The effect of drought stress on Norway spruce somatic embryo development

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INTRODUCTION

Somatic embryogenesis is a developmental process where a plant somatic cell dedifferentiate to a totipotent embryonic stem cell that has the ability to give rise to an embryo under appropriate conditions. Desiccation is the final phase of normal embryonic development in most angiosperms and appears to be important in the transition from embryogeny to the ability to germinate and form normal seedlings.

The objective of the presented study was to follow morphological and selected biochemical characteristics induced by various air humidity during desiccation of Norway spruce (*Picea abies* Karst.) somatic embryos.

Abiotic stresses, such as drought and osmotic stress can increase reactive oxidative species levels, which have a negative impact on plant survival. We described abscisic acid and malondialdehyde content to follow the level of drought stress in embryos.

Since chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39) are hydrolytic enzymes expressed as a defense mechanism against different biotic and abiotic stresses (He et al. 2018), we focused on monitoring of their activities during somatic embryo desiccation. Concurrently we observed the expression of two β – glucanase and two chitinase genes.

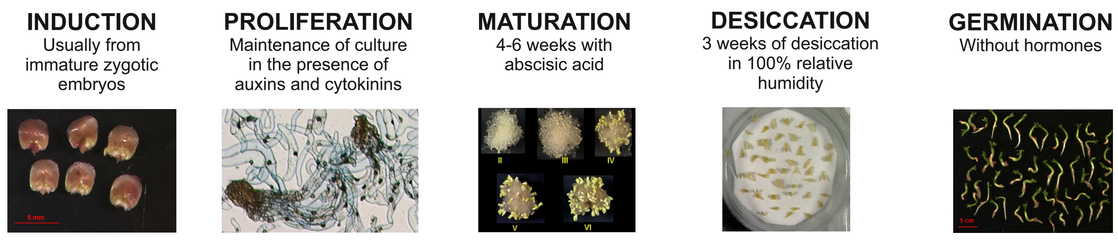
Autophagy is a catabolic pathway degrading cellular components to recycle macromolecules and ensure cellular homeostasis (Klionsky et al. 2016). Autophagy plays role both in development and reaction of plants to various stresses. Autophagy-related genes (*ATG*s) control autophagosome formation, which requires four main protein complexes (Avin-Wittenberg et al. 2018). Among them are two ubiquitin-like systems conjugating Atg12 with Atg5, and Atg8 with lipid phosphatidylethanolamine, respectively (Minina et al. 2018). We followed the expression of selected *ATG* genes in the course of somatic embryo desiccation to confirm the role of autophagy in this process.

AIM

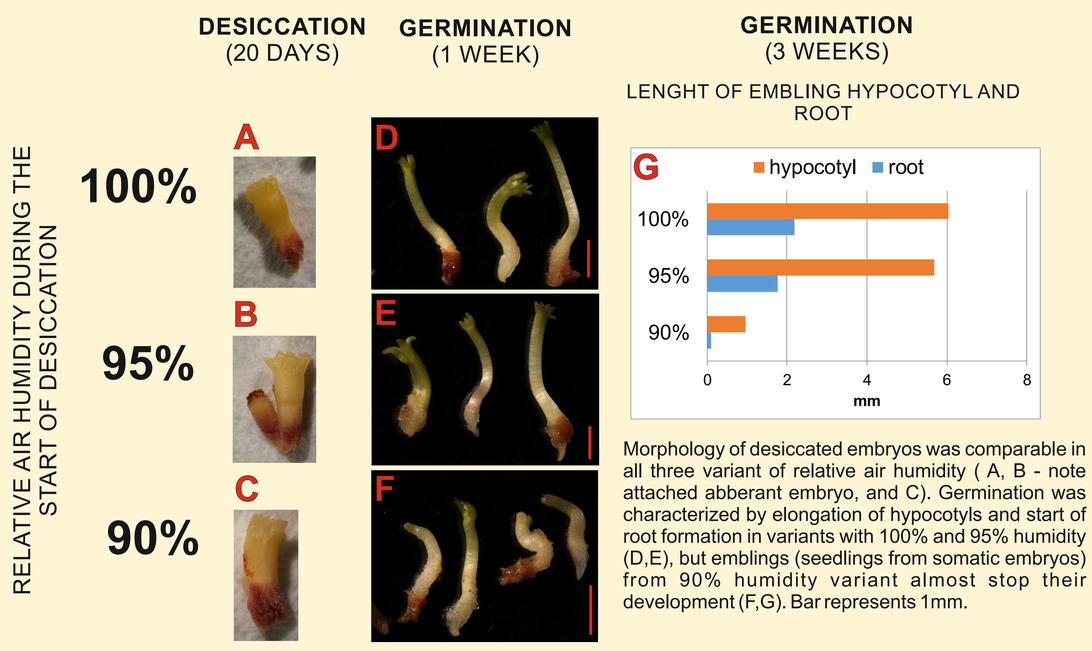
To define the effect of different air humidity in the dessication phase of Norway spruce (*Picea abies* Karst.) somatic embryo development, namely at these levels:

