IN VITRO CULTIVATION AND EX VITRO ADAPTATION OF NEPETA NUDA SSP. NUDA – CORRELATION BETWEEN REGENERATION POTENTIAL, LEAF ANATOMY, AND PLASTID PIGMENTS

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Abstract


The leaf anatomy and the amount of the plastid pigments in in vivo plants, in vitro plants, in vitro regenerated after cryopreservation plants (cryo plants) and ex vitro adapted plants of Nepeta nuda ssp. nuda were examined as markers for the assessment of the regeneration potential of the species. The anatomical study showed that under in vitro conditions leaf with thin lamina and uniformly structured assimilation parenchyma was formed. The leaf of in vivo and ex vitro plants was equally structured - bifacial. It was found that the amount of the plastid pigments is greater in in vitro, cryo and ex vitro plants as compared to in vivo plants. The highest value was measured in cryo plants. The results indicated a high regeneration potential of N. nuda ssp. nuda during in vitro cultivation and ex vitro adaptation.

Key words: medicinal plant, assimilation parenchyma, cryopreservation, chlorophyll, carotenoids

Introduction

Nepeta nuda ssp. nuda is a herbaceous, perennial plant of the genus Nepeta (catmints) from the Lamiaceae family. The plant is valuable due to its essential oil which main constituents are the terpenoids possessing antimicrobial, antiviral, and antifungal activities (De Pooter et al., 1987; Handjieva et al., 1996), as well as a high antioxidant activity (Gkinis et al., 2010). This fact is a prerequisite to focus the studies on N. nuda and the other representatives of the genus Nepeta primarily on their essential oils composition (Hussain et al., 2009; Mehrabani et al., 2004; Alim et al., 2009; Gkinis et al., 2004), as well as putative medical applications (Aly et al., 2010). As a medicinal plant, N. nuda is suitable for microvegetative reproduction aimed at fast generation of a large amount of plants with known origin (Apóstolo and Llorente, 2000) and equal in quality (Hazarika, 2006), and having also the option for a long-term storage via cryopreservation.

The objective of the present work is to compare the status of N. nuda plant variants, namely: (i) in vivo-grown control plants; (ii) in vitro cultured plants; (iii) plants, regenerated in vitro after cryopreservation; (iv) ex vitro adapted plants. In particular, the study aims at analyzing the leaf anatomy and the amount of plastid pigments in leaves as markers for the assessment of the regeneration potential of the species during the processes of in vitro propagation and cryopreservation.

Materials and Methods

Plant material

Above-ground parts from mature in vivo-grown N. nuda plants were collected in their natural habitat in the Lozen Mountain near Sofia, Bulgaria, in period of blooming (in May). The voucher specimen 105807 was deposited in the Herbarium of the Department of Botany, Faculty of Biology, Sofia University. For in vitro cultivation, mono-nodal 1-2 cm
stem segments from the in vivo-grown plants were thoroughly washed with tap water and sterilized by incubation in 0.1% HgCl₂ (w/v) for 8 min followed by three washes with sterilized distiller water. Under aseptic conditions, the explants were inoculated on basal MS (Murashige and Skoog, 1962) medium containing 3% (w/v) sucrose and 7 g L⁻¹ agar without any supplement of growth regulators. The in vitro cultivation occurred under controlled environment (16 h light/8 h dark, 60 μmol m⁻² s⁻¹ photosynthetic photon flux density, Philips TLD-33, temperature 25°C and 60-70% relative air humidity). The plant material was collected after four weeks of cultivation. For cryopreservation and ex vitro adaptation were used 4-week-old N. nuda plants with fully developed leaves and roots with absence of vitrification and callus formation.

Cryopreservation of N. nuda shoot-tips

Shoot-tips of in vitro propagated N. nuda plants were precultured in 10 ml liquid RMB₀₅ medium (containing MS salts, 1 ml L⁻¹ Gamborg’s vitamins (Gamborg et al., 1968), 1 ml L⁻¹ glycerol, 100 mg L⁻¹ myo-inositol and 2% (w/v) sucrose), and supplemented with 0.076 μM abscisic acid (ABA) and 0.5 mg L⁻¹ benzyl adenine (BA) for 7 days. Further, the explants were treated for 20 min in LS solution (2 M glycerol and 0.4 M sucrose) at room temperature. Plant shoot-tips were dehydrated in PVS₃ (50% w/v sucrose and 50% w/v glycerol) and 0.4 M sucrose) at room temperature. Plant shoot-tips were rinsed in liquid RMB₀₅ containing 1.2 M sucrose and cultivated further on semi-solid RMB₀₅ for regeneration. After 2-week cultivation in the dark and 1-week cultivation in half-intensity light, the survived cryopreserved plants were grown at the same environmental conditions as the in vitro cultured non-frozen plants. The survival rate was determined as the percentage of green growing meristems with differentiating shoots 4–6 weeks after cryopreservation compared to the initial number of frozen shoot-tips. The survived cryopreserved plants (cryo plants) were propagated on basal MS medium for several months.

