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EFFECT OF PLANT GROWTH REGULATORS ON CHLOROPLAST ULTRA STRUCTURE IN *LAMIUM ALBUM* PLANTLETS

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Abstract

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Lamium album L. stem nodal segments were grown on Murashige and Skoog's medium without growth regulators (control) and on media supplemented with 0.2 mg l^{-1} N^6-benzyladenine, 0.5 mg l^{-1} indole-3-butyric acid or both plant growth regulators. The plastid apparatus of *in vitro* formed leaflets was studied by transmission electron microscopy. The cytokinin N^6-benzyladenine negatively affected the chloroplast formation when was applied individually or in combination with the auxin indole-3-butyric acid. On the contrary, IBA in concentration of 0.5 mg l^{-1} was appropriate for development of chloroplasts with regular shape and well organized internal membrane system thus providing for the regeneration capacity *in vitro.*

Key words: in vitro-regeneration, indole-3-butyric acid, medicinal plant, N^6-benzyladenine, transmission electron microscopy, white dead nettle

Abbreviations: BA - N^6-benzyladenine; IBA - indole-3-butiric acid; PGRs - plant growth regulators; TEM - transmission electron microscopy

Introduction

More than 2 000 different species are used in Europe for production of medicinal and other herbal preparations. Almost two thirds of these species are already with limiting resources, which requires alternative way for their propagation. Biotechnological methods seem appropriate ones with their potential for multiplication, selection and protection of medicinal plants (Tasheva and Kosturkova, 2013). The direct plant regeneration technique is an effective way for rapid multiplication of plants producing important secondary metabolites or possessing other valuable traits (Gopi et al., 2006). *Lamium album* L., commonly known as "white dead nettle" is a perennial herb from Lamiaceae family widely used in folk and official medicine. Recent research reported anti-proliferative properties, great antioxidant capacity and free radical scavenging activity of different extracts of the plant (Paduch, 2008; Valyova, 2011). The success of *in vitro* cultivation depends on the quality of the explants source, the cultivating conditions as well as the plant growth regulators (PGRs) commonly supplemented to the medium. The type and concentration of the PGRs added in the culture medium controls several morphogenic responces and histological changes in plantlets (Mohamed and Alsadon, 2011), since they play a major role in the cell division and differentiation. The cytokinins are important for development of the plant photosynthetic apparatus through their direct role in the chloroplast differentiation (Colijn et al., 1982; Arigita et al., 2005). The influence of cytokinins on the leaf ultrastructure of *in vitro*-grown plants has been subject of scientific interest (Wetzstein and Sommer 1982; Olmos and Hellín, 1998; Paek and Hahn, 2000; Sudriá et al., 2001; Toma et al., 2004; Deccetti et al. 2008; Oliveira et al. 2008; Magyar-Tábori et al., 2010) while the effect of auxins on the leaf ultrastructure of micropropagated plants has been poorly examined (Sudriá et al., 2001; Toma et al., 2004). On the other hand, tissue culture conditions that promote rapid growth and multiplication of shoots often result in formation of plantlets with abnormal morphology, anatomy and physiology (Debergh et al., 1992; Olmos and Hellín, 1998; Hazarika, 2006). Structural irregularities, especially a poor development of the photosynthetic system in culture may obstruct the regeneration process and make plantlets vulnerable during

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transfer from *in vitro* to *ex vitro* conditions (Lee et al., 1985). A proper histogenesis and regular ultra structural organization of leaves is an important indicator for the accommodation capacity of *in vitro*-grown plants.

In this paper, we analyze the effect of cytokinin (BA), or auxin (IBA), or both together, on leaf chloroplast differentiation and describe their ultrastructure in order to assess the regeneration potential of *in vitro* cultured *L. album* plants.

Materials and Methods

Plant material

Plant material of *Lamium album* L. was collected at its natural habitat in the Lozen mountain, Sofia, Bulgaria. The voucher specimen SO 105183 has been deposited in the Herbarium of Sofia University „St. Kliment Ohridski". *In vitro* shoot cultures were induced from sterilized mono-nodal stem segments of the *in situ* growing wild plants. Cut explants were washed thoroughly under running tap water for 30 min. Later they were sterilized with 0.1 % (w/v) $HgCl_2$ for 8 min and washed three times with sterilized distilled water. Explants were inoculated under aseptic conditions on standard MS (Murashige and Skoog, 1962) medium containing 3% (m/v) sucrose and 7g l^{-1} agar without any supplement of growth regulators. Plants were *in vitro* cultivated under controlled environmental conditions (16h light/8h dark period, 60 µmol m^{-2} s^{-1} photosynthetic photon flux density, Philips TLD-33, temperature 25ºC and 60–70% relative air humidity). After 25 days of cultivation, explants (shoots with two axillary buds) from the regenerated plants were propagated on MS medium without growth regulators (control) and on MS media suplemented with 0.2 mg l^{-1} N6-benzyladenine (BA); 0.5 mg l^{-1} indole-3-butyric acid (IBA); 0.2 mg l^{-1} BA + 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BA + 0.05 mg l^{-1} IBA. The explants were cultivated under above mentioned laboratory conditions for five weeks.

