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MORPHOANATOMICAL STUDY OF *IN VITRO* PROPAGATED AND *EX VITRO* ADAPTED *ACHILLEA THRACICA* VELEN. PLANTS

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Abstract

Rogova, M., Ts. Ganeva, M. Stefanova, D. Koleva and V. K.-Toteva. Morphoanatomical study of *in vitro* propagated and *ex vitro* adapted *Achillea thracica* Velen. plants. *Bulg. J. Agric. Sci.*, 21: 947–950

Achillea thracica Velen. plants from their natural habitat, *in vitro* propagated plants and *ex vitro* established plants were studied. Morphological observation of the three examined variants showed that the organogenesis of *in vitro* cultured and *ex vitro* adapted plants corresponded with *in vivo* plants. Comparative histological analysis showed that the leaves in all variants were equifacial, however in *in vitro* leaves the shape and arrangement of the mesophyll cells were different compared with wild plants. The *ex vitro* plants formed leaves with the best organization of the photosynthetic parenchyma. Considering the successful organogenesis, normal histogenesis, lack of hyperhydricity and high survival rate both *in vitro* and *ex vitro*, we can conclude that for *A. thracica* a micropropagation is a suitable method for obtaining sufficient amount of plant material for *ex situ* conservation of this endangered endemic species.

Keywords: micropropagation, *Achillea,* leaf structure

Introduction

The contemporary methods of biotechnology are widely applied in propagation and long term conservation of valuable plant species. *Achillea thracica* Velen. (Asteraceae) is an endangered Bulgarian endemic plant (Stanev, 2011) protected by the Bulgarian Biodiversity Act and the Bern Convention, and it is included in the European Red List of Vascular Plants (Bilz et al., 2011). Micropropagation of *A. thracica* will ensure its rapid multiplication and preservation of the genetic potential of the initial plants. Regenerated *in vitro* plants often develop specific phenotype that could impede their *ex vitro* acclimation. The special conditions during *in vitro* cultivation result in formation of plants with abnormal morphology and anatomy, especially of the leaves (Buddendorf-Joosten and Woltering, 1994; Kozai and Smith, 1995; Pospisilova et al., 1997). Consequently, it is important to elaborate successful procedure for micropropagation of each species as there is no universal one (Tasheva and Kosturkova, 2013).

A. thracica as being one of the threatened species is a suitable subject for *in vitro* cultivation, *ex vitro* adaptation and *ex situ* conservation, respectively. In the present study morphological and anatomical indicators of *in vivo* plants, *in vitro* plantlets propagated on MS medium with vitamins Gamborg B5, and *ex vitro* plants were analyzed. The aim was to find out the possible changes in organ- and histogenesis of the leaves as markers determining the regeneration potential of the species for successful *ex vitro* adaptation.

Materials and Methods

Intact plant material of *Achillea thracica* Velen. was collected at its natural habitat in Thracian lowland, Manole village, near Plovdiv, Bulgaria. The voucher specimen SO107385 has been deposited in the Herbarium of Sofia University "St. Kliment Ohridski". *In vitro* shoot cultures were induced from the stem as previously described (Hristova et al., 2012). After 30 days of cultivation, explants from the regenerated plants were propagated on MS medium (Murashige and Skoog, 1962) with vitamins after Gamborg B5 (Gamborg et al., 1968) without growth regulators.

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For *ex vitro* adaptation 30 plants with good developed roots were taken. *In vitro* regenerated plants were washed from the agar and potted in non-sterile substrate (sand and soil in proportion 1:1). The plants were covered with plastic lids with small perforations at the top. After 2 weeks the covers were removed and 2 months later the plants were planted outdoor on the experimental field.

Leaves' fresh and dry weight of *in vivo*, *in vitro* and *ex vitro* plants, as well as diameter of the formed callus around the stem base of *in vitro* plantlets were measured and the average values were calculated.

For light microscopy (LM) leaves from the 2nd and 3rd node of *in vitro*-cultivated plants were fixed in 3% glutaraldehyde (m/v) in 0.1 M sodium phosphate buffer (pH 7.4) for 12 hours at 4°C and subjected to microscopy studies. Hand-cut transverse sections of the middle part of the leaf lamina were mounted on slides in glycerol. Microphotographs were taken using light microscope and camera Nicon Eclipse 50i (Japan).

