

## INFLUENCE OF INDOLE-3-BUTYRIC ACID ON *NEPETA NUDA* SPP. *NUDA* PLANTS REGENERATED AFTER CRYOPRESERVATION

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### ABSTRACT

*Nepeta nuda* spp. *nuda* (family *Lamiaceae*) is a medicinal plant which possesses diuretic, antispasmodic, antiasthmatic, antioxidant and sedative properties. In this study, *in vitro* propagated *Nepeta nuda* plants were successfully cryopreserved by vitrification and the recovery rate was 20%. The effect of a range of concentrations (0.1 – 1.0 mg.L<sup>-1</sup>) of the auxin indole-3-butyric acid (IBA) was tested in *in vitro* propagated *N. nuda* plants without or after cryopreservation. The number and length of shoots, callogenesis capability and root formation were determined, as well as the content of malondialdehyde (MDA) and intracellular reactive oxygen species (ROS). Significant stimulation of the length of shoots was observed in cryopreserved *N. nuda* plants compared to the non-frozen plants, and in both cases a maximal length of shoots was achieved in medium supplemented with 1.0 mg.L<sup>-1</sup> IBA. According to the preliminary analyses, the IBA treatment did not affect considerably the content of MDA and ROS in the cryopreserved *N. nuda* plants, while an increase was observed in the control non-frozen plants when higher IBA concentrations were applied. So far, this is the first report which indicates the influence of IBA on growth and environmental responses of cryopreserved *N. nuda* plants.

*Key words: cryopreservation, in vitro propagation, Nepeta nuda, MDA, ROS*

### INTRODUCTION

The genus *Nepeta* (family *Lamiaceae*) are annual and perennial herbs, most of which grow wild in Central and Southern Europe, Northern Africa, Central and Southern Asia and comprise more than 250 species. *Nepeta nuda* spp. *nuda* is a medicinal plant which possesses diuretic, antispasmodic, antiasthmatic, antioxidant and sedative properties. The leaves of *Nepeta nuda* contain variety of biologically active compounds: monoterpenes, nepetolactones, iridoids and flavonoids (Gkinis et al., 2010).

Cryopreservation is a prospective method for the long-term storage of medicinal plants because it offers preservation of the original qualities of the cryopreserved plants (Urbanova et al., 2006). Among the other cryopreservation approaches, vitrification is the one of the most applicable methodology. Its unique characteristics such as freeze avoidance, technical simplicity and low cost, ranks it on the top of the most recently used protocols, resulting in the rapidly increasing number of cryopreserved plant species over the last decade. The exposure of plants to the extreme temperature is likely to induce oxidative stress, where the ability of plants to withstand cellular damage is a vital key determinant in their survival (Skyba et al., 2010).

The aim of this study was to compare the influence of a range of concentrations (0.1-1.0 mg.L<sup>-1</sup>) of the auxin indole-3-butyric acid (IBA) on growth and environmental responses in *in vitro* propagated *N. nuda* plants without and after cryopreservation.

### MATERIAL AND METHODS

#### Plant material

Above-ground material was collected from mature plants of *Nepeta nuda* L. ssp. *nuda* harvested in the Lozen Mountain, near Sofia, Bulgaria. The voucher specimen 105807 was deposited in the Herbarium of the Department of Botany, Faculty of Biology, Sofia University. *In*

*in vitro* cultures were induced from sterilized mono-nodal stem segments of the mature growing wild plant. After sterilization, the plants were propagated under controlled environmental conditions *in vitro* (Nedelkova et al., 2011). Non-frozen and cryopreserved plants were inoculated on basal MS medium (Murashige and Skoog, 1962) supplemented with different concentrations (0.1-1.0 mg.L<sup>-1</sup>) of IBA. Plant material for analyses was collected after four weeks of cultivation. In average, 10 to 20 plants were analyzed per treatment.

#### **Cryopreservation of *N. nuda* shoot-tips**

Before preculture treatment shoot-tips of *in vitro* propagated plants were isolated and grown on the basal MS medium. After 1 month cultivation shoot-tips were subjected to cryopreservation procedure. The preculture treatment of *N. nuda* was based on 0.076 μM abscisic acid (ABA) exposure of shoot-tips in RMB<sub>0.5</sub> liquid culture medium 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 minutes on ice and finally directly immersed into liquid nitrogen (-196°C, LN). After one week of storage, thawing was performed in water bath at 40°C for 1 min. Tips were rinsed in liquid RMB<sub>0.5</sub> containing 1.2 M sucrose. Afterwards, shoot-tips were cultivated on semi-solid RMB<sub>0.5</sub> for regeneration. A 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 control unfrozen 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 frozen. The survived cryopreserved plants were propagated on basal MS medium for several months before inoculated with different concentrations of IBA.

