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COMPARATIVE LEAF STRUCTURAL ANALYSIS OF *Nepeta nuda* L. PLANTLETS, REGENERATED FROM CRYOPRESERVED SHOOT MERISTEM AND *ex vitro*-ADAPTED PLANTS

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ABSTRACT

The leaf anatomy and chloroplast ultrastructure of *Nepeta nuda* L. plantlets regenerated *in vitro* from cryopreserved shoot apical meristem and *in vitro*-micropropagated plantlets were studied comparatively to assess whether cryoprocedure affected leaf morphogenesis. Both postcryo and in vitro plantlets failed to develop a distinguishable palisade layer, making the mesophyll appear homogeneous. Significant damage to the chloroplast envelope and substantial thylakoid ruptures were also observed. We assumed that the specific in vitro conditions more likely affected the structures than the cryotreatment itself. Light and transmission electron microscopy observations were also carried out on newly formed leaves of *ex vitro*-adapted plants. The examined leaf features were similar to those in the *in situ* plants – bifacial leaf lamina, double-layered palisade parenchyma, loosely arranged spongy parenchyma cells, and chloroplasts with intact envelope and evenly distributed throughout the stroma internal membrane system. The obtained histological and ultrastructural results revealed the retained morphogenetic potential of *N. nuda* plants and proved cryopreservation as a suitable method for long-term storage.

Key words: chloroplast, cryopreservation, leaf anatomy, light microscopy, mesophyll, transmission electron microscopy

INTRODUCTION

Nepeta nuda L. (syn. *N. pannonica* L.) is an herbaceous perennial plant inhabiting mainly Europe, Southwest Asia, and Central Asia. The species is well known in folk medicine in Bulgaria and the Balkan Peninsula. Several studies have proven the antimicrobial, fungal static, antioxidant, anti-inflammatory, cholesterol-lowering, cardioprotective, and insecticidal activity of *in situ* and *in vitro* cultivated *N. nuda* plants [Baranauskienė et al. 2019, Baser et al. 2020, Hinkov et al. 2020]. Acimovic et al. [2020, 2022] summarized that *N. nuda* is a "highly promising species for application in food and pharmaceutical industries, as well as in agriculture for the development of natural pesticides". However, the increased demand for plant raw materials threatened the natural habitats of the species, so it became a starting point for biotechnological research. In the last few decades, cryopreservation of plant material combined with *in vitro* culture has been established as a successful method for the long-term preservation of plant species with valuable economic qualities or conservation importance [Brunáková and Cellárová 2016, Halmagyi et al. 2017, Coelho et al.

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2020, O'Brien et al. 2021]. Germplasm cryostorage has been reported for over 100 plant species in recent decades [Kaviani and Kulus 2022]. Currently, the scientific interest in this field has become even more comprehensive. Cryopreservation – the storage of plant germplasm in liquid nitrogen – is especially applicable for species in which micropropagation can be initiated from shoot tips [Zhang et al. 2023]. The shoot tips comprise organized structures and are genetically programmed to develop into "true-to-type" plants. Cryopreservation for long-term conservation of *in vitro* germplasm results in the exposure of tissues to physical, chemical, and physiological stresses that cause cryoinjury, so it is of great importance to assess the genetic stability of plants surviving cryostorage to determine if they are "true-to-type" after the procedure [Roque-Borda et al. 2021]. Moreover, cryopreservation induces processes associated with reactive oxygen derivatives, which may manifest later in regenerated plant tissues, causing cytological and histological alterations [Ren et al. 2021, Whelehan et al. 2022] and leading to problems for shoot development and whole plant recovery after cryostorage treatments [Kulus et al. 2018]. Some histological and genetic studies on surviving cryotreated shoot tips confirmed their potential for regeneration was preserved, but they did not further research the extent to which this potential is achieved [Halmagyi et al. 2017]. Ganeva et al. [2009] supposed that the cell and tissue structure of differentiated leaves from plants cultivated *in vitro* from successfully cryoprotected plant material was insufficiently investigated. Later studies of *in vitro* regenerated plants after cryopreservation found that in the most critical photosynthetic organs, the leaves, there are deviations in the mesophyll structure and chloroplast ultrastructure, and these alterations are species-specific, which confirms the need to continue the research. These works revealed that examinations at the histological, cellular, and subcellular levels were necessary for evaluating plant regeneration capacity in vitro and during *ex vitro* acclimatization [Stoyanova-Koleva et al. 2013, 2015]. Nevertheless, many open questions that refer to the structural and functional status of the plants regenerated from recovered after cryo-treatment meristem remain and deserve further analysis.

