The effect of different air humidity during desiccation on the development of Norway spruce somatic embryos

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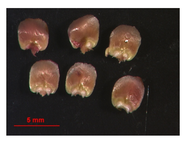
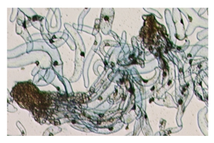
INTRODUCTION and AIMS

Somatic embryogenesis is characterized by series of phases (see below), where plant somatic cells dedifferentiate and under appropriate conditions they are able to give rise to an embryo. The phase of desiccation appears to be important in the transition from embryogeny to seedling formation.
The objective of the presented study was to follow morphological (1), selected biochemical (2) and transcriptional (3) characteristics induced by various air humidity during desiccation of Norway spruce (*Picea abies* L. Karst.) somatic embryos.
Polyamines, low molecular mass polycations, are ubiquitous molecules present in almost all cells. They play an important role in diverse plant growth and developmental processes as well as in adaptation to environmental stresses.
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 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. 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. Among them there 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.

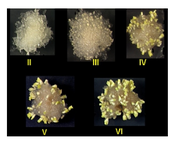
INDUCTION
From immature zygotic embryos



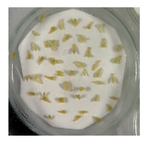
PROLIFERATION
Maintenance of culture in the presence of auxins and cytokinins



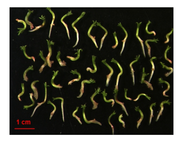
MATURATION
4-6 weeks on medium supplemented with abscisic acid



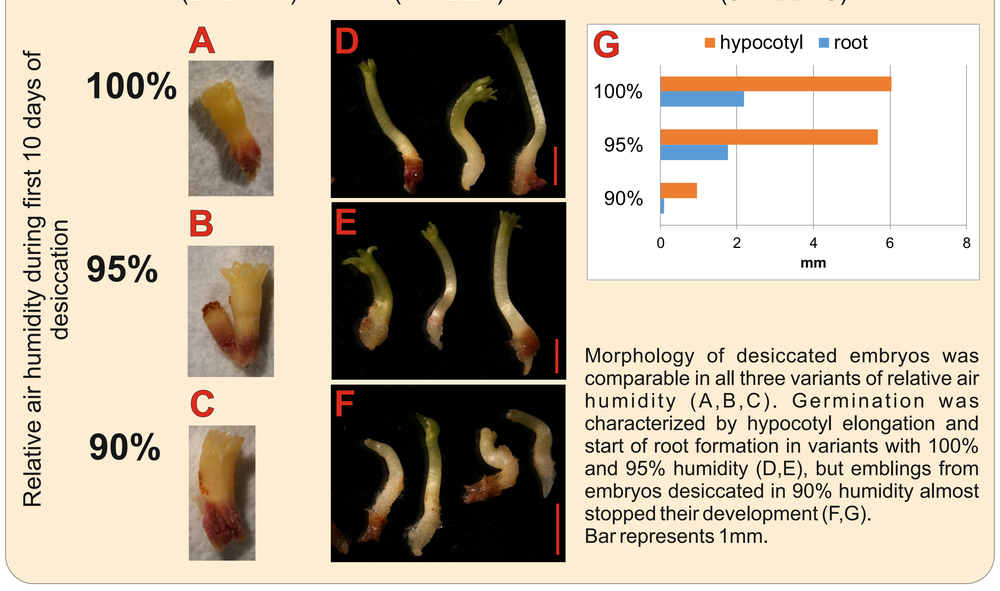
DESICCATION
3 weeks of desiccation in 100% relative humidity



