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Effect of N^6-benzyladenine and indole-3-butyric acid on photosynthetic apparatus of *Orthosiphon stamineus* plants grown *in vitro*

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Abstract

The leaf structure and chloroplast ultrastructure of kidney tea (*Orthosiphon stamineus* Benth.) was studied in *in vitro* culture on standard MS medium supplemented with or without plant growth regulators (PGRs). The cytokinin N^6-benzyladenine (BA) negatively affected the structure of the palisade parenchyma and chloroplast ultrastructure and increased the stomatal frequency of the adaxial epidermis. The auxin indole-3-butyric acid (IBA) did not modify the morphology of regenerated leaf tissues as well as the chloroplast ultrastructure. The effect of both PGRs applied in combination was manifested in well-differentiated mesophyll parenchyma, typical chloroplast ultrastructure and increased stomatal frequency on both leaf surfaces. This protocol can be suggested for further *ex vitro* propagation.

Additional key words: auxins, chloroplast ultrastructure, cytokinin, leaf anatomy, medicinal plant, micropropagation.

Introduction

Orthosiphon stamineus Benth. (*Lamiaceae*) is a common medicinal plant from South East Asia. At the beginning of the 20th century this plant was introduced to Europe where it became a popular herbal health tea, called Cat's whiskers, Java tea or kidney tea (Wagner 1982, Jagarath and Ng 2000). Commercial interest in kidney tea has been increasing and micropropagation has become a viable alternative for reproducing this plant because of many difficulties encountered with its conventional propagation. 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 (Hazarika 2006), which makes them vulnerable during transfer from *in vitro* to *ex vitro* conditions. The vulnerability of plants during acclimatization is a consequence of lack of epicuticular wax, poor mesophyll differentiation, and insufficient stomatal regulation of water loss (Brainerd and Fuchigami 1981). A reduced leaf photosynthetic activity caused by low chlorophyll content and poor development of chloroplasts also limit the successful transition from *in vitro* to *ex vitro* conditions (Amâncio *et al*. 1999). Therefore, the leaf structural and/or functional changes may significantly affect the success of acclimatization (Magyar-Tábori *et al*. 2010). Morphogenesis during micropropagation can be manipulated by the *in vitro* conditions such as irradiance, temperature, air humidity and osmotic potential and contents of minerals, sugars and plant growth regulators (PGRs) in the medium (Davies 1987, Mohamed and Alsadon 2011). Cytokinins regulate leaf development by affecting cell division, photosynthesis, chloroplast development, senescence and assimilate partitioning (Binns 1994) and are very important for development of the plant photosynthetic apparatus through their direct role in chloroplast differentiation (Chernyad'ev 2000, Kulaeva 2002, Arigita *et al*. 2005). Auxins are known to promote cell elongation, cell division and formation of adventitious roots (Dimitrova *et al*. 2010). The influence of cytokinins on the leaf structure of *in vitro* grown plants often has been subject of scientific research (Wetzstein and Sommer 1982, Olmos and Hellín 1998, Paek and Hahn 2000, Sudriá *et al*. 2001, Toma *et al*. 2004, Deccetti *et al*. 2008, De Oliveira *et al*. 2008, Magyar-Tábori *et al*. 2010), while the effect of auxins has been less investigated (Sudriá *et al*. 2001, Toma *et al*. 2004).

The present study aims to examine the structural organization of the photosynthetic apparatus of *in vitro*

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

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propagated *O. stamineus* plants and to establish the role of the cytokinin N^6-benzyladenine (BA) and the auxin indole-3-butyric acid (IBA) in the regenerative processes at tissue, cellular and subcellular levels.

Materials and methods

Orthosiphon stamineus Benth. stock plants were maintained and subcultured *in vitro* every 5 weeks on a standard full-strength Murashige and Skoog (1962; MS) medium, supplemented with 2 % (m/v) sucrose and 8.0 g dm^{-3} agar. The growth conditions were: temperature 22 ºC, 16-h photoperiod, photosynthetic photon flux density 60 μmol m^{-2} s^{-1} (white fluorescent tubes). Axillary buds with a small piece of stem were transferred onto standard MS medium (control) and media supplemented with 0.1 mg dm^{-3} 6-benzyladenine (BA), 0.9 mg dm^{-3} indole-3-butyric acid (IBA), and their combination.

After 35 d of cultivation under the same environmental conditions segments (1 mm^2) from the middle part of fully expanded leaves were taken from the 2nd or 3rd nodes, fixed in 3 % (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 ºC and used in microscopy studies.

