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The biochemical characteristics of the physiological activity of beech and spruce embryos

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Introduction

It is necessary to find convenient and measurable criteria for the evaluation of seeds/embryos quality before germination because of a long time interval between the preparation of seeds or embryos (before germination) and obtaining the seedlings or emblings. The success of all treatments before germination is assessed by the quality of the newly formed plants only. The aim of our experiments is to estimate the potential of seeds harvested from different sources and/or of the somatic embryos developed in different embryogenic cultures just before germination, to develop into high quality plants. During the search of a suitable method we selected 3 substances to measure: 1) ABA (abscisic acid) as the phytohormone which controls the dormancy in seeds and which regulates the maturation of somatic embryos; 2) IAA (indolyl-3-acetic acid) as the phytohormone which regulates the growth and the development of embryos and whole seedlings and 3) fumarase as the enzyme which is often correlated with the dormancy and the germination of seeds. Fumarase is the key enzyme in mitochondrial metabolism. Its activity indicates the respiration rate and the ability of seeds to use stored reserves.

Methods

ABA and IAA

The weighted plant material (around 0.1 g FW) was milled on a DNA mill and extracted in a modified Bieleski solution. The extract was centrifuged and dried on a rotary vacuum concentrator at room temperature.
Dried samples were dissolved in a 15 vol % solution of acetonitrile in water, injected into HPLC and precleaned on C-18 with gradient elution and fractionation on a fraction collector. The fraction at time 23.05 min was collected for 1 min and dried. After drying the collected fraction was derivatized by diazomethane solution in ether, dried, and dissolved in 100 µl of acetone. Of the redissolved sample 8 µl was injected into GC-MS/MS and analyzed by Ion trap in MS/MS scan mode (MS1: full scan 50-300 amu; MS2 IAA: precursor 130.1 amu, product full scan 65-200 amu; MS2 labelled IAA: precursor 136.1 amu, product full scan 70-200 amu; MS2 ABA: precursor 190.2 amu, product full scan 65-200 amu; MS2 labelled ABA: precursor 194.2 amu, product full scan 70-200 amu) (Kosova et al 2012).

Fumarase

The seeds stored at -80ºC were used. The weighed 5 pieces of embryonal axis (or an adequate amount of somatic embryos) was milled on a DNA mill and extracted by extraction buffer. The solution was centrifuged and the pure extract was filtered by a 0.2 µm microfilter. A quantity of 150 µl of filtered extract was added to a reaction mixture (HEPES buffer, malate dehydrogenase, NADP+, $MgCl_2$, KH_2PO_4). After 3, 6, 9 and 12 minutes of reaction the increase of absorbance at 340 nm (NADPH formation) is measured in a 50 mm cuvette. It is proportional to the amount of fumarase in the sample (method adapted according Hatch 1978 and Shen and Oden 2000).

The optimization of the enzymatic reaction was realized in two steps: 1) The timing of the reaction using pig fumarase from Sigma and the optimum for measurement were assessed to be 340nm during 1 – 18

minutes of reaction; 2) The effect of fumarase concentration on absorbance. The maximum of the sensitivity measurement is between 0 and 0.25 IU of fumarase.

Material

Beech seeds and spruce somatic embryos were used for verification of these three methods. We compared the quality of dormant and non-dormant beech seeds and the success of the maturation process of spruce somatic embryos, i.e. we tried to define the different quality of somatic embryos at the end of maturation.

Results

The endogenous level of ABA

The ABA content in dormant and non-dormant beech embryos was measured in the embryonal axis, cotyledons and in the whole embryo from the freshly harvested dormant seeds and from the stored seeds just before germination. We found relatively high endogenous ABA in beech embryos; the highest level of ABA was detected in the embryonal axis. The difference between the ABA content in dormant and non-dormant embryos is more than 50%. The endogenous ABA decreases during the whole process of seeds stratification (13 weeks), but the ABA level decreases more rapidly at the beginning of stratification (Fig. 1).