GERMINATION
Without hormones

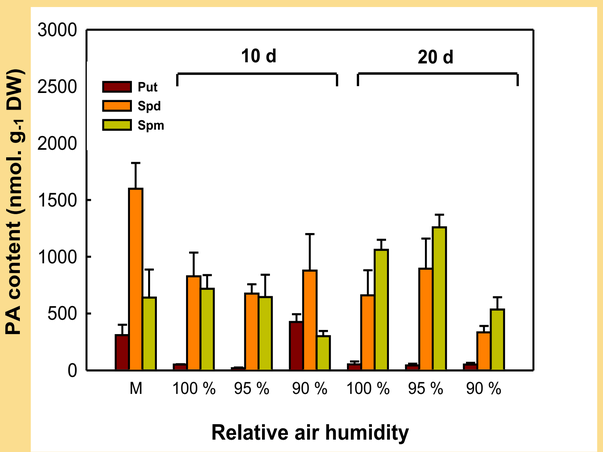


1) MORPHOLOGY AND GERMINATION



Morphology of desiccated embryos was comparable in all three variants of relative air humidity (A,B,C). Germination was characterized by hypocotyl elongation and start of root formation in variants with 100% and 95% humidity (D,E), but emblings from embryos desiccated in 90% humidity almost stopped their development (F,G).
Bar represents 1mm.

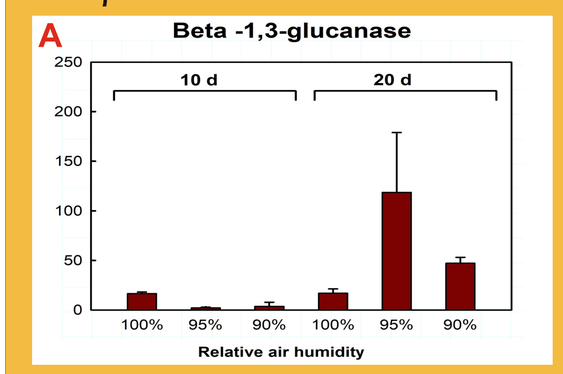
2) POLYAMINES

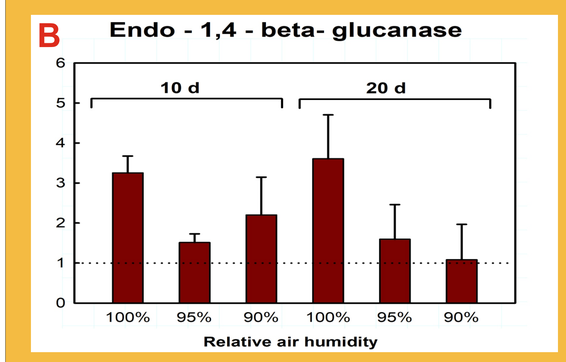


Content of free polyamines was analyzed in somatic embryos at the end of maturation (M) and during desiccation under different relative air humidities. Embryos were desiccated in 100% (control) and reduced humidity (95% and 90%) for 10 days, the rest of desiccation took place in 100% air humidity. In control variant desiccation led to decrease in proportion between putrescine and higher forms of polyamines (spermidine and spermine). 90% humidity in the start of desiccation caused the increase of putrescine level, which was subsequently lowered after rehydration in the end of desiccation. Rehydration increased the level of spermin in all variants.