In this study, we aimed to evaluate the morphogenetic capacity of *N. nuda* plantlets regenerated *in vitro* from cryopreserved shoot tips and then adapted *ex vitro* to assess whether this method was suitable for long-term storage of *N. nuda* germplasm. To achieve this goal, we examined and compared the leaf histological features and the chloroplast ultrastructure of plantlets regenerated *in vitro* from cryopreserved meristems, *ex vitro*-adapted plants, and in situ plants.

MATERIAL AND METHODS

Plant material

Nepeta nuda L. plants in the flowering phase were collected from a natural locality in Lozen Mountain [42.584722, 23.5167524], Bulgaria. The voucher specimen SO-105807 was deposited in the Herbarium of Sofia University "St. Kliment Ohridski."

Plant multiplication and maintenance *in vitro*, cryopreservation by the vitrification method, postcryo *in vitro* regeneration, and *ex vitro* adaptation were carried out in the Laboratory of Plant Biotechnology, Department of Plant Physiology, Faculty of Biology, University of Sofia "St. Kl. Ohridski" [Dragolova et al. 2014, Dragolova et al. 2015]. The examined variants were as follows: 1) *in situ N. nuda* plants (i*n situ* plants); 2) *in vitro* cultivated 5-week-old plantlets (*in vitro* plantlets); 3) 5-week-old plantlets recovered *in vitro* from cryopreserved meristems (postcryo plantlets); 4) plants adapted *ex vitro* in a greenhouse from *in vitro* cultivated plantlets approximately three months after transferring them to soil (*ex vitro* plants); and 5) plants adapted *ex vitro* in a greenhouse from regenerated *in vitro* after cryopreservation plantlets approximately three months after transferring them to soil (postcryo *ex vitro* plants).

Light microscopy (LM)

Ten fully expanded leaves of the third or fourth nodes were collected from five plants of each variant. Small segments (4–5 mm^2) from the middle part of the leaf lamina were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. Handmade transverse sections were mounted on slides in glycerol. Histological observations were carried out by an Amplival 4 light microscope (Carl Zeiss, Jena, Germany). A light microscope and Nicon Eclipse 50i camera (Tokyo, Japan) with NIS-Elements BR 2.30 software took representative microphotographs with high resolution.

Statistical analysis

Ten microphotographs of each variant were used to measure the thickness of the leaf lamina (LL), palisade parenchyma (PP), spongy parenchyma (SP), adaxial (AdE), and abaxial (AbE) epidermises. Thirty measurements for each parameter and each variant were made with ImageJ software. The statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the comparison of group means (Tukey test, $p < 0.05$) with the program SigmaPlot.

Transmission electron microscopy (TEM)

For ultrastructural analysis of the mesophyll chloroplasts, leaf segments (1–2 mm^2) were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and postfixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100% v/v), the samples were embedded in Durcupan (Fluka, Switzerland) and sectioned with a Reichert-Jung (Wien, Austria) ultramicrotome. The ultrathin (50–60 nm thickness) samples were mounted on copper grids and double-stained with uranyl acetate and lead citrate. A JEM-2100 (JEOL Ltd. Tokyo, Japan) electron microscope performed observations and documentation of the results.