- **basic morphology of somatic embryos and their ability to germinate**
- **activities of β-1,3-glucanases and chitinases and expression of selected β-glucanase and chitinase genes**
- **expression profiles of selected autophagy-related genes (*ATG*s)**

PHASES OF CONIFEROUS SOMATIC EMBRYOGENESIS

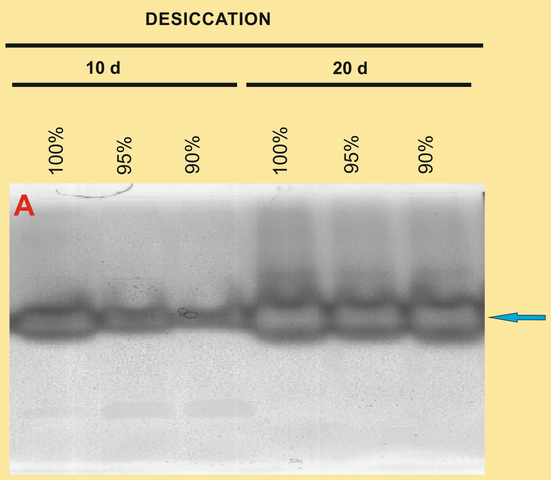


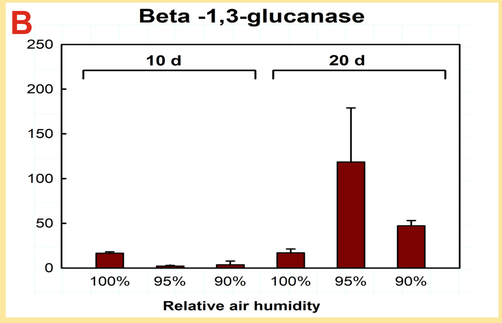
MORPHOLOGY OF EMBRYOS AFTER DESICCATION IN DIFFERENT RELATIVE AIR HUMIDITY



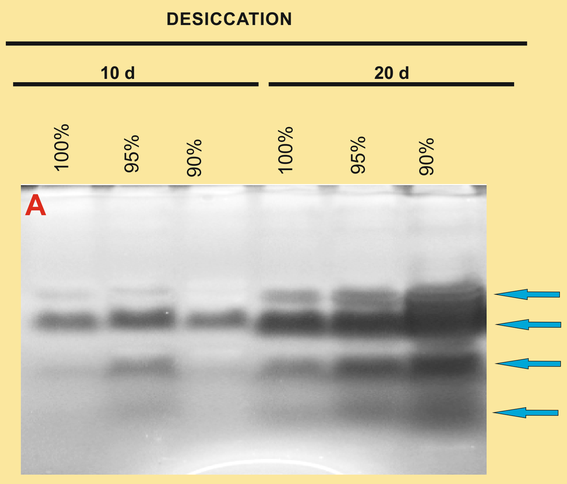
Morphology of desiccated embryos was comparable in all three variant of relative air humidity (A, B - note attached abberant embryo, and C). Germination was characterized by elongation of hypocotyls and start of root formation in variants with 100% and 95% humidity (D,E), but emblings (seedlings from somatic embryos) from 90% humidity variant almost stop their development (F,G). Bar represents 1mm.

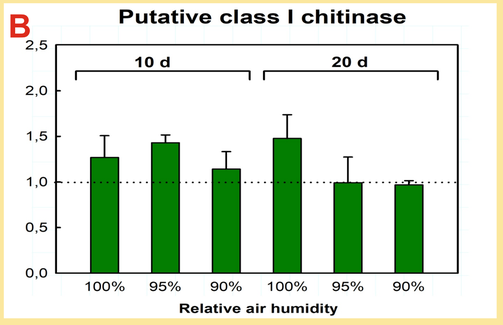
β-1,3-GLUCANASES





CHITINASES





Activities of total β- 1,3 glucanases and total chitinases and relative expresssion levels of their selected genes were analyzed in somatic embryos desiccated 10 days in control (100%) and reduced humidity (95% and 90%) and after the rest of desiccation (another 10 days = 20 d) which took place in 100% air humidity.

