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Histo-anatomy and *in vitro* morphogenesis in *Hyssopus officinalis* L. (Lamiaceae)

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The sequence of histogenesis and organogenesis in the calus of *Hyssopus officinalis* nodal explants was studied during treatment with growth regulators. This process represents an important indicator of the accommodation capacity of regenerated plantlets to the *ex vitro* conditions. The cell multiplication rate was stimulated in Murashige and Skoog medium supplemented with indole-3-butyric acid (1 mg L^{-1}). The study was carried out from the start of the culture until the development of shoots. Leaf and stem anatomy was influenced by growth regulators. Combinations between auxine and citokinine have induced callus development at the basis of the regenerated shoots, revealing different degrees of vitrification in stems and leaves.

Key words: morphogenesis, histogenesis, hyperhydricity, micropropagation, organogenesis, anatomy, *Hyssopus officinalis*, Lamiaceae

Introduction

Hyssop (*Hyssopus officinalis)* is a well-known and widely used medicinal and aromatic plant. As a medicinal plant, hyssop has also been used as a carminative, emmenagogue, stimulant, stomachic, and tonic. The constituents of hyssop that contribute to its healing qualities include up to 2% volatile oils, comprising mainly pinocamphone, isopinocamphone, pinenes, camphene and terpenine; a glycoside called hyssopin, tannins, flavonoids, isolic acid, oleonolic acid, a principle called marrubiin as well as resin and gum (VARGA et al. 1998).

In the case of medicinal plants, micropropagation procedures are nowadays used for obtaining a large number of valuable individuals starting from a donor plant with high productive capacity.

The studies devoted to the *in vitro* morphogenetic reaction with labiatae species are quite numerous (ONISEI et al. 1989, PATTANAIK and CHAND 1996, ANDRADE et al. 1999, FRACARO and ECHEVERRIGARAY 2001). A study concerning the *in vitro* morphogenesis of *Hyssopus officinalis* is still absent in the literature.

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Plants cultured *in vitro* grow usually under very different conditions as compared to those grown in open air. Low irradiance and high relative humidity strongly alter their structure (DONNELLY and VIDAVER 1984, MAJADA et al. 2000).

Hyperhydration is a major problem in the tissue culture industry since it can affect shoot multiplication and culture vigor, limiting the application of *in vitro* techniques for mass propagation (GEORGE 1993). A histo-anatomical investigation of regenerated plantlets before the translation in *ex vitro* conditions could be useful for several reasons: the most advantageous combinations of growth regulators (which induced less significant structural modifications) could be detected in an incipient stage of the experiment; thus, we can explain why some regenerated plantlets with an approximately normal aspect are inadaptable to *ex vitro* conditions; the theoretical importance lies in the possibility of highlighting the influence of different growth regulators on the histogenetic process.

Leaf and shoot anatomy of *in vitro* grown plants was demonstrated elsewhere, on some species such as raspberry (DONNELLY and VIDAVER 1984), African violet (REDWAY 1991), rose (JOHANSSON et al. 1992), tobacco (RADOCHOVÁ et al. 2000). The authors investigated the role of the medium from the culture vials, the light or the carbon source on the leaf and the shoot structure.

This paper reports on the histological events leading to nodule formation and shoot regeneration from axillary bud cultures of *Hyssopus officinalis* L. The influence exercised by the hormonal balance was especially considered. In a complementary paper we have analyzed the seedling structure of *Hyssopus officinalis* grown *in vivo* (GOSTIN and TOMA 2000). The data obtained being further used in the present paper to spotlight the anatomical differences between the *in vivo* and *in vitro* grown plantlets.

Material and methods

Plant material and culture conditions

The donor plants used for initiating the *Hyssopus 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 minutes. After rinsing with sterile water, the explants were inoculated in 100 ml Erlenmayer flasks, containing ~ 13 ml culture medium. The basal medium was composed according to MURASHIGE-SKOOG (1962). Different combinations of growth regulators were used. 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 hours daylight during a period of 8 weeks.

Growth regulators treatments

The effects of different auxins (IAA indole-3-acetic acid, NAA naphtyl acetic acid, IBA indole-3-butyric acid) and one cytokinin (BAP benzylamino purine) on the histological structure of the callus, leaves, and shoots were tested. Three combinations of growth regulators were used in this experiment (Table 1). For each variant 10 Erlenmayer flasks were used. In each flask 3–4 nodal explants was inoculated.