RESULTS

The transverse section of *in situ N. nuda* leaves revealed bifacial leaf lamina with true-to-type organized mesophyll (Fig. 1A1). The palisade parenchyma was double-layered, composed of cells with a typical cylindrical shape and many peripherally located chloroplasts. The cells were longer in the upper layer than in the lower layer. The spongy parenchyma consisted of 3–4 rows of loosely arranged cells with irregular shapes. Despite the large intercellular spaces, the spongy tissue was 23% less thick than the palisade parenchyma. Both adaxial and abaxial epidermises were composed of ordinary cells, stomata, and two types of trichomes – non-glandular and glandular (Fig. 1A2). The outer walls of the ordinary epidermal cells of the adaxial epidermis were convex. Additionally, the thickness of the adaxial epidermis was greater than that of the abaxial epidermis. According to the measurements, the in situ plants developed the thickest leaf blade, palisade, and spongy parenchyma (Tab. 1).

Predictably, there were significant differences in the histological organization and the measured features between the in situ plants and both types of in vitro plantlets but not between them (Fig. 1B, 1C, Tab. 1). The leaf lamina was approximately threefold thinner in the *in vitro* plantlets and three and a half times thinner in the postcryo plantlets. In both *in vitro* variants, the palisade parenchyma consisted of only one row of concise cells with round or irregular shapes. The spongy tissue comprised 2–3 rows of compactly arranged oval-shaped cells. However, the thickness of the spongy parenchyma was 66% greater than that of the palisade parenchyma. The observed mesophyll organization was difficult to accept as bifacial. The epidermises were thinner than in the in situ leaves, and usually, the outer walls of the adaxial ordinary cells were flat and even slightly concave.

The leaf histological organization of the *ex vitro*-adapted plants' two variants was very similar and slightly different from that of the *in situ* plants (Fig. 1D, 1E). The palisade parenchyma was also double-layered, but the cells in the lower row were relatively short and often hourglass-shaped, especially in the postcryo *ex vitro* plants. However, the thickness of the palisade parenchyma was almost the same as that in the in situ plants. In both *ex vitro* variants, the spongy parenchyma was almost twofold thinner than the palisade tissue. Compared with the in situ leaves, the spongy cells were more compact, reducing the volume of the intercellular spaces. The latter decreased the lamina thickness of the *ex vitro* leaves compared with the in situ leaves (Tab. 1). The thickest abaxial epidermises were measured in both *ex vitro* variants, although marked differences in the structure of the epidermis in comparison with the *in situ* plants were not observed.

TEM analysis of the mesophyll cells from the in situ plants identified chloroplasts with a typical oval shape. The internal membrane system occupied the entire volume of the stroma and was composed of medium-high grana (7–15 lamellae) connected by parallel-oriented long stromal thylakoids. Starch grains were observed in the stroma. Plastoglobules were absent (Fig. 2A).

The chloroplasts in the *in vitro* plantlets were elongated to oval-shaped. The chloroplast envelope was impaired. The thylakoid membranes in the central zone of the stroma were destroyed, so it was not easy to distinguish apparent granal stacking. The granal thy-