Handmade transversal cuttings were mounted on slides in glycerol. Observation and measurements were carried out by *Amplival 4* microscope (*Carl Zeiss*, Jena, Germany). The thickness of the leaf lamina, mesophyll, palisade and spongy parenchyma, adaxial and abaxial epidermis were measured. The number of stomata [mm^{-2}] was counted on the middle part of both leaf surfaces. Fifteen measurements for each parameter and each treatment were made. Mean values were compared using one-way *ANOVA* and Kruskal-Wallis *ANOVA* on ranks test. All pairwise multiple comparison procedures were made by means of Holm-Sidak test and Tukey test, respectively. Microphotographs were taken using light microscope and camera *Nicon Eclipse 50i* (Tokyo, Japan).

For transmission electron microscopy (TEM), the leaf segments were post fixed 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) and cross-sectioned with *Reichert-Jung* (Wien, Austria) ultramicrotome. Observation was performed by *JEOL 1200 EX* (Tokyo, Japan) electron microscope.

Results

The leaves of *in vitro*-cultivated *O. stamineus* plants were dorsiventral, hypostomatous (Fig. 1*A*), with average thickness of the leaf lamina 170.50 ± 20.8 μm (Table 1). The thickness of the assimilation parenchyma took up to 87 % of the leaf lamina and was composed of one layer palisade cells which manifested normal symplast contact and 3 to 4 layers of spongy parenchyma cells. Epidermal tissues consisted of pavement cells with more or less sinuous undulated anticlinal walls, restricted to the abaxial side diacytic stomata (124 per mm^2) with different shape and size of the subsidiary cells, and two type of hairs – non-glandular uniseriate and glandular capitate with bicellular or four-cellular heads (Fig. 1*B,C*). BA influenced the palisade parenchyma and epidermal tissue structure. The form of the palisade cells was atypical, with undulated cell walls (Fig. 1*D*) thereby reducing the symplast contact and increasing the intercellular spaces. BA also increased the number of stomata on the adaxial epidermis (85 per mm^2) compared with the control leaves. The number of stomata on the abaxial epidermis was lower (72 per mm^2) and only about 50 % of the control leaves. Thus the leaf of plantlets growing on BA was amphistomatic (Fig. 1*E,F*). On the other hand, the average thickness of the spongy paren-

Table 1. Thickness [μm] of the leaf lamina and its tissues and stomata density [mm^{-2}] of *O. stamineus* plants cultivated *in vitro* on control MS medium without PGRS or media with 0.1 mg dm^{-3} BA or 0.9 mg dm^{-3} IBA or combination of both PGRS. Means ± SE of 15 measurements per treatment. Within each row means followed by the same letter were not significantly different at $P \leq 0.05$.

Parameter	Control	BA	IBA	BA + IBA
Leaf lamina	170.50 ± 20.38a	157.67 ± 20.95a	154.83 ± 18.01a	126.33 ± 11.29b
Mesophyll	147.83 ± 10.30a	134.00 ± 16.20b	124.00 ± 18.54b	96.33 ± 11.98c
Palisade parenchyma	52.33 ± 8.99a	52.50 ± 5.59ab	52.50 ± 4.01ab	39.83 ± 4.38c
Spongy parenchyma	87.00 ± 13.37a	83.00 ± 16.40a	70.83 ± 12.63b	61.67 ± 7.30b
Adaxial epidermis	16.33 ± 2.29a	17.33 ± 1.76ab	16.67 ± 1.81ab	14.33 ± 1.76ac
Abaxial epidermis	20.33 ± 3.11a	21.33 ± 2.08a	20.00 ± 2.11a	17.17 ± 2.08b
Stomata number, adaxial	1.15 ± 3.03a	85.06 ± 11.69b	6.32 ± 8.29a	76.44 ± 23.69b
Stomata number, abaxial	163.79 ± 17.24a	72.41 ± 24.12b	100.57 ± 21.28c	237.93 ± 18.37d