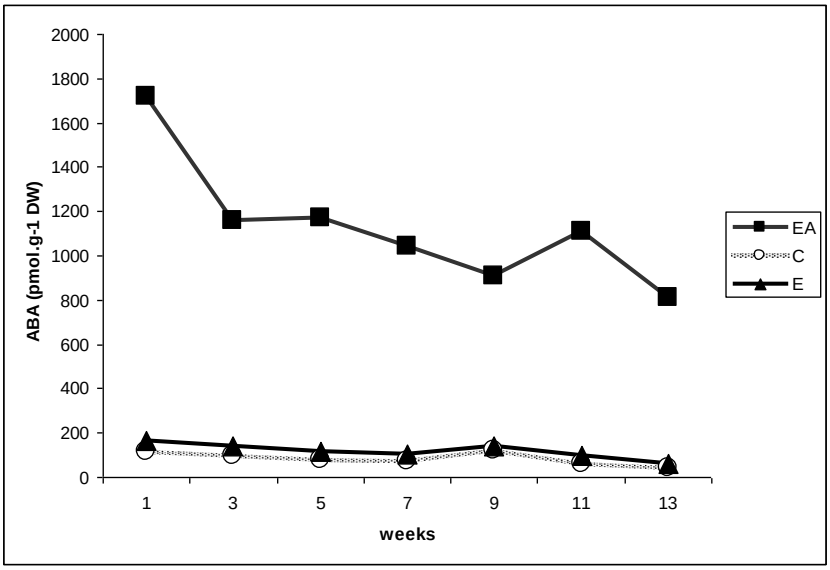

Fig. 1*: The changes in endogenous ABA (in pmols of ABA in 1 g of dry weight of beech embryos) during 13 weeks of stratification (EA = embryonal axis; C = cotyledons; E = whole embryos)*

The endogenous ABA in spruce somatic embryos increases during 4 weeks of embryos maturation (Vágner et al 1999). (Maturation is carried out on the medium supplemented by ABA.) During the last two weeks of maturation and during the whole desiccation the ABA level decreases. The somatic embryos are able to germinate when the ABA content is low.

The endogenous level of IAA

The IAA content in dormant and non-dormant beech embryos was measured in the same samples as ABA. We found extremely low IAA levels in beech embryos; the highest level was in the embryonal axis. The endogenous IAA fluctuates during the whole process of stratification. No differences between the level of IAA at the start and after 13 weeks of stratification were found. No difference between the IAA content in dormant and non-dormant embryos was detected (Fig. 2).

The endogenous IAA in spruce somatic embryos increases during the 2nd and in the 3rd week of maturation when the root and shoot pole of embryos are formed. (Vágner et al 1999) The 2nd maximum of IAA

content is found at the end of desiccation just before germination. We can speculate that the transient increase of IAA together with low ABA content is necessary for successful germination of spruce somatic embryos.

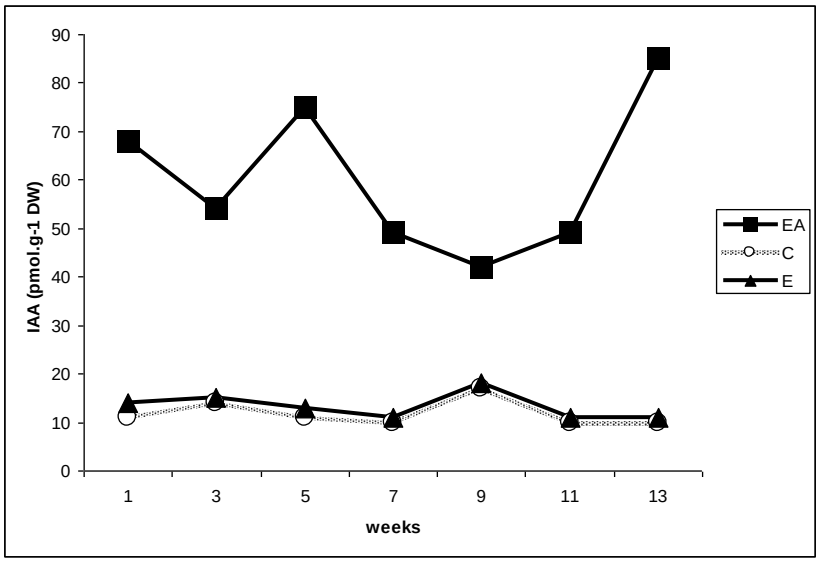

Fig. 2: *The changes in endogenous IAA (in pmols of ABA in 1 g of dry weight of beech embryos) during 13 weeks of stratification (EA = embryonal axis; C = cotyledons; E = the whole embryos)*

The fumarase activity

In the first step the method was verified using embryonal axis from stored seeds and from dead seeds of beech. The enzyme fumarase is active in living cells only, i.e. the activity measured in dead embryonal axis represents the base level for measurement. It is possible to differentiate the living and dead embryos using this method.

The fumarase activity in embryonal axis from dormant and non-dormant beech seeds_differs during the whole measurement (3, 6, 9, 12 min. of reaction). The lower fumarase activity is observed in embryonal axis from dormant seeds (Fig. 3).