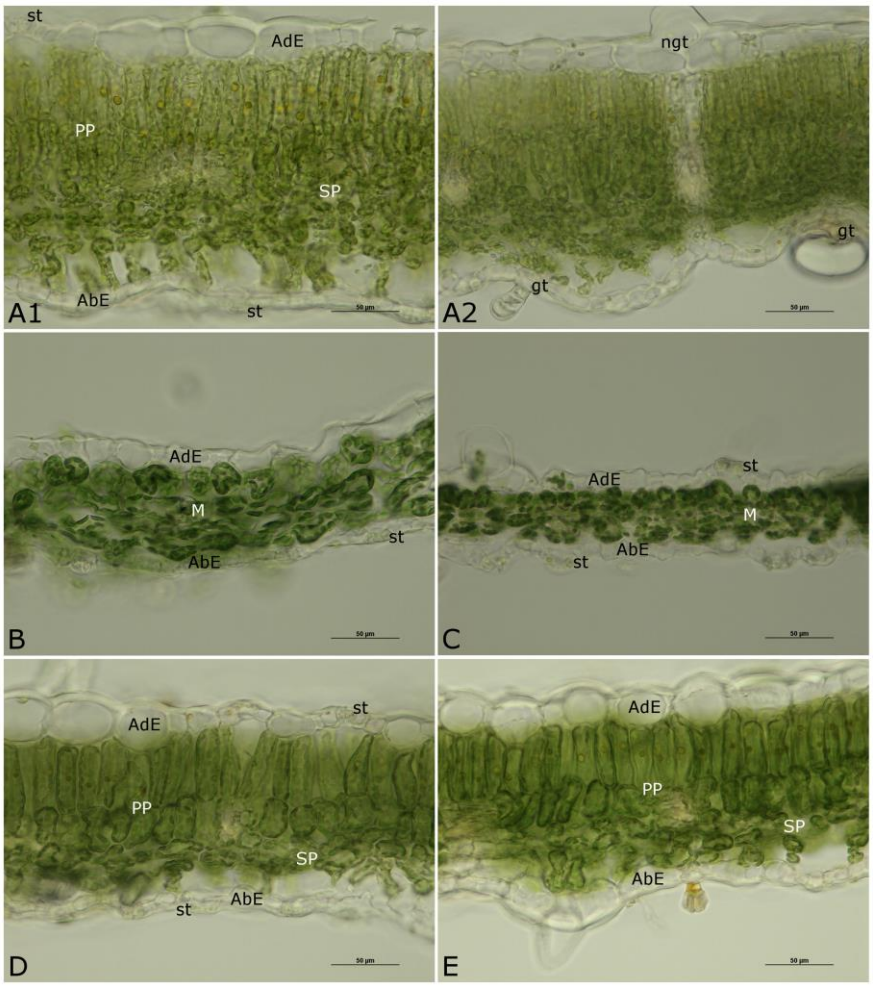

Fig. 1. . Leaf anatomy of *N. nuda*. Transverse section of the middle part of the leaf blade: A1, A2 – *in situ* plants, B – *in vitro* plantlets, C – postcryo plantlets, D – *ex vitro* plants, E – postcryo *ex vitro* plants; AdE – adaxial epidermis, AbE – abaxial epidermis, PP – palisade parenchyma, SP – spongy parenchyma, ngt – non-glandular trichome, gt – glandular trichome, st – stoma

Table 1. Morphometrical data of the leaf anatomical features of the *N. nuda* variants

***N. nuda* variants**	**Leaf anatomical features***				
	leaf lamina	palisade parenchyma	spongy parenchyma	adaxial epidermis	abaxial epidermis
in situ plants	202.60 ±12.13^{a}	89.67 ±7.90^{a}	77.93 ±11.54^{a}	24.23 ±5.17^{a}	11.81 ±1.50^{a}
in vitro plantlets	74.12 ±10.34^{b}	20.27 ±2.19^{b}	30.94 ±6.06^{b}	11.84 ±3.15^{b}	7.71 ±1.51^{b}
postcryo plantlets	57.10 ±6.15^{b}	15.13 ±2.43^{b}	21.53 ±3.02^{c}	10.49 ±2.40^{b}	7.86 ±1.58^{b}
ex vitro plants	171.48 ±18.60^{c}	89.16 ±9.57^{a}	44.74 ±11.22^{d}	23.63 ±7.43^{a}	13.98 ±2.90^{a}
post-cryo *ex vitro* plants	162.99 ±12.22^{c}	77.47 ±6.44^{c}	45.52 ±7.99^{d}	24.52 ±5.63^{a}	14.64 ±3.35^{a}

* Values of the thickness (µm) were expressed as the means ± standard deviations. Statistically significant differences (Tukey's test, $p < 0.05$) between the variants are indicated by different letters.