Tab. 1. Variants and concentrations of growth regulators used in this experiment

Variants	Growth regulators (mg L^{-1})			
	BAP	IAA	IBA	NAA
BN1	1.00	–	–	0.5
BA1	1.00	0.5	–	–
IBA1	–	–	1.00	–

Histo-anatomical analysis

Culture explants were removed after 6 and 8 weeks of culture (when the shoots are completely developed). For the anatomical analysis, callus, leaves, and shoots fragments were fixed in FEA (formol, ethanol 70% and acetic acid 1:19:1) and dehydrated with ascending ethanol series. 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 red-ruthen and methyl-blue and mounted in Canada Balsam. The drawings were made after obtaining permanent preparates, using a projection mirror on a MC 1 microscope.

Results

Plantlets regenerated on BN1 medium

Shoots regenerated after a 6 week culture from vegetative buds on BN1 have a relatively well-developed callus at their basis. At this level, the epidermis becomes disorganized under the pressure of the newly formed tissue. This is due to the divisions occurring in the cortical parenchyma.

In the subepidermal region, the cells are periclinally divided. A tissue similar in aspect with phellogene (a subero-phellodermic generator area) is thus obtained (Fig. 1A). The divisions are oriented in parallel to the explant's surface. In the external part of the callus placed in direct contact with the medium the neo-formations are lacking any organization (Fig. 1B).

The cells of the non-differentiated, meristematic tissue are small, elongated, concentrically arranged. From them, tracheids will be most frequently formed. Only seldom, and only in certain vascular formations, may phloem elements also occur.

After two more weeks of culture, generating zones and, further on, vascular formations occur, as well, in the callus internal area. The meristematic cells have sizes comparable with those from the vegetative apex, from which they are differentiated by the presence of relatively large vacuoles. In most cases, in the center of the meristematic area (formed of concentrically-arranged cells) woody vessels, and, sometimes, phloem elements on their external side, are differentiated (Fig. 1C).

In its median area, the shoot structure (Fig. 2) is similar to that of *in vivo* cultivated plantlets (GOSTIN and TOMA 2000), with the following differences: the contour of the cross-section is not square, yet oval; the epidermal cells are smaller, the cuticle is thinner;

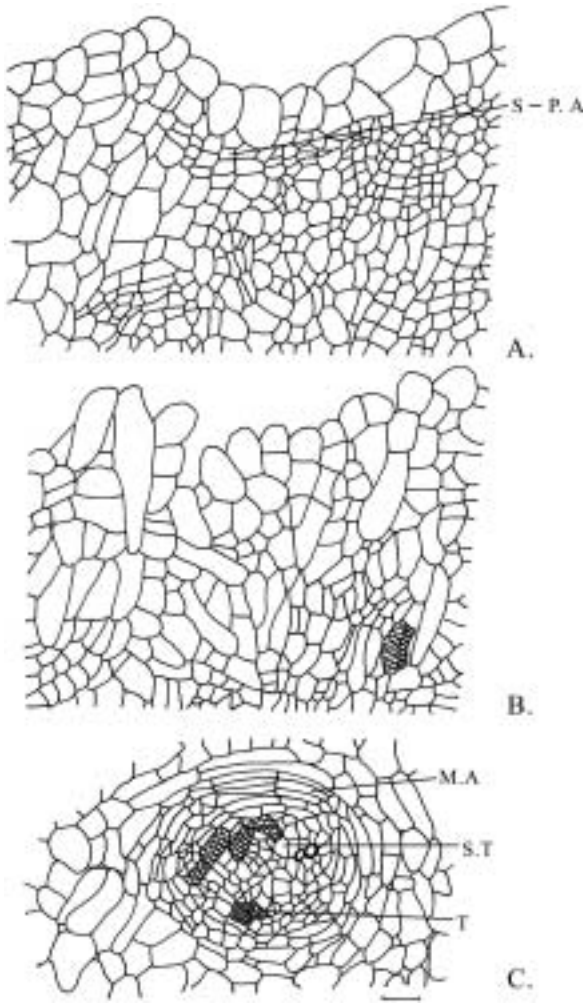

Fig. 1. Transection of the callus provided from shoots basis on BN1 medium: A – subero – phelodermic area; B – callus with low histogenetic potential; C – vascular elements: M.A – meristematic area, S.T – sieve tube, S-P.A – subero-phelodermic area, T – tracheide (bar = 50 μm)

no differentiation of the subepidermal collenchyma belts is to be observed; the cortex evidences aeriferous cavities, resulted from the disorganization of some parenchymatic cells; wood has a primary structure; the tector and glandular hairs are much rarer, even on the superior internodes while, in the case of plantlets cultivated under normal conditions, their density is very high.

The apical zone of the stem has a typically meristematic structure; there are no differences between it and the same region from the stem cultivated *in vivo*.