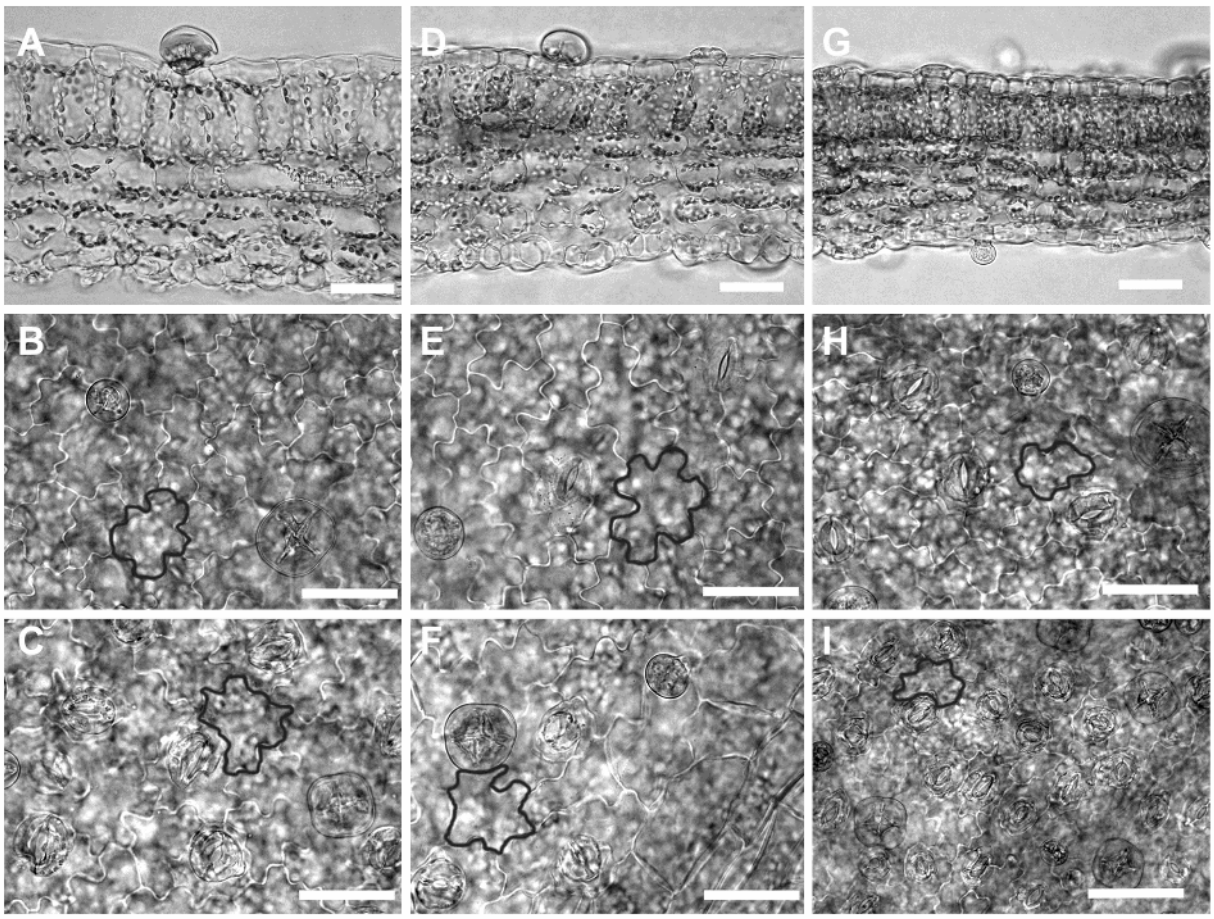

Fig. 1. Leaf cross-section (*A*,*D*,*G*), adaxial (*B*,*E*,*H*) and abaxial (*C*,*F*,*I*) epidermes of *O. stamineum* plants cultivated *in vitro* on MS medium without PGRs (control; *A*,*B*,*C*) or with 1 mg dm^{-3} BA (*D*,*E*,*F*) and BA + 0.9 mg dm^{-3} IBA (*G*,*H*,*I*). *Scale bars* = 50 μm. Epidermal pavement cells are highlighted with *black border*.

chyma was about 20 % less in plantlets grown on medium with IBA than in control plantlets, which resulted in thinner leaf lamina (154.83 ± 18.01 μm). Under the combination of BA and IBA, the average thickness of assimilation parenchyma was about 35 % lower in comparison to the control plants but the proportion between tissues was unchanged. The combination of BA and IBA induced higher number of stomata on the adaxial (76 per mm^2) as well as on the abaxial (238 per mm^2) epidermis. Stomata on the adaxial epidermis were regularly-shaped with typical form of the guard cells and pore (Fig. 1*H*). Stomata on the abaxial epidermis were unevenly distributed, indefinitely orientated with different guard cells size and aperture (Fig. 1*I*).