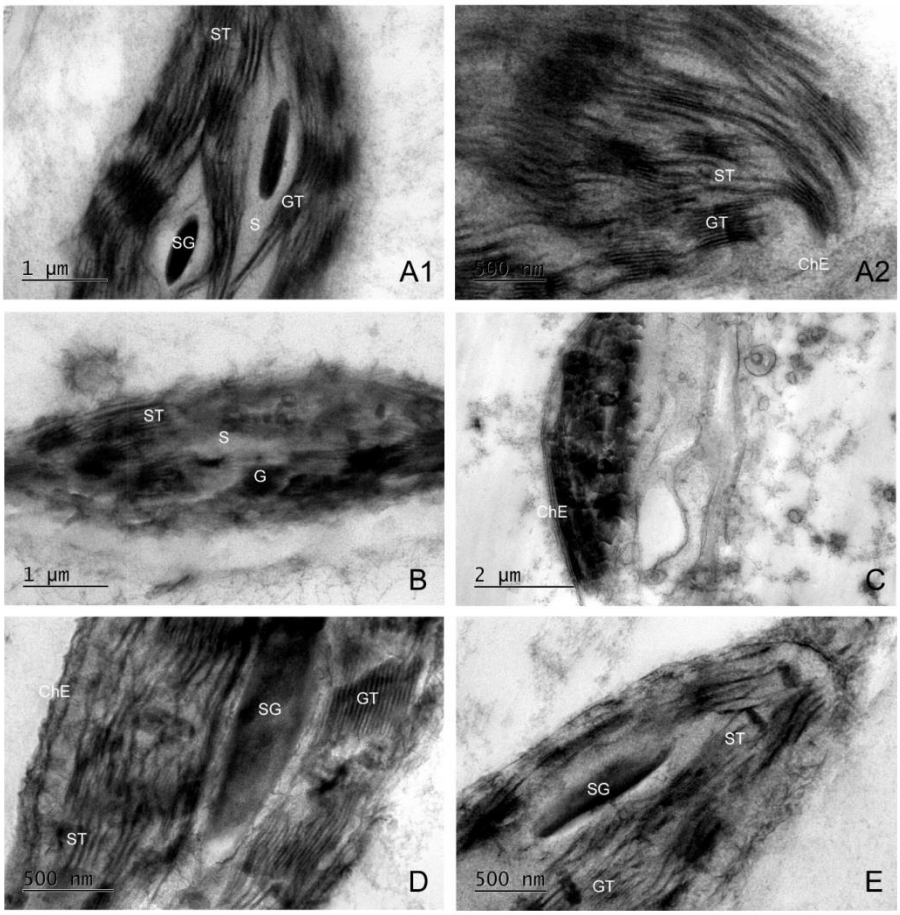

Fig. 2. Mesophyll chloroplast ultrastructure of *N. nuda* after various treatments. A1, A2 – *in situ* plants, B – *in vitro* plantlets, C – postcryo plantlets, D – *ex vitro* plants, E – postcryo *ex vitro* plants; ChE – chloroplast envelope, GT – granal thylakoids, S – stroma, SG – starch grain, ST – stromal thylakoids

lakoids in the periphery were fused, while the stromal thylakoids were partially preserved. (Fig. 2B). The chloroplasts in the postcryo plantlets' mesophyll cells were also elongated to oval. The chloroplast envelope membranes appeared intact, while the thylakoid membranes were almost completely fused or destroyed. Only a few stromal thylakoid membranes in the chloroplast periphery seemed partially preserved (Fig. 2C).

The *ex vitro*-adapted plants had typical oval-shaped chloroplasts. The chloroplast envelope was entire, and the internal membrane system was evenly distributed throughout the chloroplast stroma. The grana were of different heights and consisted of 3–4 to 17–20 thylakoids. Few stromal thylakoids were observed. Single starch grains were present in the stroma (Fig. 2D). In the postcryo *ex vitro* plants, the chloroplast structure was also somewhat restored during *ex vitro* adaptation. The internal membrane system occupied the entire volume of the stroma except for the space in which there were starch grains. The grana were low, some thylakoids were fused, and the stromal thylakoids were few and loosely situated in the stroma (Fig. 2E).

DISCUSSION

In fully secured shoot tips, the dome meristem and the leaf primordia can survive storage in liquid nitrogen [Kulus et al. 2019]. The possible injuries that might occur at the cellular and subcellular levels during cryopreservation may become evident sometime later and