The structure of the primordia and the young leaves is presented on figures 3A and 3B (the hyperhydricity phenomenon being hardly manifested). The palisade is uni-layered. Sometimes, at mature leaves, the mesophyll is non-differentiated, being formed exclu-

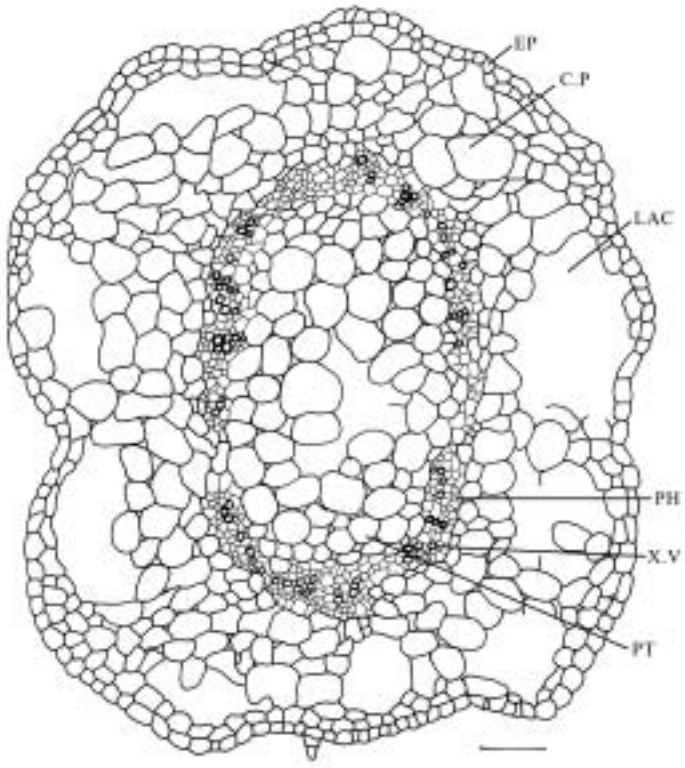

Fig. 2. Transection of a shoot regenerated on BN1 medium (median level): C.P – cortical parenchyma, EP – epidermis, LAC – lacune, PH – phloem, PT – pith, X.V – xylem vessel (bar = 50 μm)

sively of spongy parenchyma, or the palisade parenchyma is reduced (Fig. 3 C); the cuticle is thinner than that of the plants cultivated under normal conditions.

The quite rare stomata are situated above the epidermis level (Fig. 3 D). The middle vein is not so prominent, its thickness being comparable to that of the foliar limb, while its conducting tissue is less developed. No mechanical tissue is noticed in front of the middle vein. The tector and glandular hairs are much rarer, the latter demonstrating a bicellular pedicle and a unicellular gland.

Plantlets regenerated on the BA1 medium

The contour of the stem cross-section is circular; the epidermis persists on the explant exterior part, although it is interrupted here and there, as a result of intense proliferation of the cortical parenchyma's cells. In some epidermal cells, division walls with different orientation may be observed.

Internal proliferation is more reduced than in the previously described situation. The procambium suffers some additional divisions, yet the central cylinder maintains its individuality.