The chloroplasts of plantlets cultivated on standard MS medium without PGRs had typical lens shape and well-structured internal membrane system (Fig. 2*A*). Grana thylakoids represented different height (from 3 to 5 thylakoids) and were connected with great number of stroma thylakoids. There were few small starch grains in the stroma, but plastoglobuli were missing. In plantlets cultivated in medium supplemented with BA, oval-shaped chloroplasts with large contact area between them were observed (Fig. 2*B*). BA treatment resulted in bigger volume of the internal membrane system with atypical spatial orientation. Centrally situated thylakoids seemed to be damaged. Stroma thylakoids were almost missing and grana without distinct thylakoids were observed. Peripherally the internal membrane system was typically structured. IBA did not affect the structure of the plastid apparatus (Fig. 2*C*). Under combination of BA and IBA, chloroplasts with typical shape and well-developed internal membrane system were formed. The thylakoids were regularly-structured but covered a small part of the whole chloroplast volume (Fig. 2*D*). In the stroma different number (3 - 4) relatively big starch grains were observed. These chloroplasts differed from the control equivalent by the great amount of starch and relatively small proportion of the internal membrane system.

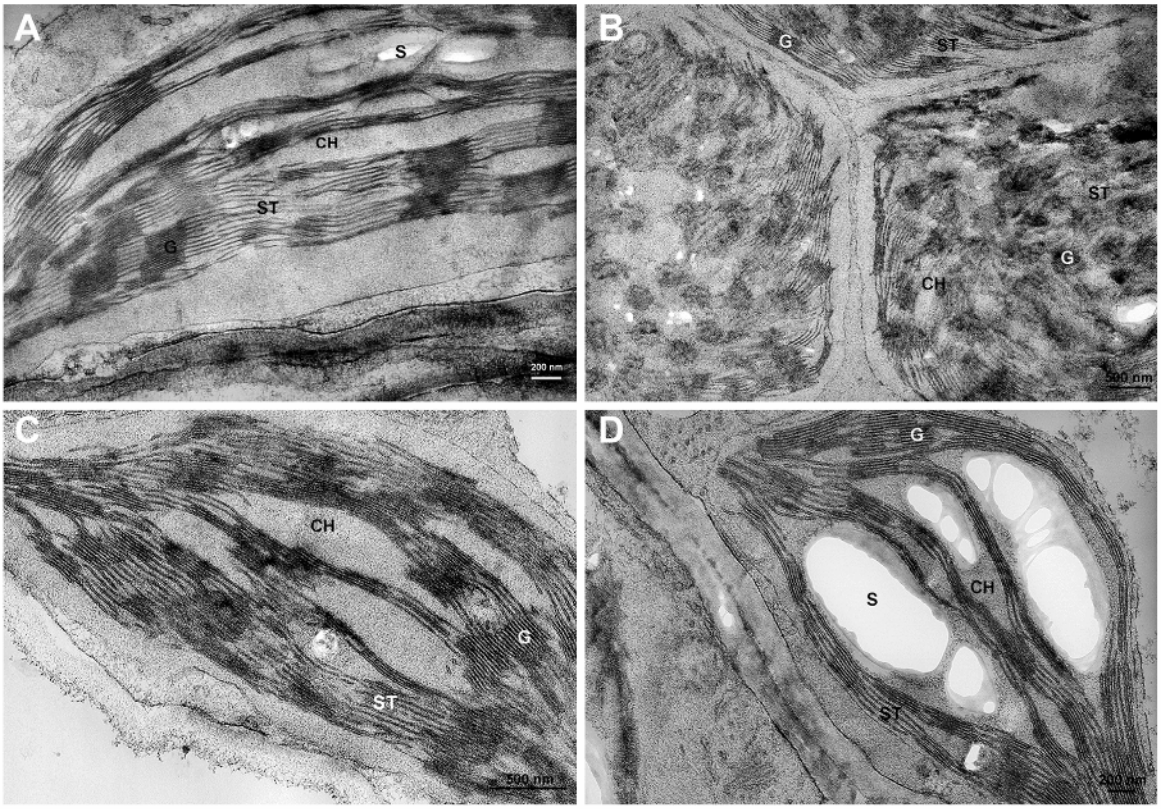

Fig. 2. The ultrastructure of chloroplast from control plants (*A*), plants cultivated on medium with BA (*B*), IBA (*C*) and BA + IBA (*D*). G - granum, CH - chloroplast, S - starch, ST stromal thylakoids.