Ex vitro adaptation of N. nuda plants

For ex vitro adaptation, the regenerated in vitro-grown and cryopreserved N. nuda plants with well-developed root system were removed from the culture tubes. The agar from the medium was washed away from the roots. The roots were treated with an aqueous solution of potassium permanganate (KMnO₄) for sterilization. The plants were transferred into plastic pots containing a mixture from sterile soil: coconut filling:sand = 2:1:1. The adaptation was maintained in a growth chamber (POL-EKO APARATURA SP.J.A. Polok Kowalska KK 350 STD 1400 W) for 20 days under controlled conditions by changing the relative humidity. Next, the adapted plants were transferred to a standard growth-room for a month followed by a transfer to the greenhouse for another month, and subsequent transfer outside to the natural habitat in the field of Lozen Mountain, near Sofia. After one year of adaptation to the field conditions, well-developed ex vitro plants of regenerated in vitro-grown and cryopreserved plants were harvested during blooming in June.

Light microscopy

For histological analysis mature leaves from in vivo-grown plants, fully expanded leaves of the 2nd and 3rd nodes from 4-week-old in vitro cultured plants (non-frozen and cryo), and well developed leaves from ex vitro adapted in greenhouse plants were collected. Small segments were taken from the middle part of the leaves and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. Handmade transverse cuttings were mounted on slides in glycerol. Histological observations and documentation of the results were carried out by light microscope and camera Nicon Eclipse 50i (Tokyo, Japan).

Plastid pigments assay

Chlorophyll (a and b) and carotenoid content were determined according to Arnon (1949). Leaf material (30 mg) of each variant was homogenized in 80% aqueous acetone and the samples were centrifuged. The absorbance of the obtained pigment extract was measured at 663, 645 and 452.5 nm using Shimadzu UV 1800 spectrophotometer. The pigments quantity was calculated as previously reported (McKinney, 1941). The statistical significance between the plant variants was assessed by using t-test at $P \leq 0.05$ and $P \leq 0.001$.

Results

Leaf anatomy

The leaves of N. nuda in vivo-grown plants were bifacial and amphistomatous. The stomata density was higher at the abaxial leaf surface (Figure 1A). The assimilation parenchyma consisted of double-layered palisade parenchyma and 3-4 layered spongy parenchyma. The palisade cells in the upper layer were more elongated than these in the lower layer and had a regular cylindrical shape. The spongy cells had a typical irregular shape and only the ones adjacent to the abaxial epidermis cells were slightly elongated. The epidermis characterized with pavement epidermal cells, stomata and two types of trichomes – non-glandular and glandular. Both adaxial and abaxial epidermises formed multicellular non-glandular trichomes composed of a file of cells ending
Plastid pigments

The quantitative analysis of plastid pigments in leaves of *in vivo*-grown control *N. nuda* plants showed that the amount of chlorophyll *a* was 1.05 ± 0.14 mg.g⁻¹ fresh weight (FW), chlorophyll *b* - 0.78 ± 0.02 mg. g⁻¹ FW, and the carotenoids content was 0.07 ± 0.02 mg.g⁻¹ FW (Table 1). It was established that the pigment content in leaves of all the other variants was significantly higher in comparison with the *in vivo*-grown *N. nuda* plants. The amount of chlorophyll *b* showed the least increase (from 0.78 to 0.97 mg. g⁻¹ FW), while the amount of chlorophyll *a* was greater (from 1.05 to 1.84 mg. g⁻¹ FW), and the highest increase was observed in the amount of carotenoids (from 0.07 to 0.22 mg. g⁻¹ FW) (Table 1). The top amount of chlorophyll *a* was found in leaves of *cryo* plants - 75%, whereas in *ex vitro* regenerated plants (non-frozen and *cryo*) it reached up to 52% and 43%, respectively, in comparison with the *in vivo*-grown plants (Table 1). The amount of chlorophyll *b* ranged between 1% and 6% and was greatest in leaves of *cryo* plants by 24% (Table 1). In the case of carotenoids, the strongest peak in the amount was observed again in leaves of *cryo* plants - three times greater in comparison with the *in vivo*-grown plants (Table 1).