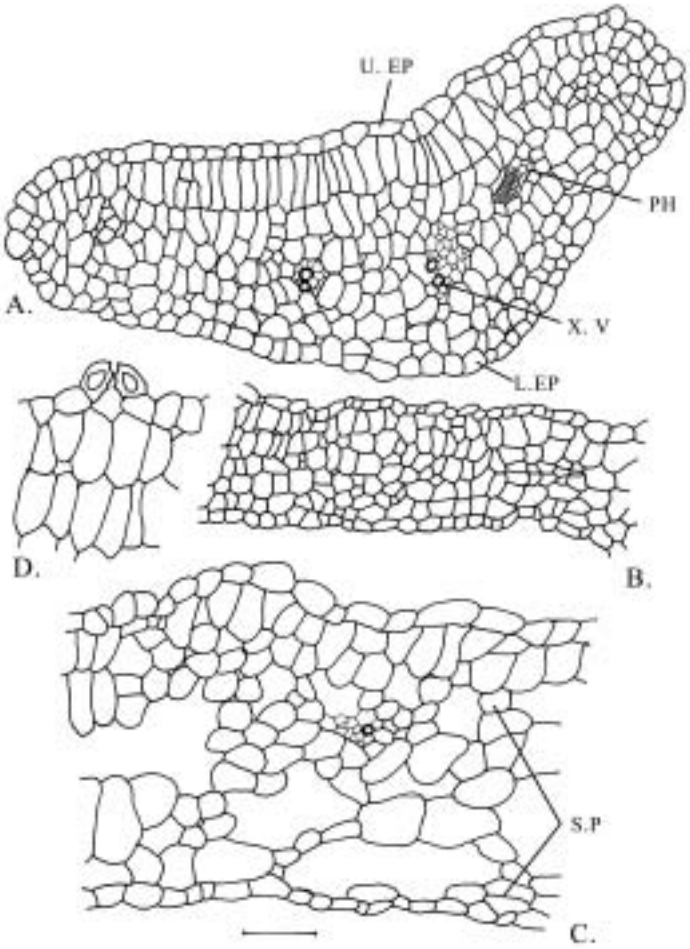

Fig. 3. Histogenesis of the leaves (BN1 medium) (transections): A – young primordia, B – very young leaf, C – mature leaf, D – mature leaf – stomata situated above the epidermic level: L.EP – lower epidermis, PH – phloem, S.P – spongy parenchyma, U.EP – upper epidermis, X.V – xylem vessel (bar = 50 μm)

At a higher level, as a result of meristematic tissue organized proliferation, the central cylinder increases its volume, which causes cortex disintegration. Several epidermal cells demonstrate division among the periclinal walls. The pith cells are partially disintegrated, which results in aeriferous cavities.

The terminal internodes of the stem are much closer to the normal structure (Fig. 4). Some differences do exist, such as: the development of the axillary buds is much more reduced than normally; sometimes, they may be completely absent from the leaf axilla, which never occurs under normal conditions. No mechanical tissues are observed, while the conducting tissues are weakly developed. In the external cortical region, divisions with a different orientation could take place. The tector and glandular hairs are either very rare or absent.

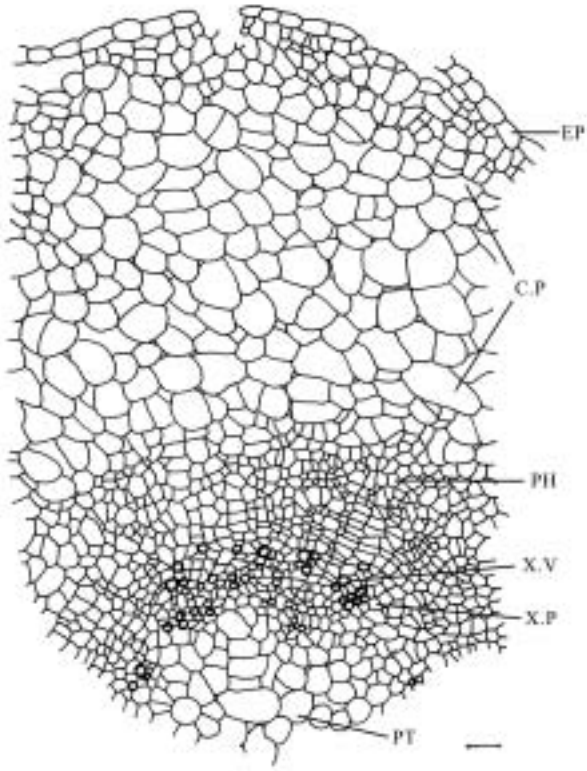

Fig. 4. Transection of the shoot regenerated on BA1 medium: C.P – cortical parenchyma, EP – epidermis, PH – phloem, PT – pith, X.P – xylem parenchyma, X.V – xylem vessel (bar = 50 μm)

The aspect of the leaves from the shoots is modified: they are thick, dark-green in color, with a well-developed spongy parenchyma. The cells are large with thin walls and extensive intercellular spaces.

Plantlets regenerated on the IBA1 medium

The base of the shoots has a different structure from that of the previously analyzed ones (Fig. 5). The cortex cells grow and begin to lose contact between them. Division walls are rare, which means that cortex volume does not grow too much, inducing only a partial disintegration of the epidermis. The conducting tissues remain almost intact, while the vascular bundles have a normal structure. The primary wood is formed of vessels arranged in radial rows, which are separated by cellulosic parenchyma cells. The procambium is formed of 1–2 cell layers (no proliferation of the procambium being noticed as a result of auxin action). The phloem is formed of sieve tubes and companion cells, and the only ordered divisions occur on the internal side of the vascular bundles.

The leaves are a quite normal structure, with no significant vitrification features. The mesophyll is differentiated into a unilayered palisade and a multilayered spongy parenchyma. However, the cuticle is thinner than that of the normal leaves.