A After SDS-PAGE separation one majority isoform was detected (30 kDa), which activity increased in desiccation. Low humidity in desiccation reduced its activity (10d) and was restored after embryo rehydration at the end of desiccation (20d).

B Relative expression of beta - 1,3 – glucanase homologue gene was up regulated during desiccation in all variants of air humidity, mostly in 100% variant; relative expression after rehydration of embryos (20 days) was massively up regulated in stressed variants. All data were compared relative to expression levels at the end of maturation (value1).

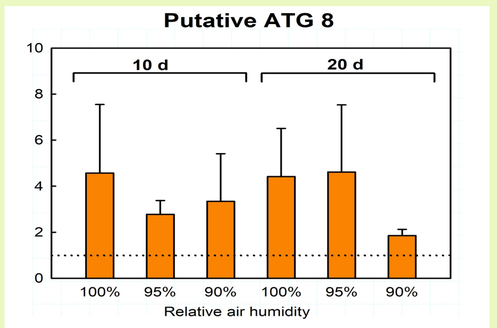
C Relative expression of endo - 1,4 - beta - glucanase homologue gene was slightly up regulated during desiccation in 100% air humidity, its relative expression levels remained unchanged in all stressed variants as compared to expression levels at the end of maturation (value1).

A After SDS – PAGE separation one major isoform of chitinases was detected. after 10 d of desiccation was not significantly affected by different humidity. After embryo rehydratation the activity of major isoform increased and three new isoforms of chitinases appeared. These isoforms were highly detectable in embryos desiccated in 100 % and 95% air humidity. After desiccation in the lowest humidity their activities were reduced.

B The level of relative expression of putative class I chitinase gene remained unchanged during desiccation in all air humidity conditions as compared to expression levels at the end of maturation (value1).

C Relative expression of class IV chitinase Chia4-Pa was highly (about 20 times) increased during desiccation in 100% air humidity, this effect was further pronounced after rehydration of stressed embryos.

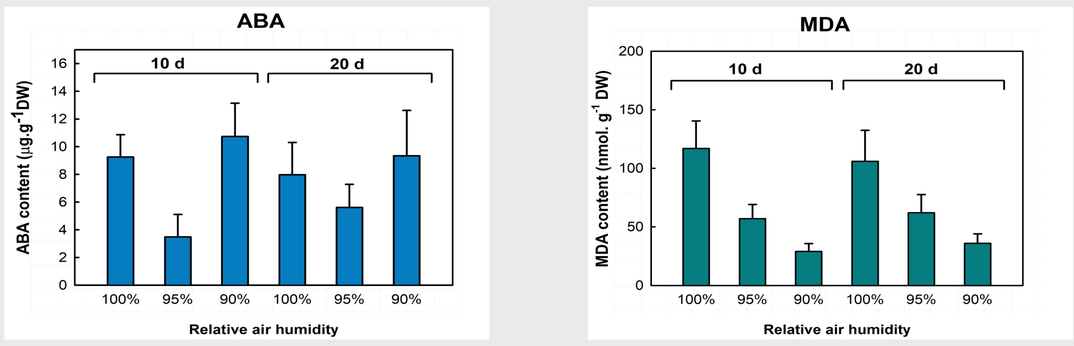
AUTOPHAGY-RELATED GENES



Relative expression of putative *ATG* 8 and *ATG* 12 gene was analyzed in somatic embryos dessicated 10 days in control (100%) and reduced humidity (95% and 90%) and after the rest of desiccation (another 10 days = 20 d) which took place in 100% air humidity.

The expression of putative *ATG* 8 gene was slightly up regulated (2-5 times) in embryos of all desiccation variants as compared to expression levels at the end of maturation (value1).

The level of putative *ATG*12 gene expression was increased by reduced air humidity (90%) in desiccation, after rehydration was oppositely down regulated. Relative expression of putative *ATG*12 gene in embryos desiccated in 95% air humidity was converse – during stress effect remained unchanged, after rehydration highly increased as compared to expression levels at the end of maturation (value1).