Methods

For TEM analysis segments (1 mm^2) from the middle part of fully expanded leaves were taken from the 2nd or 3rd nodes and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4ºC. The samples were postfixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethylalcohol (from 25 to 100%), the samples were embedded in *Durcupan* (Fluka, Buchs, Switzerland). Ultrathin sections were cut from the palisade parenchyma using a *Reichert-Jung* (Wien, Austria) ultra microtome, contrasted with lead citrate (Reynolds, 1963), and examined using an electron microscope (*JEOL 1200EX*, Tokyo, Japan).

Results

The leaves of *in vitro* regenerated on standard MS medium *L. album* plants (control plants) display well developed assimilative parenchyma. The chloroplasts in the mesophyll cells are elliptical to oval shaped in transverse section. Large associative regions between chloroplasts and between chloroplasts and mitochondria are visible (Figure 1A, B). The internal membrane system consist of grana with different height (from 5-8 to more than 35 thylakoids per granum) linked with relatively long stroma thylakoids. Large stroma areas without starch inclusions were observed (Figure 1A, B). Part of the thylakoid membranes and the chloroplast envelope, mainly in oval shaped plastids, are undulated. Some of the stroma thylakois are partially fragmentated (Figure 1B). The internal membrane system of the chloroplasts in BA treated plantlets totally differs from the control ones. Great part of the grana and stroma membranes is merged and destructed, especially in the central area of the chloroplasts (Figure 1C). Only small part of the periferally situated thylakoids remains stacked in grana. The chloroplasts in IBA treated plantlets are flattened and ellongated, which restricts the associative areas between them. The internal membrane system is properly organized, parallel to the long axis of the plastids (Figure 1D). The grana are of middle height and stroma thylakoids are long and well structured. There are not any anomalies in the thylakoid membrane development. Small starch inclusions are noticed in the stroma. BA and IBA supplemented together to the culture medium affect the leaflet plastid structure in negative way. Some anomalies in the chloroplast organization are observed. The thylakoids are abundant but irregularly orientated (Figure 1E, F). The grana thylakoids are merged and the membranes are fragmented to almost complete destruction, especially in the plantlets cultured in combination of 0.2 mg l^{-1} BA and 0.05 mg l^{-1} IBA (Figure 1F). Only in the periferal zone of the chloroplasts there are partially retained thylakoids.

Discussion

Structural organization of the chloroplasts in in vitro cultivated plants is very important indicator for evaluating the regenerative potential of the species. Proper development of the plastid apparatus is a nessesary condition providing normal ontogenetic process in micropropagated plants. A lot of TEM studies indicate wide structural variety of chloroplasts in in vitro cultured plants (Wetzstein and Sommer, 1982; Sudria et al., 2001; Majada et al., 2002; Jausoro et al., 2010). Every fluctuation of the experimental conditions causes ultrastructural deviations in plantlets (Lee et al., 1985; Olmos and Hellín, 1998; Louro et al., 1999; Serret and Trillas, 2000;