Results

Morphological characteristics

A. thracica is herbaceous perennial plant with non-branching stem and simple pinnate leaves. After 30 days *in vitro* cultivation plantlets with well developed adventitious roots and simple pinnate leaves, morphologically similar to the wild *in vivo* plants were obtained. The average value of the dry biomass decreased significantly compared to the *in vivo* plants: it was only 9.0 ± 0.2% in the *in vitro* shoots while the stems of the *in vivo* plants contained 30.1 ± 0.1% dry biomass. It is worth to mention that no hyperhydricity was observed, and the plantlets reached normal dry biomass (31.0 ± 0.1%) shortly after their *ex vitro* adaptation. Low callus formation (4.6 ± 1.5 mm in diameter) occurred around the stem along with the roots, which did not proliferate. The *ex vitro* adapted plants showed the same morphology of the vegetative organs as that of the *in vivo* plants. All of the regenerated *in vitro* plants were successfully adapted *ex vitro*.

Leaf structure

Leaf laminas of the wild growing plants were flat and equifacial with 2-layered palisade parenchyma beneath the adaxial and the abaxial epidermis and 2-3 layers spongy parenchyma in the middle (Figure 1). Palisade cells had small contact areas and untypically large intercellular spaces while the spongy cells were rounded and densely placed. Both adaxial and the abaxial epidermis consisted of uniform pavement cells, regularly arranged stomata, uni-cellular capitate glandular and uniseriate nonglandular trichomes.

The leaves of the *in vitro* plants were equifacial too, but unlike the *in vivo* plants the palisade parenchyma cells were very short and round (Figure 2). The spongy cells were tangentially flattened and densely arranged. However, no differences in the epidermis were observed. Without morphometric analysis it was obvious that the thickness of the lamina was approximately twice reduced compared with the *in vivo* plants.

The *ex vitro* plants developed equifacial leaves as in the *in vivo* ones (Figure 3). The palisade parenchyma consisted

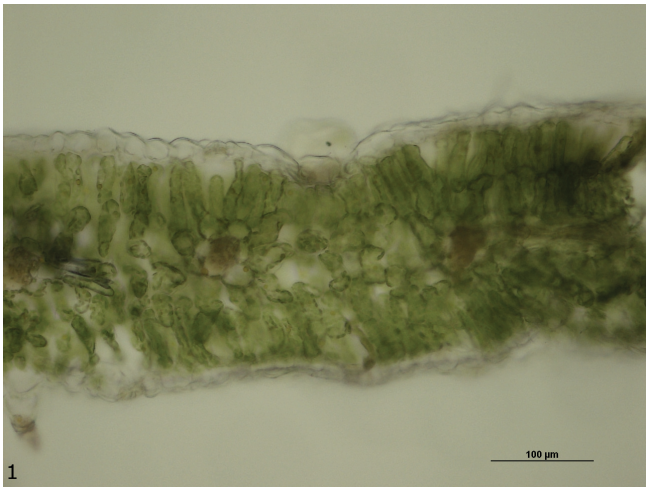

Fig. 1. *A. thracica* leaf structure of wild growing plants (bar=100µm)

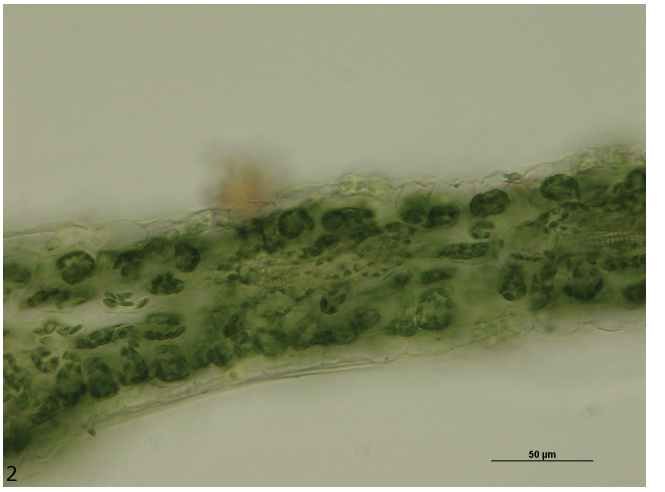

Fig. 2. *A. thracica* leaf structure of *in vitro* plants (bar=50µm)