#### **MDA determination and ROS imaging**

Plant material (0,3 g, fresh weight) was homogenized in 3 ml 0.1% (w/v) TCA (trichloroacetic acid) on ice, centrifuged at 14,000 g for 20 min. MDA was determined from the supernatant according to Dhindsa et al. (1981) following TCA/TBA (thiobarbituric acid) addition and the heat/cool cycle. The absorbance was measured at 532 nm and 600 nm and MDA concentration calculated from its molar extinction coefficient 155 mM<sup>-1</sup>cm<sup>-1</sup>.

Intracellular ROS were detected using 2',7'-dichlorofluorescein diacetate (DCF-DA), following the protocol of Sakamoto et al. (2005). DCF-DA is non-fluorescent in reduced form and readily permeates the membrane. Once in the cell, non-specific esterases cleave its acetate groups and the dye becomes membrane impermeable becoming trapped inside the cell and the cellular compartments. DCF-DA is converted to the fluorescent form when oxidized by hydrogen peroxide, hydroxyl radicals and various free radical products that are downstream from hydrogen peroxide. To determine intracellular ROS, after exposure to experimental treatments, leaf segments were washed with distilled water and incubated for 60 min in the presence of 10 μM DCF-DA. The fluorescence of DCF-DA stained samples was determined in the abaxial side of *N. nuda* leaves by mrc Scientific Instruments microscope, model I 3201LFD, and magnification 40x.

### **RESULTS AND DISCUSSION**

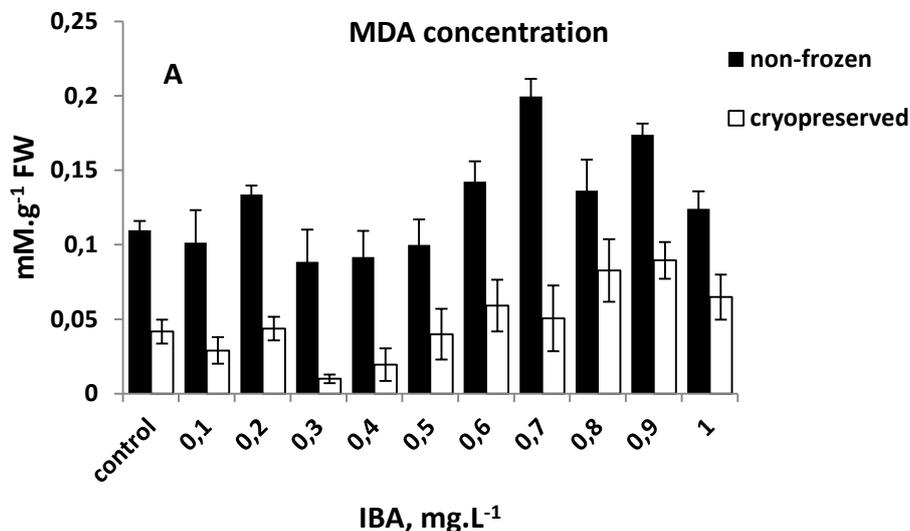
Cryopreservation in LN is a three-step process comprised of pre-culture, cryoprotection and cooling/freezing with recovery of the plant. Each of the steps can have impact on the survival rate and on the genetic stability (Yordanova et al. 2011). *N. nuda* plants were successfully cryopreserved by vitrification and the recovery rate of *N. nuda* plants was 20%. Interestingly, supplementation of IBA (0.1-1.0 mg.L<sup>-1</sup>) in the culture medium increased the length of shoots in cryopreserved *N. nuda* plants compared to non-frozen control. In the cryopreserved plants all concentrations of IBA (0.1-1.0 mg.L<sup>-1</sup>) enhanced shoot length, especially at high concentration of IBA (0.8-1.0 mg.L<sup>-1</sup>) where the maximal length of shoots (11.21 cm±1.63) was achieved in medium supplemented with 1.0

mg.L<sup>-1</sup> IBA (Table 1). All concentrations of IBA resulted in increased root formation and suppressed callus proliferation. Similarly, in the non-frozen control *N. nuda* plants the maximal length of shoots (5.81 cm±0.95) was obtained in medium supplemented with 1.0 mg.L<sup>-1</sup> IBA as previously described by Nedelkova et al. (2011). The results demonstrated that after cryopreservation, the *N. nuda* plant growth is stimulated.

Variants	Length of shoots (cm)	Number of shoots	Root formation	callus formation
control	7.25±1.44	2.00±0.0	+	-
0.1 IBA	7.35±1.72	2.00±0.0	+	-
0.2 IBA	7.80±1.08	2.00±0.0	+	-
0.3 IBA	8.55±1.70	2.00±0.0	+	-
0.4 IBA	8.50±1.58	2.00±0.0	+	-
0.5 IBA	8.55±1.70	2.00±0.0	+	-
0.6 IBA	8.80±1.89	2.00±0.0	+	-
0.7 IBA	9.00±1.14	2.00±0.0	+	-
0.8 IBA	10.31±1.28	2.00±0.0	+	-
0.9 IBA	10.07±1.62	2.00±0.0	+	-
1.0 IBA	11.21±1.63	2.00±0.0	+	-

Table 1. Influence of different concentrations of IBA on length and number of shoots, root formation, and callogenesis of cryopreserved *N. nuda* plants.

