NAA (10.74 μM); (e) -IBA medium supplemented with IBA (4.92 μM) and (f) -KN medium supplemented with kinetin (4.65 μM) and NAA (2.68 μM) (bar = 10 mm)

formed (Fig. 2b). In some of these structures, phloem elements may also be noticed. The development of such structures begins with the occurrence of some tracheids around which a generative area was organized (Fig. 2c).

The stem structure of the plantlets from B05 medium was analyzed (Fig. 2d). In its median area, the shoot's structure has an oval contour; the epidermal cells are smaller, the cuticle is thinner; No mechanical tissues are observed, while the conducting tissues are weakly developed; wood has a primary structure. Aeriferous cavities resulted from the disorganization of some parenchymatic cells are visible in the cortex; the tector and glandular hairs are much rarer, even on the superior internodes whereas in the case of plantlets cultivated under normal conditions, their density is very high (Fig. 2e).

The plantlet shoot structure regenerated on KN medium is quite close to the normal one; as compared with the variants cultivated on B05 media, a decrease in the number and dimensions of aeriferous cavities occurring in cortex and pith may be noticed (Fig. 2f). The primary wood is formed of vessels arranged in radial rows, which are separated by cellulosic parenchyma cells; the xylem vessels consist of thick and lignified walls. At the basis