could directly affect morphogenesis during subsequent regeneration and *ex vitro* acclimatization [Halmagyi et al. 2017, 2022]. For example, histological studies of regenerated *Hypericum rumeliacum* and *Orthosiphon stamineus* plantlets after cryopreservation reported cell plasmolysis, the collapse of the photosynthetic tissue, and destruction of some intracellular membranes [Ganeva et al. 2009]. However, in our study, such severe damage to leaf tissues was not observed in the postcryo *N. nuda* plantlets. The anatomical characteristics of the postcryo leaves were strongly reduced thickness of the lamina and its tissues, dense uniformly structured mesophyll – poorly differentiated palisade parenchyma, and devoid of intercellular spaces spongy parenchyma. Compared to the *in situ N. nuda* leaves, these characteristics were significantly different but not specific – the same histological organization had the leaves of *N. nuda in vitro* plantlets. In our opinion, that structure was probably a consequence of the specific *in vitro* conditions. Moreover, similar morpho- and histological variations, such as a thin palisade layer and dense spongy parenchyma, have been observed in some other species propagated in vitro, which was an adverse effect of the high sugar content and addition of plant growth regulators in the medium [Sáez et al. 2012, Suárez et al. 2019].

Transmission electron microscopy of the chloroplasts expanded the analysis of mesophyll organization and provided information about the regenerative capacity of proplastids in the leaf primordia and the extent to which they could differentiate into normal chloroplasts. In *N. nuda* postcryo plantlets and *in vitro* plantlets, the chloroplasts were slightly flattened and smaller compared with the *in situ* ones. Although the shape remained typical, significant damage to the chloroplast envelopes and substantial thylakoid ruptures were observed. The specific *in vitro* cultivation conditions had an apparent adverse effect on the structural organization of the membranes. According to Sáez et al. [2012], impaired chloroplast ultrastructure was probably a consequence of cultivation under insufficient irradiance *in vitro* or exogenous sucrose in the medium.

In *N. nuda* cryo-plantlets, the damage to the chloroplasts was more prominent. That was not a unique phenomenon. Similar structural disorders, such as altered chloroplast shape, destroyed envelope, a relatively small volume of the thylakoid system, and even destroyed granal and stromal thylakoids or specific spatial orientation without any destruction of the thylakoid membranes, were observed in the leaf mesophyll of *Hypericum plantlets* regenerated *in vitro* after cryopreservation [Skyba et al. 2012, Stoyanova-Koleva et al. 2013, Koleva et al. 2015, Stoyanova-Koleva et al. 2015]. We presumed that cryo treatment of the apical meristem negatively affected the proplastids and that they could not differentiate into chloroplasts with proper structure.

Halmagyi et al. [2017, 2022] considered that the histological and ultrastructural analysis of shoot tip cells of recovered plants after *ex vitro* acclimatization will bring added value to the existing studies. Proceeded by the authors, TEM observations on tomato shoot apex cells from acclimatized plants showed a regular ultrastructure, which suggested regular histo- and organogenesis. Our study of *N. nuda* went further by examination of *ex vitro* differentiated leaves. Despite the atypical structure of the mesophyll *in vitro* and the fact that the chloroplasts in both variants were entirely damaged, *N. nuda* plants showed high plasticity and regenerative ability and adapted *ex vitro*. The histological analysis of the newly formed leaves of the *ex vitro* plants revealed that their anatomical structure corresponded to that found in plants *in situ*. The only significant difference between the in situ and the *ex vitro* plants was the smaller lamina thickness, palisade, and spongy parenchyma in the latter variants.

According to Khoshravesh et al. [2022], the average structural characteristics of the mesophyll suggested the probability of photosynthetic efficiency and better water regulation capacity of the plants. The chloroplast ultrastructure of *N. nuda ex vitro* and postcryo *ex vitro* plants was generally similar to that of the *in situ* plants. As a confirmation of preserved photosynthetic capacity, starch grains in their stroma were observed.

CONCLUSIONS

We could assume that the impaired mesophyll structure and chloroplast integrity *in vitro* were not a result of cryo-damage of the meristematic cells but most likely were caused by the cultivation conditions themselves. The structural changes of the leaves in vitro were overcome in the *ex vitro* adaptation process. The observed leaf organization and chloroplast ultrastructure of all *ex vitro*-adapted plants could be considered a structural marker for regular histogenesis

of the newly formed leaves and a sign of remarkable phenotypic plasticity of the species. Cryopreservation of N. nuda meristems with high recovery capacity could benefit large-scale micropropagation projects for this plant of great medicinal interest.

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