Among the possible ‘stressor’ candidates, oxidative stress caused by the formation of ROS represents a major response to temperature stress in plants (Skyba et al., 2010). Significant increase in the content of MDA (Fig. 1, A) and ROS determined by staining with DCF-DA in control non-frozen *N. nuda* plants was observed on MS medium supplemented with higher, 0.6 up to 1.0 mg.L<sup>-1</sup>, IBA concentrations (Fig. 1, B-D). On the contrary, in cryopreserved *N. nuda* plants was observed only slight increase in the content of MDA (Fig. 1, A) and ROS (Fig. 1, B-G) on MS medium supplemented with 0.8 and 0.9 mg.L<sup>-1</sup> IBA compared to non-treated plants and the control non-frozen plants inoculated with the same concentrations of IBA. In cryopreserved *Hypericum rumeliacum* plants supplementation of cytokinin 0.1 mg/l 6-benzylaminopurine (BA) in the culture medium has been shown to result in increased accumulation of ROS and MDA, as well as decreased level of phenolic content and antioxidant activity in comparison to plants propagated on cytokinin-free medium (Yordanova et al., 2011). Further experiments are foreseen to measure the total content of phenols and flavonoids, and of total antioxidant activity in the tested *N. nuda* samples.



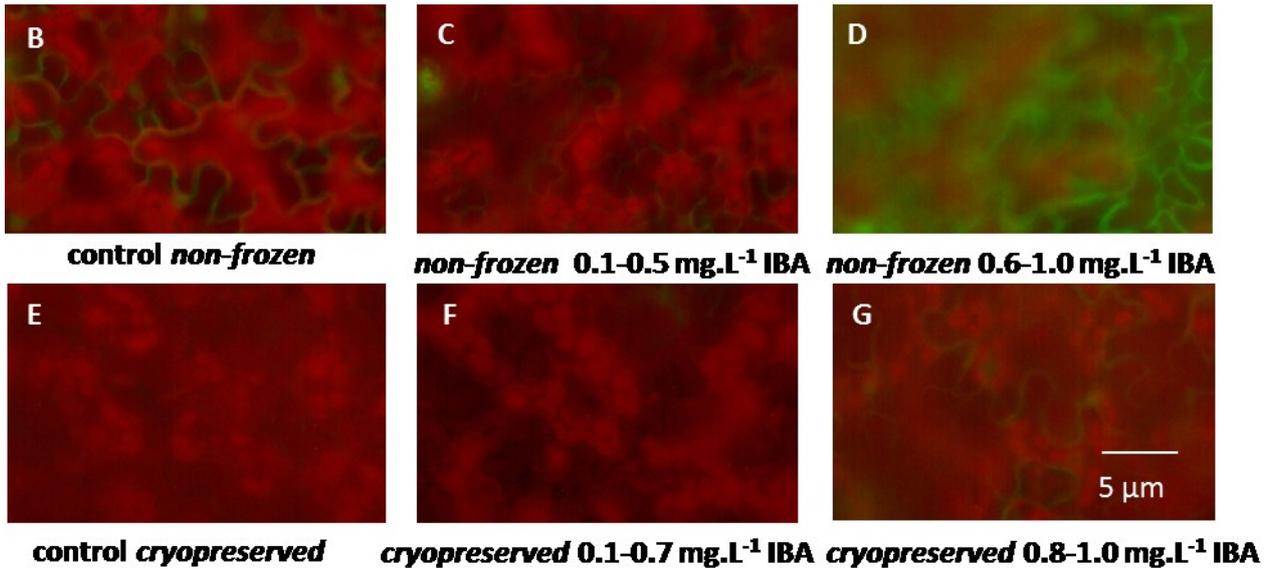


Fig.1. Concentration of MDA (A) and ROS accumulation in abaxial part of leaves of non-frozen and regenerated after cryopreservation *N. nuda* plants. (B-G) Representative images of epifluorescence of ROS after staining with DCF-DA. The green fluorescence indicates ROS; chlorophyll fluorescence appears in red. Scale bar = 5 μm.

## CONCLUSION

Despite the preliminary results achieved in the presented work, further experiments are needed for optimization of the cryopreservation procedure and assessment of biologically active compounds, antioxidant activity, as well as genetic stability. Nevertheless the cryopreservation approach seems to be prospective method for maintenance of biodiversity of medicinal plants.

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