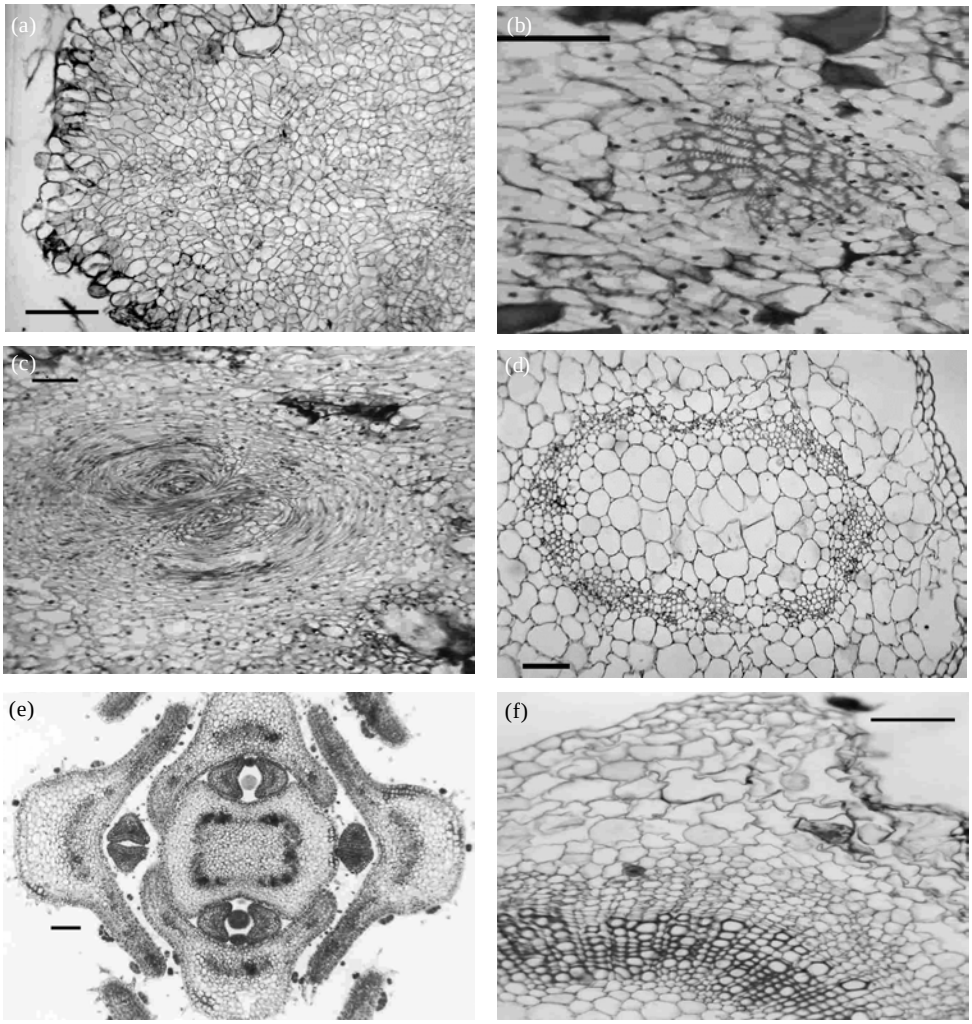


Fig. 2: Cross sections: (a) Callus provided from shoots basis on N2 medium (NAA-10,74 μM); the initial divisions occur in all directions; (b, c) Vascular formations from the callus (N2 medium); (d) Stem structure of the plantlets from B05 medium; (e) Stem structure of a normal plantlets (grown *in vivo*) and (f) Shoot regenerated on KN medium (median level); the cortical parenchyma is quite compact and the vascular tissues are well developed; (a-d, f- bar = 50 μm, e bar = 100 μm)

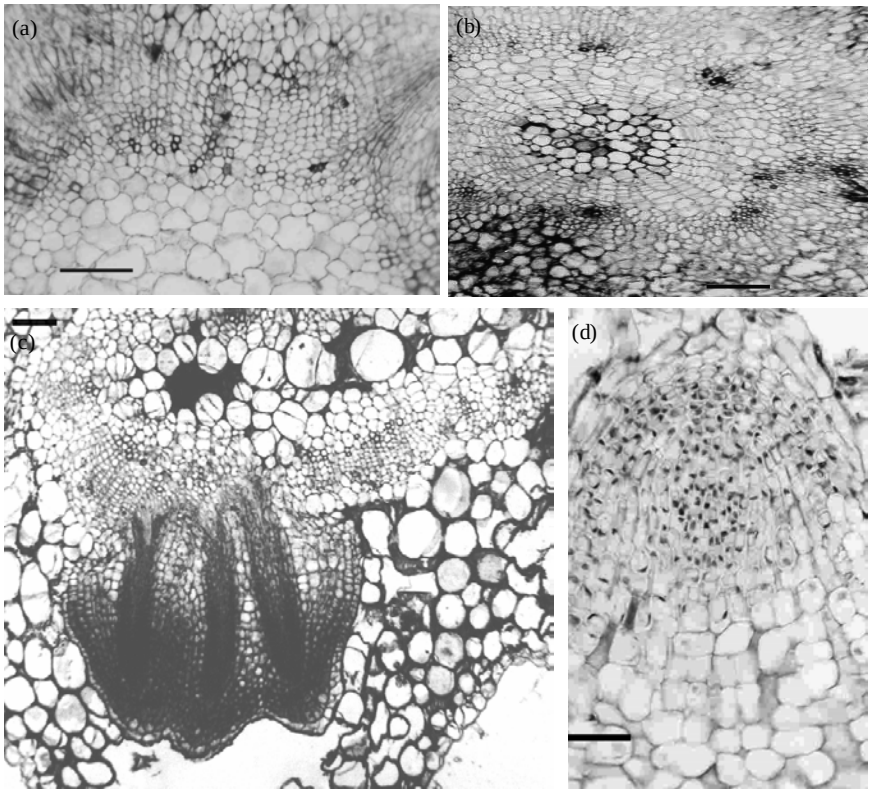


Fig. 3: Cross sections: (a) Basis of the shoot regenerated on KN medium (kinetin-4.65 μM and NAA-2.68 μM); vascular connection between the shoot and the adventitious root could be observed, (b) Basis of the regenerated on KN medium; tangential division of theparenchymatic cells around the pith could be observed; (c) Adventitious roots are formed from direct organogenese and (d) Longitudinal section through an adventitious root (bar = 50 μm)

of the explant some tangential divisions around the pith could be observed (Fig. 3a). The callus is missing at this level. The conducting tissues remain almost intact, while the vascular bundles have a normal structure.

At the basis of the regenerated plantlet adventitious roots are formed from direct organogenesis. This is proven by the vascular connection between the young root and the conducting tissue of the explant (Fig. 3c). The root structure demonstrates no significant modifications as compared with the plantlets cultivated *in vivo* (Fig. 3d).

## DISCUSSION

The morphogenetical capacity of *Salvia officinalis* was tested on MS medium supplied with growth regulators (auxins and cytokinins, alone or in combination).