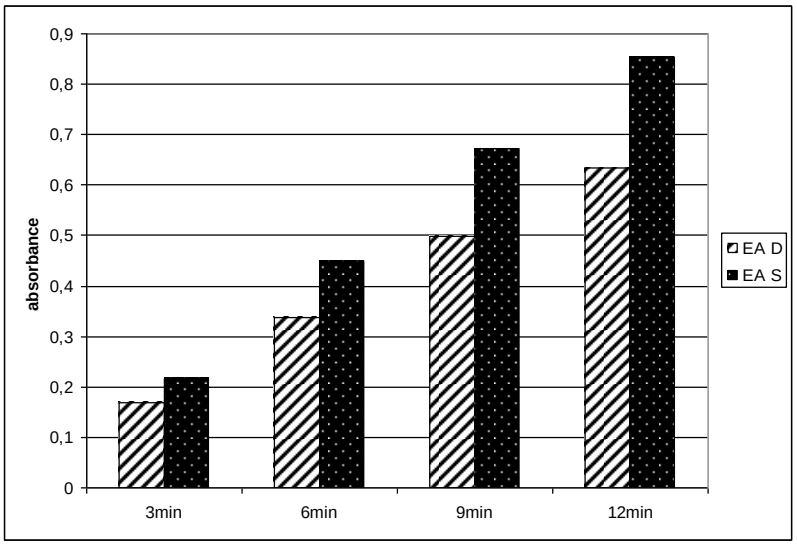

Fig. 3*: The fumarase activity in embryonal axis from dormant beech seeds and from seeds after 15 weeks of stratification (EA D = embryonal axis from dormant seeds; EA S = embryonal axis from non-dormant seeds, after 15 weeks of stratification)*

The fumarase activity in matured somatic embryos of spruce was measured in the embryos of different quality and in the rest of the embryonic suspensor mass (consisting of dead cells and embryos). We did not find any activity of fumarase in ESM. The differences in fumarase activity among other somatic embryos are rather small. The higher fumarase activities were found in growing somatic embryos; ie. in developing globular embryos and in malformed embryos, where callogenesis starts. The lower fumarase activity was found in somatic embryos at the end of successful maturation (Fig. 4).

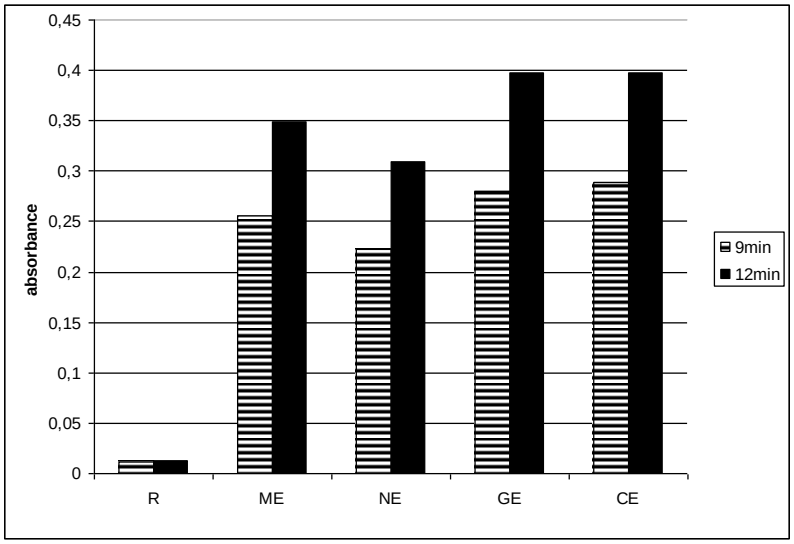

Fig. 4: *The fumarase activity in different maturated somatic embryos after 9 and 12 min. of reaction (R = rest of embryonic suspensor mass; ME = maturated embryos; NE = non-maturated embryos; GE = globular embryos; CE = malformed embryos with callus formation)*

Conclusions

1) The endogenous level of ABA can indicate the depth of dormancy and the effect of stratification on beech embryos. ABA can also characterize the ability of somatic embryos to germinate. This accurate method of ABA determination could be successfully used for embryo evaluation.

2) The endogenous level of IAA does not correlate with the dormancy of beech embryos and/or stratification. The endogenous level of IAA increases during polarization of spruce somatic embryos. The increase of the IAA level correlates with the start of somatic embryo germination. The IAA content can characterize the specific steps in the embryo development, but it is not the marker of deep of dormancy.

3) The fumarase activity can clearly distinguish the living and dead cells or tissue. After the rigorous optimization of the method (it is very sensitive to external conditions) it could be a promising method for testing dormancy. The fumarase activity is not a suitable criterion for the evaluation of spruce somatic embryos quality.

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