Discussion

The results of the histological study have shown that *N. nuda* is a species, for which the *in vitro* and *cryo* conditions of cultivation influence the thickness of the leaf lamina in the newly formed leaves as well as the histogenesis of the assimilation parenchyma. It has been found out that the *in vitro* conditions significantly influence the ontogeny (Hazarika, 2006), and the effect on the leaf histogenesis could be quite negative (Mills, 2009). In general, the *in vitro* plants form leaves with small (Knöss, 1999) and thin lamina (Zobayed et al., 2012). Formation of thinner leaves is considered to be a universal trait of the *in vitro* cultivated plants (Mayer et al., 2008; Saez et al., 2012) and *N. nuda* is a species that reacted

<table>
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<tr>
<th>Variants</th>
<th>Chl. a</th>
<th>Chl. b</th>
<th>Carotenoids</th>
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<tbody>
<tr>
<td><em>N. nuda</em> in vivo</td>
<td>1.05±0.14</td>
<td>0.78±0.02</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td><em>N. nuda</em> in vitro</td>
<td>1.27**±0.09</td>
<td>0.79±0.03</td>
<td>0.15***±0.02</td>
</tr>
<tr>
<td><em>N. nuda</em> cryo</td>
<td>1.84***±0.19</td>
<td>0.97***±0.10</td>
<td>0.22***±0.05</td>
</tr>
<tr>
<td><em>N. nuda</em> ex vitro</td>
<td>1.60***±0.17</td>
<td>0.83±0.05</td>
<td>0.16***±0.06</td>
</tr>
<tr>
<td><em>N. nuda</em> ex vitro after cryo</td>
<td>1.50***±0.03</td>
<td>0.83***±0.01</td>
<td>0.13***±0.01</td>
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Fig. 1. Leaf lamina cross-section of *N. nuda* in vivo plants (*A, B*), *in vitro* regenerated plants (*C*), *in vitro* regenerated cryopreserved plants (*D*), *ex vitro* adapted *in vitro* cultured plants (*E*), and *ex vitro* adapted *in vitro* regenerated cryopreserved plants (*F*)
showed that a direct relation between anatomical structures in the leaves of in vitro cultured plants differed from the same leaf tissue in in vivo-grown plants (Zhao et al., 2006; Dousseau et al., 2008; Jarda et al., 2011; Stefanova et al., 2011). It is very common for in vitro cultivation that instead of bifacial leaves a uniform assimilation parenchyma to be structured. Our studies on in vitro and cryo N. nuda plants revealed the same morphological characteristics of the newly formed leaves.

The anatomical study of in vitro and cryo plants regenerated and subsequently adapted to ex vitro conditions has shown that the ex vitro N. nuda plants have the same histological organization of the leaves as in vivo-grown plants. The typically structured assimilation parenchyma and epidermal tissues in ex vitro formed leaves are indicators of the high regeneration potential of N. nuda during cultivation. At cellular and tissue level of organization these are indicators of genetic stability of the anatomical features (Zhao et al., 2006) which is an essential but not always a sufficient precondition for successful regeneration of plants.

At a physiological level, photosynthesis is strongly affected by exposure to cold and the photosynthetic pigments are highly sensitive to the deleterious effect of freezing. Low temperature causes a significant reduction in chlorophyll $a$ and chlorophyll $b$, as well as significant changes in the carotenoid composition (Partelli et al., 2009). In a study of regenerated Hypericum tetrapetalum plants after cryopreservation (Georgieva et al., 2014) no significant changes were observed in the amount of photosynthetic pigments, which according to the authors showed that this species is more tolerant to freezing procedures. In another study with H. rumeliacum plants after cryopreservation, Danova et al. (2012) also have found an increase in the amount of chlorophyll $a$ and chlorophyll $b$. Significant enhancement in the amount of carotenoids was found in cryopreserved H. rumeliacum plants, which have a protective role for the oxidative stress (Georgieva et al., 2014). It is well documented that carotenoids may act as antioxidants, which functions include membrane protection against free radicals’ damage and their abundance increases at low temperatures (Ivanov et al., 2006). It could be suggested that the accumulation of plastid pigments in in vitro propagated (non-frozen and cryo) and ex vitro adapted N. nuda plants is a kind of compensatory mechanism associated with the cultivation conditions.

**Conclusion**

As many previous research reports, the present study also showed that a direct relation between anatomical structures of leaves of in vitro and cryo N. nuda plants and the level of plastid pigments could not be made. The question that remains is: why is it so high the content of chlorophyll $a$ and carotenoids in these plants, if the histogenesis of assimilation parenchyma is disturbed? The answer of this question could be found in further studies on the structural organization of the plastid apparatus and the putative compensatory mechanism at subcellular level.

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**References**


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