Discussion

The leaf architecture and organization of the plastid apparatus are important indicators of the regulatory role of BA and IBA on the regeneration capacity of *in vitro* cultivated plants. According to some authors (Wetzstein and Sommer 1982, Magyar-Tábori *et al*. 2010) leaves of *in vitro* grown plants were generally characterized by poor mesophyll differentiation consisted of an absent or poorly developed palisade and spongy parenchyma with rounded interspersed cells. Microscopic analysis of the *in vitro* regenerated *O. stamineus* leaves showed a normal course of the histogenesis. The leaf architecture was not changed and was characterized with typical bifacial structure with well-differentiated palisade and spongy parenchyma. This was observed for both plantlets grown without PGRs in the medium (control) or in media supplemented with PGRs. In contrast to the reported effect of cytokinins on leaves of greenhouse-grown plants where they promoted an increase of the mesophyll parenchyma volume (Ron'zhina 2003), the BA effect on the photosynthetic tissue of *in vitro* cultivated *O. stamineus* plants was rather negative. Moreover, BA and IBA supplemented in the medium, decreased the number of glandular trichomes in *Lavandula dentata* plantlets (Sudriá *et al*. 2001), not established effect for *O. stamineus* plants. The results of our study verified the view of many authors who emphasized the role of genotype for the regenerative processes during the *in vitro* cultivation (Fasolo *et al*. 1989, Korban *et al*. 1992, Sarwar and Skirvin 1997). Of special interest is the role of some hormones as regulators of stomatal conductance (Chernyad'ev 2000). Usually *in vitro* regenerated plantlets had high stomata frequency (Capellades *et al*. 1990, Paek and Hahn 2000, Deccetti *et al*. 2008). In the current study high stomata frequency was observed only as a result of combined effect of BA and IBA.

Results obtained by Dobránszki *et al*. (2005) indicated that the type and concentration of hormones applied before the regeneration phase can modify the organogenetic potential of leaf tissues, and therefore also the regenerative process. The authors asserted that the structural characteristic of leaves altered by cytokinins was related to the organogenetic ability of explants. Anatomical changes detected after pre-treatment of shoots might be used as an indicator for regeneration potential. In the case of *in vitro* grown *O. stamineus* plantlets the observed modifications in the leaf structure seem to reflect the effect of BA and IBA on the

regenerative processes of the photosynthetic apparatus, which can be used further to predict plantlet growth properties.

The data obtained by TEM-analysis revealed that the structure of the chloroplasts might be strongly dependent on PGRs content in the medium. Cytokinins controlled the growth and division of chloroplasts and the formation of their membrane system and components of the electron transport chain (Chernyad'ev 2000). According to this author the low concentration of cytokinins (10^{-8} -10^{-5} M) regulated formation of chloroplasts by inducing the synthesis of chloroplast proteins and photosynthetic pigments in greenhouse-grown plants. The evidence for the role of cytokinins in chloroplast development and/or chloroplast gene expression was published by Kulaeva (2002). It has been established that in the presence of cytokinins different types of chloroplasts were differentiated: *1*) chloroplasts with low chlorophyll content, often abnormal with disorganized or limited formation of grana and abundant stroma (Magyar-Tábori *et al*. 2010), *2*) oval-shaped chloroplasts which lacked starch grains (Dobránszki *et al*. 2005), or *3*) chloroplasts with a well-developed grana system and great accumulation of starch grains (De Oliveira *et al*. 2008). The present study recorded some of the above mentioned characteristics only when BA was added in the culture medium. The atypical form and structure of the inner membrane system was considered to be directly related to BA, but not specific for this hormone. We observed similar structural organization in *in vitro* cultivated *Petunia hybrida*, but under the influence of IBA (Ganeva *et al*. 2009). The chloroplasts of *O. stamineus* plantlets were not affected by IBA under the present experimental conditions. The combination of BA and IBA was optimal for the plastid apparatus differentiation – chloroplasts with starch grains and well-organized internal membrane system were formed. Chloroplast differentiation and sugar metabolism in the explants' leaf tissue are essential for shoot regeneration *in vitro*. The relationship between starch accumulation in the tissues and regeneration potential had been reported for many species (Fortes 2000, Li 2000, Chen 2005).

In summary, the results of our study showed that combined application of BA and IBA in the MS medium favored proper differentiation of the mesophyll parenchyma and chloroplasts of *in vitro* propagated *O. stamineus* plants.

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