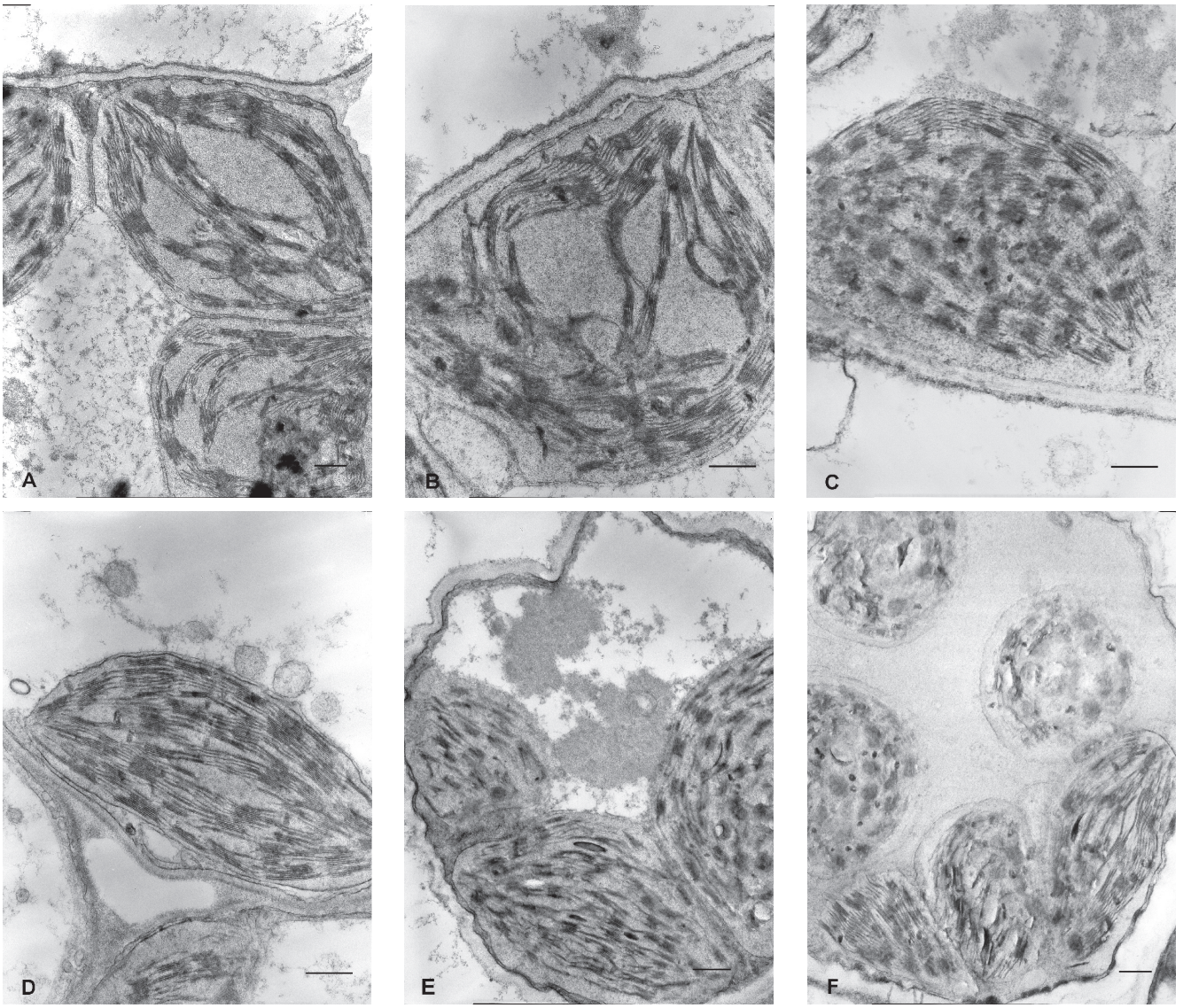

Fig. 1. The chloroplast ultrastructure in control plants (A,B), plants cultivated on medium with 0.2 mg l^{-1} BA (C), 0.5 mg l^{-1} IBA (D), 0.2 mg l^{-1} BA + 0.5 mg l^{-1} IBA (E), and 0.2 mg l^{-1} BA + 0.05 mg l^{-1} IBA (F). Scale bar = 500 nm

Oliveira et al. 2008). According to Laetsch and Stetler (1965), the chloroplasts in some tissue cultured plants can differentiate to the same extend as the chloroplasts in normal leaf tissue. However, many studies indicate that specific in vitro conditions negatively affect the development of the plastid apparatus, which is manifested in altered shape of the chloroplasts, irregular orientation of the thylakoid system, plastoglobuli accumulation (Wetzstein and Sommer, 1982; Lee et al., 1985; Majada et al., 2002). Such abnormalities are considered to be responsible for diminishing the photosynthetic capacity of affected plantlets (Fontes et al., 1999). In the current experimental conditions, the TEM analysis is very informative for the role of PGRs on the development of the plastid apparatus in *in vitro* cultured *L. album* plants. Standard medium and especially medium supplemented with IBA induce regular chloroplast ultra structure. On the contrary, the presence of BA in the medium disturbs the proper development of the photosinthetic apparatus in *L. album* plantlets. According Polanská et al. (2007), the chloroplasts are indifferent towards cytokinin regulation. The authors do not observe any

structural changes except fluctuation in starch amount and plastoglobuli accumulation. Moreover, BA can stimulate the formation of thylakoid membranes in tissue-cultured petunia (Colijn *et al.* 1982). However, in *L. album* BA (applied alone or together with IBA) alters the chloroplast shape and causes destruction of the thylakoid membranes. This is in accordance with findings of Mazari and Camm (1993) for *Pinus ponderosa* and Magyar-Tábori et al. (2010) for apple culture. Wilhelmova and Kutík (1995) found out that BA treated tobacco plantlets had small flattened chloroplasts with high grana stacking and profound accumulation of starch inclusions. This is evidence, which gives a proof that BA causes large scale of ultra structural variations in plantlets from different species. This is a reason to assume that the structural answer to PGRs' treatment depends on the genotype of the plant species. TEM analysis of *L. album* plantlets pointed out IBA as auxin favourable for differentiation of the chloroplasts. This corresponds with findings of Lucchesini et al. (2006) who describe chloroplasts with regularly structured thylakoid system, single starch grains and few plastoglobuli in *Myrtus communis* plantlets, regenerated on IBA supplemented medium. In agreement with Aloni (1995), we can assume that for micropropagated *L. album* IBA is obligate PGR providing for successful regeneration on sub cellular level of organization.

Conclusions

The results of our study showed that the growth conditions apparently affect chloroplast formation. BA (applied alone or in combination with IBA) is inappropriate PGR while IBA in a concentration of 0.5 mg l^{-1} is required for the proper organization of the plastid apparatus in *in vitro* cultured *L. album* plants. These results provide reasonable basis for the improvement of the standard protocol for micropropagation and *ex vitro* adaptation of this useful medicinal plant.

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