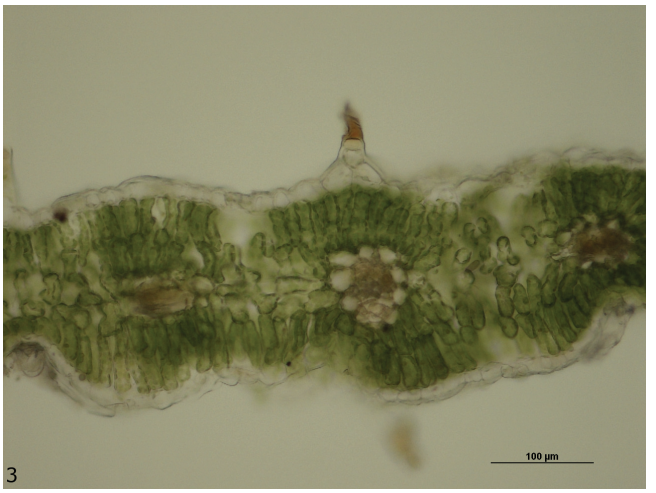

Fig. 3. *A. thracica* leaf structure of *ex vitro* plants (bar=100µm)

of 2 layers cylindrical cells with normal symplast contacts but more tightly arranged in comparison with *in vivo* plants. The morphology and arrangement of the spongy parenchyma cells resemble the *in vivo* plants' leaves. The structure of the epidermis was similar to the other experimental variants. There was no apparent difference in the lamina thickness compared to the *in vivo* plants.

Discussion

In the present study *in vitro A. thracica* plants formed well developed root system and morphologically identical to the *in vivo* plants leaves. That was a prerequisite for the established successful *ex vitro* adaptation of all *in vitro* regenerated plants. Many previous studies shows that the adaptation process from *in vitro* to *ex vitro* conditions often is critical for plants' survival (Pospíšilová et al., 1999; Apóstolo et al., 2005) or even insoluble problem (Hazarika, 2006). In spite of the low dry biomass of the *in vitro* shoots, no hyperhydricity was observed, and the plantlets recovered their dry biomass after the *ex vitro* adaptation. Such considerable temporary diminution of the dry biomass is normal for some species under the conditions of *in vitro* cultivation. *Ex vitro* adaptation of the endangered endemic species *A. thracica* was successful as all of the *in vitro* regenerated plantlets survived.

Numerous studies report that changes in morphology and anatomy could occur in all *in vitro* plants' organs but the most important ones are the histogenesis deviations in the leaves (Magyar-Tábori et al., 2010). They could modify the morphogenetic potential of this organ and affect the regeneration effectiveness. The light microscopy analysis of the leaves displayed that in *in vitro* conditions *A. thracica* plants formed leaves with reduced thickness and with considerable deviations in the histogenesis palisade parenchyma. As it was noticed, the *in vitro* leaves of many other species had smaller sizes (Knöss, 1999), thinner lamina (Sáez et al., 2012) and often different structure of the photosynthetic tissues compared to the leaves from plants grown in nature (Rady and Ali, 1999; Dousseau et al., 2008; Jarda et al., 2011). It is well known that the successful *in vitro* regeneration and *ex vitro* adaptation are related to the histogenesis of the photosynthetic tissues. The normally structured mesophyll of the *ex vitro A. thracica* plants is an important marker demonstrating high regeneration potential of the cultivated species. The observed conservative structure of the epidermis could be an indicator for its genetic stability (Zhao et al., 2006). However, the authors (Zhao et al., 2006) pointed out that it was essential but not sufficient prerequisite for successful regeneration of the plants.

The results of the present morphoanatomical study of wild growing, *in vitro* and *ex vitro A. thracica* plants showed no divergences in organ- and histogenesis of the leaves and the accomplished *ex vitro* adaptation was normal. Consequently, that could provide successful *ex situ* conservation of this species.

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