The influence of different concentrations of growth regulators on some species of *Salvia* genus was previously investigated by other researchers. In *Salvia officinalis* Tawfik and Mohamed (2007) obtained greater number of shoots from callus differentiated on BA (4.4 or 8.8 μM) medium with 0.45 mM ascorbic acid added. Shoots developed roots on MS medium supplemented with 4.9 μM of indole-3-butyric acid. Cuenca and Amo-Marco (2000) obtained a maximum shoot proliferation from nodal explants for two species of endemic *Salvia* from Spain using MS medium supplemented with 6-γ-γ-dimethylallylaminopurine at 4.9 μM for *S. blancoana* and the same medium supplemented with kinetin at 4.6-9.3 μM for *S. valentine*; in this case, a higher concentration of BAP inhibited shoot formation after 4 weeks of culture. We have tested the effects of different concentrations of BAP on shoots proliferation in *Salvia officinalis*. A medium concentration of BAP (2.22 μM) induced a good growth rate; the resulted plantlets have normal aspect, the number of leaves, the shoots and leaves length are superior as compared with the variants which contain a lower (0.89 μM) or higher (4.44 μM) amount of BAP (Table 2). Our experiment confirms earlier research concerning the

influence of BAP concentration on the morphogenesis process. This fact proves the necessity of BAP for shoot development and the optimum level to be supplemented to the culture media depends on the endogenous level of cytokinins.

In the presence IBA or NAA in the culture medium, without association with a cytokinine, the explants response was not satisfactory. In this experiment IBA alone did not promote root initiation, although it has been successfully used in other *Salvia* species, Arikat *et al.* (2004) obtained a high rooting percentage in *Salvia fruticosa* cultivated on MS medium supplemented with 2.7 μM IBA. At the basis of the plantlets only a pale green callus could be observed. Mascarello *et al.* (2006) conclude that in vitro multiplication of *Salvia elegans*, *S. sinaloensis*, *S. cinnabarina* and *S. jamensis* is better mediated by a low level of BA. Kinetin did not sufficiently increase the multiplication rate. The morphogenetical response of the species under the influence of growth regulators is conditioned by plant-specific peculiarities and their genetic pattern. We can affirm these because in a earlier study (Toma *et al.*, 2004), we analyzed the influence of growth regulators on morphogenesis and anatomy of *Hyssopus officinalis* (from Lamiaceae family). In that case the MS medium supplied with 4.92 μM IBA provides shoots with no vitrification features and a good growth rate.

The combination between kinetine (4.65 μM) and NAA (2.68 μM) produced shoots with the highest number of leaves (9.79) and with a good growth rate; NAA induced direct rizogenesis. The histo-anatomical study completes the data concerning the response of *Salvia officinalis* at the *in vitro* culture. This is necessary to confirm the absence of structural changes when the aspect of the regenerated plantlets is quite normal.

The histological analysis revealed the absence of the organogenetic process from the callus obtained on N2 medium. That confirms a non-rizogenetic action of NAA in *Salvia officinalis*.

The stem structure exhibits (in all variants) in various degrees a thin cuticle, aeriferous cavities in the cortical parenchyma and a weakly developed vascular tissue. These aspects were earlier reported for other species (*Rosa* and *Solanum*, respectively) by other authors (Johansson *et al.*, 1992; Picoli *et al.*, 2001). In all analyzed samples, the cuticle was thinner than that of the plants cultivated under normal conditions. This seems to be a common feature for all in vitro cultivated plant species. Johansson *et al.* (1992) demonstrated the develop-mental differences in the cuticle of rose plants which occur in various micropropagation stages. Therefore, in the case of shifting from *in vitro* to *ex vitro* cultures, the cutin is gradually synthesized, with the decrease of relative humidity of the medium in which the plant lives.

The glandular hairs were rare and weakly developed in both stem and leaves epidermis. This may be determined by the culture conditions and by the presence of growth regulators in the medium. Sudriá *et al.* (1999) demonstrated that the presence of IBA in the culture medium reduced with 44% the number of glandular hairs at *Lavandula* and blocked it in the presecretory stage. We can conclude that some modifications are common for all *in vitro* cultivated plants and are induced by the specific growth conditions. Some hormonal balances could decrease the structural changes and these are appropriate for obtaining plantlets with a better *ex vitro* accommodation.

In the case of *Salvia officinalis* the most convenient results (both from morphological and histo-anatomical point of view) were obtained on KN1 medium.

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