Endogenous level of abscisic acid (ABA) and malondialdehyde (MDA) content in somatic embryos dessicated 10 days in control (100%) and reduced humidity (95% and 90%) and after the rest of desiccation (another 10 days= 20 d) which took place in 100% air humidity.

ABA content signifficantly decreased in embryos dessicated in 95% air humidity after 10 d of dessication and remained lowered after rehydratation. No stress reaction was found in embryos dessicated in 90% air humidity.

Meassured rates of lipid peroxidation (which are determined by monitoring changes in the levels of MDA) can be related to the antioxidant activity balance within a given cell or tissue. Observed decline in embryos dessicated in 95% and 90% air humidity may coincide with decreased of total metabolic activity under these conditions.

CONCLUSIONS

- **90% relative air humidity in desiccation negatively affected germination of embryos**
- **reduced relative air humidity in desiccation decreased the activity of β-1,3 glucanases and chitinases, abundance of distinct chitinase isoforms varied in embryos desiccated in different air humidity**
- **relative expression of selected β-glucanase genes was up regulated during desiccation**
- **relative expression of putative class I chitinase gene remained unchanged, whereas relative expression of class IV chitinase Chia4-Pa was increased**
- **relative expression of *ATG* 8 and *ATG* 12 was upregulated, indicating the role of autophagy in the process of desiccation**

DESICCATION OF SOMATIC EMBRYOS IS METABOLICALLY ACTIVE PROCESS AFFECTED BY RELATIVE AIR HUMIDITY

Cultivation:

Embryogenic cultures of Norway spruce (*Picea abies* L. [Karst.]) were cultivated on solidified (proliferation) resp. liquid (maturation) GD medium (Gupta and Durzan 1986) as described elsewhere (Vágner et al. 1998). The proliferation medium contained 5 mM 2,4-D, 2 mM BA and 2 mM kinetin, maturation medium was supplemented by 20 μM ABA and 3.75% (w/v) PEG 4000. The fully developed embryos were desiccated in three different levels of air humidity (90%, 95%, 100%) for 10 days, the rest of desiccation (another 10 days) took place in 100% air humidity. Germination medium without phytohormones was supplemented with active charcoal.

Morphology of embryos:

Light transmission microscop Jenaval (Karl Zeiss) with DS-5M Nikon camera was used to monitor the effect of drought stress in desiccation phase of embryo development. Obtained images were processed by Lucia image analysis system.

Biochemical analysis:

Total proteins were extracted from embryos (Hurkman and Tanaka 1986). Aliquots (20 μg) were separated in 12.5 % polyacrylamide gels to detect total enzyme profiles under standard conditions. For detection of chitinases, the gels contained 0.01 % (w/v) glycolchitin as the enzyme substrate. The glucanase activities of protein fractions were detected in gels with 0.01 % (w/v) laminarin.

The malondialdehyde (MDA) content of the samples was determined using an NWLSS-Malondialdehyde Assay kit (Cat. no. NWK-MDA01, Northwest Life Science Specialties, LLC, Vancouver, Canada) as described in detail by Cvikrova et al. (2013).

ABA was extracted as describe in (Tureckova et al., 2009) and quantified by a LC/MS system .

Gene expression analysis:

The relative transcript levels of the genes of interest were analyzed by real-time PCR. RNA was isolated from 0,1g of frozen embryos by RNeasy Plant Kit (Qiagen) and subjected to DNaseI (Thermo Scientific). cDNA was prepared using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative RT-PCR was carried out in 12 μl PCR mixture containing 6 μl of PCR MasterMix (Generi Biotech); 3,5 μl of nuclease free water; 0,5 μl of mixture of forward and reverse primer (initial concentration 10 μM) and 2 μl of cDNA. *Picea abies* alpha-tubulin gene (GenBank: X57980.1) was used as reference gene for normalization of expression of genes of interest. Primers for gene of interest were designed based on gene homology (tBLASTn): Beta - 1,3 – glucanase (GenBank: L49179.1), endo - 1,4 - beta- glucanase (GenBank: JF343550.1), putative class I chitinase (GenBank: AY450922.1), class IV chitinase Chia4-Pa (GenBank: AY270016.1), putative ATG8 (GenBank: EF677386), putative ATG12 (GenBank: BT108874). The relative transcript level expression was analyzed by the modified 2-ΔΔCT method using individual amplification efficiency for each gene (Schefe et al., 2006; Livak and Schmittgen, 2001) and compared relative to expression levels in the end of maturation (value1).

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