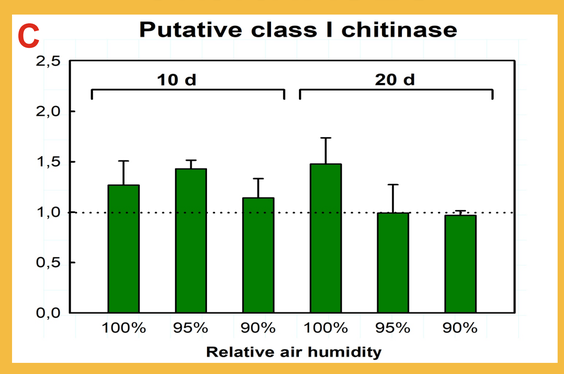
3) TRANSCRIPTION OF SELECTED GENES

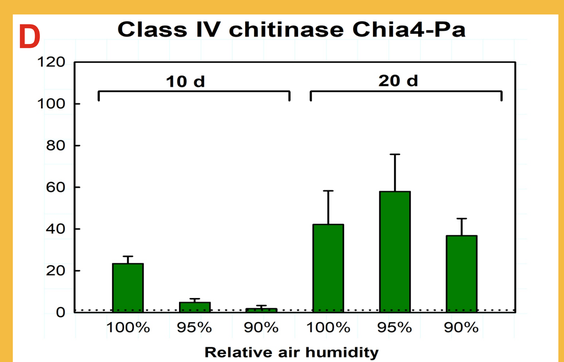
β - GLUCANASES



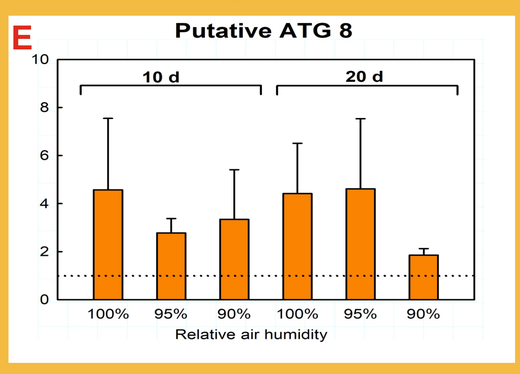


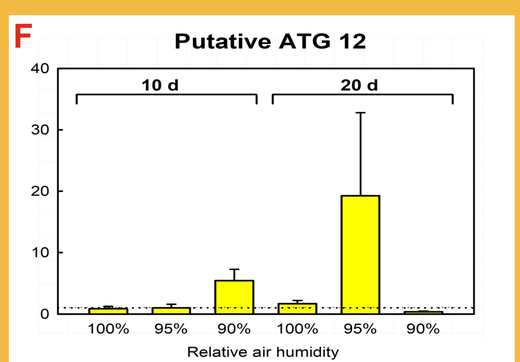
CHITINASES





AUTOPHAGY - RELATED





Relative expresssion levels of selected genes were analyzed in somatic embryos desiccated for 10 days in 100% (control) and reduced humidity (95% and 90%), and after the rest of desiccation which took place in 100% air humidity. All data were compared relative to expression levels at the end of maturation (value1).

A Relative expression of beta - 1,3 – glucanase homologue gene was up-regulated during desiccation in all variants, mostly in 100% variant; relative expression after rehydration of embryos (20 d) was massively up-regulated in stressed variants (95% and 90%).

B 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 almost unchanged in all stressed variants as compared to expression levels at the end of maturation.

C The level of relative expression of putative class I chitinase gene remained unchanged during desiccation of embryos of all variants.

D 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.

E The expression of putative *ATG* 8 gene was slightly up-regulated (2-5 times) in embryos of all variants.

F 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.

MATERIAL AND METHODS

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 on dry filter paper placed in small Petri dishes surrounded by wet paper in bigger Petri dish. Three different levels of air humidity (100%, 95%, 90%) were used for 10 days of desiccation, 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 microscope Jenaval (Carl 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 NIS-Elements image analysis system.

Polyamine content:

Polyamines were extracted and benzoylated according to the method of Slocum et al. (1989). Detection and quantification of benzoylamines were carried out using an HPLC/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 at the end of maturation (value1).

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CONCLUSIONS

CONTROL (100% relative air humidity):
- **the level of free polyamines lowered, higher forms of polyamines were favoured**
- **relative expression of selected β-glucanase genes was up-regulated**
- **relative expression of class IV chitinase Chia4-Pa increased**
- **relative expression of *ATG* 8 was up-regulated**

LOW RELATIVE AIR HUMIDITY (95% and 90%):
- **germination of embryos was negatively influenced by 90% relative air humidity**
- **putrescine level raised in 90% variant**
- **expression levels of most monitored genes were affected, some of them up-regulated (β-1,3-glucanase, *ATG* 12), some down-regulated (endo-1,4-β-glucanase)**

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

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