*Bulgarian Journal of Agricultural Science, 21 (No 5) 2015, 1027-1032*
*Agricultural Academy*

# *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

DRAGOLOVA, D., M. STEFANOVA, M. DIMITROVA, D. KOLEVA, M. ZHIPONOVA and V. KAPCHINA-TOTEVA, 2015. *In vitro* cultivation and *ex vitro* adaptation of *Nepeta nuda* ssp. *nuda* – correlation between regeneration potential, leaf anatomy, and plastid pigments. *Bulg. J. Agric. Sci.*, 21: 1027–1032

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

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stem segments from the *in vivo*-grown plants were thoroughly washed with tap water and sterilized by incubation in 0.1% $HgCl_2$ (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 7g.$L^{-1}$ 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 25ºC 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 $RMB_{0.5}$ 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) for 90 min on ice, and finally directly immersed into liquid nitrogen (-196˚C). After one week of storage, thawing was performed in water bath at 40ºC for 1 min. The shoot-tips were rinsed in liquid $RMB_{0.5}$ containing 1.2 M sucrose and cultivated further on semi-solid $RMB_{0.5}$ 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 ($KMnO_4$) for sterilization. The plants were transferred into plastic pots containing a mixture from sterile soil: coconut fillings: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

with acute apical cell. The glandular trichomes were two types – capitate (composed of a single basal cell, a stalk cell, and a head cell), and peltate (composed of a single large basal cell, a single flattened stalk cell, and a head of four secretory cells). The peltate trihomes were located only on the abaxial epidermis slightly below the leaf surface (Figure 1B). The essential oil accumulated in the space between the head cells and the detached cuticle. The histological analysis detected essential oil droplets also in the palisade cells.

The *in vitro* plants formed leaves with approximately two-fold thinner leaf lamina and different organization of the assimilation parenchyma in comparison to the leaves of the *in vivo*-grown plants (Figure 1C). The leaf blade was composed of one layer very short palisade cells and two layers of tightly packed spongy cells. Due to this morphological characteristic of the photosynthetic cells the assimilation parenchyma looked uniformly structured. Almost the same tissue organization was represented in the leaves of plants regenerated after cryopreservation (Figure 1D). In both *in vitro* growth variants the regenerated plants formed leaves without oil droplets in the photosynthetic cells. The histological analysis showed that the *in vitro* and *cryo* conditions did not significantly influence the histogenesis of the epidermal tissues.

The anatomical study of leaves from regenerated plants adapted to *ex vitro* conditions showed that both *in vitro* and *cryo* plants formed leaves with equal thickness of the lamina and same histological organization as the *in vivo*-grown control plants (Figure 1E, F). The assimilative parenchyma of all *ex vitro* plants was typically structured. It was formed of two layers regular cylindrical palisade cells with essential oil inclusions and three layers of spongy cells. There were no differences in the structural organization of the epidermal tissues in leaves between *ex vitro* adapted plants and *in vivo*-grown control plants. Thus, the described leaf organization could be considered as a structural marker for regular histogenesis of the newly formed leaves of all *ex vitro* adapted regenerated plants.

***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., 1999). 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 1**
**The amount of pigments content (mg.g$^{-1}$ FW) in leaves of *in vivo*, *in vitro*, *cryo*, *ex vitro* и *ex vitro* after *cryopreservation N. nuda* plants. Statistical significance changes compared to the control *in vivo*-grown plants were calculated by *t*-test, asterisks indicate *P* value: * *P*≤0.05, ** *P*≤0.01 and *** *P*≤0.001.**

| Variants | Chl. a | Chl. b | Carotenoids |
|---|---|---|---|
| *N. nuda in vivo* | 1.05±0.14 | 0.78±0.02 | 0.07±0.02 |
| *N. nuda in vitro* | 1.27**±0.09 | 0.79±0.03 | 0.15***±0.02 |
| *N. nuda cryo* | 1.84***±0.19 | 0.97***±0.10 | 0.22***±0.05 |
| *N. nuda ex vitro* | 1.60***±0.17 | 0.83*±0.05 | 0.16***±0.06 |
| *N. nuda ex vitro after cryo* | 1.50***±0.03 | 0.83***±0.01 | 0.13***±0.01 |