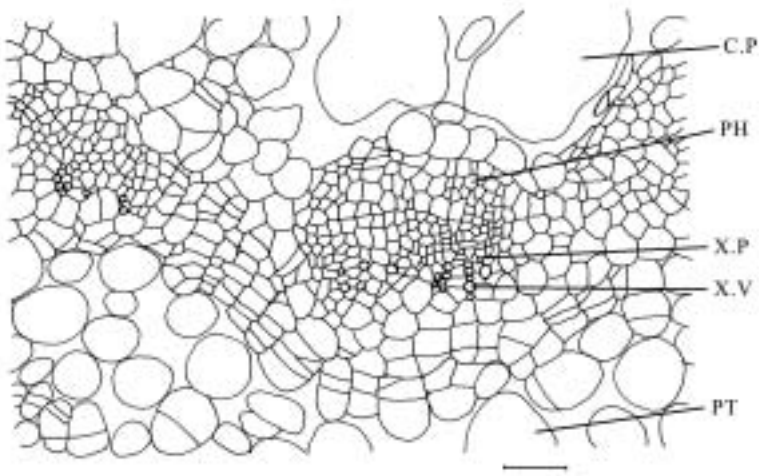

Fig. 5. Transection of the shoot regenerated on IBA1 medium: C.P – cortical parenchyma, PH – phloem, PT – pith, X.P – xylem parenchyma, X.V – xylem vessel (bar = 50 μm)

Discussion

Our observations revealed that the anatomy in the culture conditions is similar to plantlets cultivated on media with different combinations between growth regulators; the latter is determined by the growth regulator combinations and is specific to all the analyzed samples.

The stem structure presents (at all variants) thin cuticle, aeriferous cavities and weakly developed vascular tissues. Our results agree with those of PICOLI et al. (2001), which investigated stem anatomy in non-hyperhydric and hyperhydric eggplant, to identify structural changes associated with this phenomenon. They noticed smaller and more organized cells in non-hyperhydric organs and a more differentiated vascular system when compared with its hyperhydric counterpart.

At the basis of IBA1 regenerated shoots, the callus is absent. That is a desirable phenomenon because the presence of an abundant callus could be an inconvenience at the moment of the «ex vitro« accommodation of the young plantlets.

The callus from the basal part of the *Hyssopus* shoots cultivated on a BN1 medium and on a BA1 medium may demonstrate the different influence exercised by the two auxins on the differentiation process. Although at the same concentrations, it is only NAA that has induced dedifferentiation and redifferentiation processes at the level of the callus. The relatively low amount of auxin is associated with the organization at a diffuse cambium shape of cells resulted from the division of the subepidermal cells. The higher dose of auxins leads to anarchical proliferations; the neo-formations thus resulting are lacking any organization (GAUTHERET 1959).

The palisade parenchyma is reduced and the spongy parenchyma is well developed in all analysed variants. Sometimes, the spongy parenchyma represents the only assimilating

tissue of the leaf. DONNELLY and VIDAVER (1984) found a reduction of palisade parenchyma size, compared with the spongy parenchyma in the case of plantlet leaves obtained from *in vitro* cultures. They observed that, as a result of the specific conditions in the culture vessels (low amount of light, high humidity), strawberry leaves modify their structure and reduce their palisade parenchyma. Also, according to RADOCHOVA et al. (2000), decrease of the palisade parenchyma/spongy parenchyma ratio might be caused by the presence of high amounts of sacharose in the culture medium, which shifts plant metabolism from photoautotrophy to photomixotrophy.

The structure of the young leaves of plantlets regenerated on media with different hormonal balances is similar to that of leaves of plantlets grown under normal conditions; on mature leaves, vitrification signs – more or less intense – are obvious, depending on the hormonal balance applied. Also, the leaves from the shoots regenerated on a BA1 medium manifest the highest rate of vitrifications (with the mesophyll formed entirely by spongy parenchyma and with large aeriferous spaces). On the other hand, the leaves from the shoots regenerated on IBA1 medium have a quite normal structure, with one layer of palisade parenchyma under the upper epidermis. This fact suggests that this medium is most favorable for *in vitro* multiplication of this species.

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 developmental 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 cutine 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 leaf epidermis. This may be determined by the culture conditions and by the presence of growth regulators in the medium. SUDRIA et al. (1999) demonstrated that the presence of IBA in the culture medium reduced the number of glandular hairs at *Lavandula* by 44%, and blocked them in the presecretory stage.

In conclusion, our results are in agreement with those previously described by other authors, concerning the influence of the culture media on the histo-anatomy of regenerated plants. The most favorable growth regulator was IBA, in the presence of which hyperhydricity was reduced and the basal callus was missing. In both the stems and the leaves, a less modified histo-anatomy was observed.

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