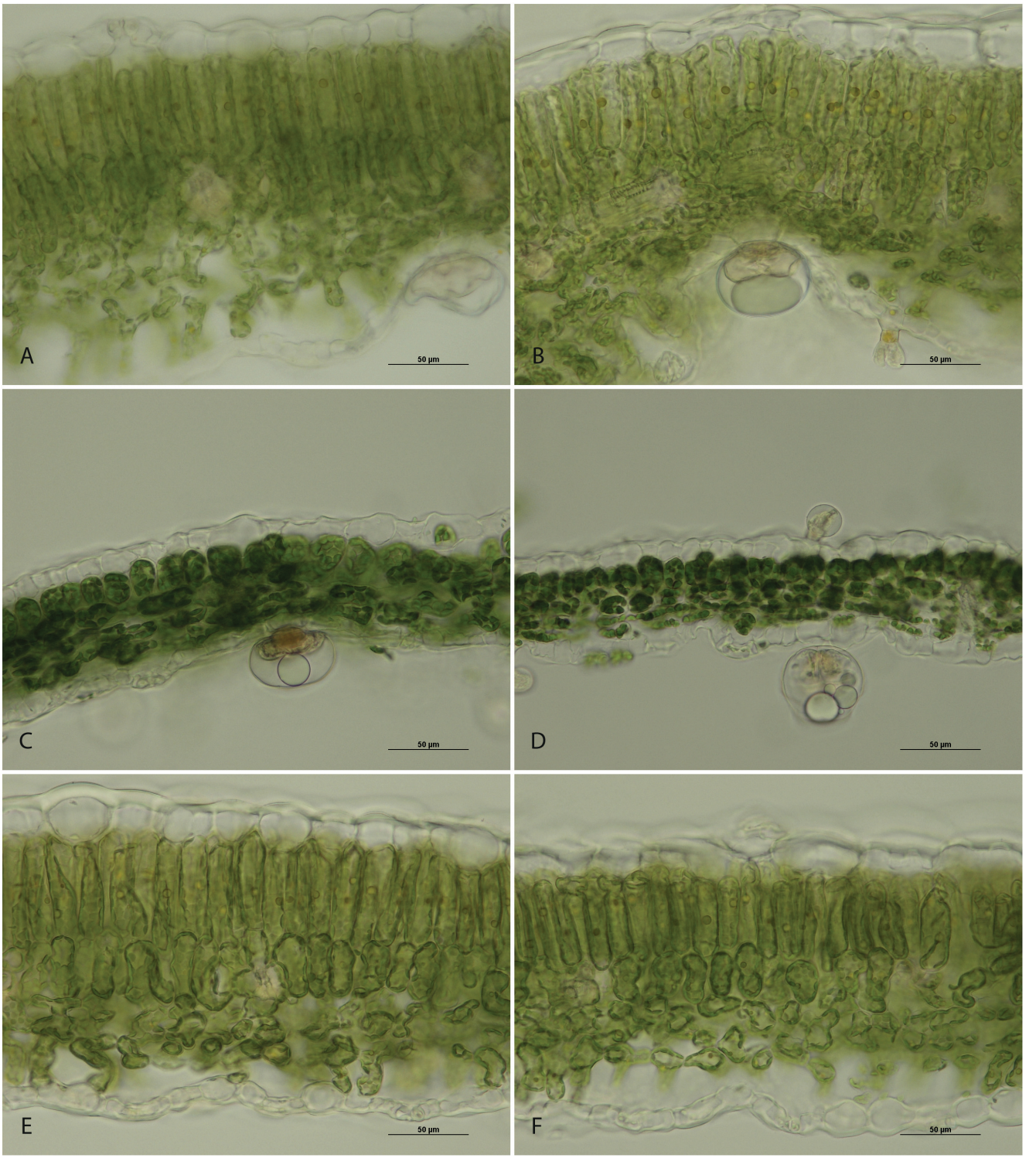


**Fig. 1. Leaf lamina cross-section of *N. nuda in vivo* plants (*A*, *B*), *in vitro* regenerated plants (*C*), *in vitro* regenerated cryopreserved plants (*D*), *ex vitro* adapted *in vitro* cultured plants (*E*), and *ex vitro* adapted *in vitro* regenerated cryopreserved plants (*F*)**

similarly in the process of cultivation. For many species it has been registered that the architecture of the assimilation parenchyma 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* duing 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 tetrapterum* 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 has 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 caroteneoids 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.

***Acknowledgments***

The investigation was financially supported by grant 34/15 of Sofia University.

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*Received January, 6, 2015; accepted for printing August, 14, 2015*