MULTIPLICATION AND EX VITRO ADAPTION OF ACHILLEA THRACICA PLANTS

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Abstract

Introduction: *Achillea thracica* Velen. (Asteraceae) is perennial Bulgarian endemic species with valuable medicinal properties. The plants from genus *Achillea* have long history of use in ethnomedicine under the form of extracts, decoctions, ointments for treatment of genital problems, diseases of the digestive system, inflammatory diseases, hemorrrhages, ulcers and infected wounds. In the present work we aimed to access effective protocol for micropropagation of the Thracian yarrow grown in medium with different concentration of phytohormones and comparative determination of the stress factors in *in vitro* cultivated and *ex vitro* adapted plants.

Material and methods: Successful micropropagation was achieved on basal MSB5 medium with 30 g/L sucrose and 7 g/L agar. To stimulate better rooting, the effect of different concentrations of auxin indole-3-butyric acid (IBA) on the *in vitro* multiplication of plants was examined. The stress levels on the in vitro propagated and ex vitro adapted plants were estimated by determination of concentrations of malondialdehyde and hydrogen peroxide in plant cells.

Results: All tested concetrations of IBA stimulated root formation but more effective was MSB5 medium supplemented with 1.0 mg/L IBA. *Ex vitro* adaption was accomplished in growth chamber with 100% survival. Analyses of the concentration of MDA and H_2O_2 of the plants showed that *in vitro* grown plants had the lowest concentrations of both stress markers.

Conclusion: The successful initiation of *in vitro* and *ex vitro* cultures is an alternative biotechnological approach for preservation of *A. thracica* and would allow further analysis of metabolite profile.

Key words: *Achillea thracica, endangered species, micropropagation protocol, ex situ conservation*

Introduction:

Achillea genus includes more than 130 species most of which are perennial medicinal plants growing wild in Southeast Europe and Southwest Asia. Thracian yarrow (*Achillea thracica* Velen.) is a perennial herbaceous plant belonging to the *Asteraceae* family [1, 2]. *A. thracica* is a local Bulgarian endemic species, distributed in Thracian lowland, protected by the Red Data Book of the Republic of Bulgaria [3] and in the 1997 IUCN Red List of Threatened Plants [4].

The use of the micropropagation technique is of a high importance for conservation, rapid multiplication and genetic improvement of medicinal plants. Effective protocols for *in vitro* propagation have been developed to investigate the effect of different concentrations of plant growth regulators on *in vitr*o multiplication of *Achillea millefolium* [5], *Achillea filipendulina* cv. Parker [6] and *Achillea asplanifolia* [7].

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The aim of the present study was to access successful protocol for *ex situ* conservation of the Thracian yarrow cultivated and comparative determination of concentration of the stress factors in *in vitro* cultivated and *ex vitro* adapted plants.

Material and methods:

Plant material: *In vitro* shoot culture was induced by ripe dry seeds from *Achillea thracica* plants (*in situ* plants) grown in their natural habitat near to Manole village, Plovdiv, Bulgaria which were collected with official permission from the Bulgarian Ministry of Environment and Water. The seeds were sterilized and cultivated *in vitro* as previously described [8] (Fig. 1. A.). Then the effect of different concentrations of the auxin indole-3-butyric acid (IBA) (0.1, 0.5 and1.0 mg/L) on *in vitro* multiplication of Achillea thracica was studied.

Ex vitro adaption: Further, micropropagated *A. thracica* plantlets were transferred on hormone free MS-B5 medium. For *ex vitro* adaption, the regenerated *in vitro* cultivated *A. thracica* plants with well-developed root system were removed from the culture tubes. The agar from the medium was washed away from the roots. The roots were treated with an aqueous solution of potassium permanganate (KMnO4) for sterilization. The plants were transferred into plastic pots containing a mixtureof sterile soil:perlite:sand=2:1:1. The adaption was maintained in a growth chamber for 21 days under controlled conditions by changing the relative humidity from 90% to 60%. Next, the adapted plants were transferred to a standard for a month, and subsequent transfer to normal garden soil in the experimental field. After that they continued to develop normally (Fig. 1. B.).

Estimation of Malondialdehyde (MDA): Free radicals generate the lipid peroxidation process in an organism. MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA [9]. The concentration of Malondialdehyde in *in vitro* propagated and *ex vitro* adapted *A. thracica* plants was estimated with the method of Dhindsa et al. 1981 [10].

Estimation of concentration of H2O2: Hydrogen peroxide (H2O2) concentration was measured by the method of Velikova et al. 2000 [11].

Statistics: The data is presented on the figures as average values from two independent experiments. For verification of the results the Student's t-criteria at p=0.001 was used.

Results and Discussion:

Plant hormones regulate physiological and biochemical processes in plants by controlling their primary and secondary metabolism [12, 13, 14]. IBA belongs to the group of auxin-growth regulators responsible for plant root differentiation, cell expansion and proliferation, apical dominance. After one month of cultivation on MS medium [15] the regenerated *A. thracica* plants showed relatively low growth index and significant yellowish above ground parts. The replacement of vitamin mixture in the MS culture medium with B5 vitamin solution [16] led to the development of plants with plentiful green leaf biomass but still the root system was poorly developed.

After 20 days of cultivation of different concentrations of IBA, shoots developed directly from explants on all tested auxin concentrations. The most effective in shoot proliferation was MSB5 media supplemented with 1.0 mg/L IBA and 100% of explants showed direct rooting and produced approximately 12 well developed roots per explant (Table 1). All concentrations of IBA stimulated root formation and callus formation at the base of shoot tips. The effective response of auxin in stimulating root developing was reported early in micropropagated *A. filipendulina* plants achieved on MS medium supplemented with 2 mg/L BA and 1 mg/L IAA where the ratio of auxin to cytokinin has to be constant at about 0.3–0.5 in order to ensure shoot regeneration [6].

Ex vitro acclimated plants showed 100% survival rate. The number of successfully adapted plants to outdoor conditions is often too small due to poorly developed root structure [17], heterotrophic way of life of *in vitro* plants, the function of their stomatal apparatus, etcetera [18]. The reported high rate of adapted plant suggests the creation of a successful adaptation protocol.

To estimate how the different growth conditions affected the plant development, the levels of stress markers - lipid peroxidation and H2O2 has been estimated. For this purpose fresh leaves from 1 year old *ex vitro* adapted plants and 28 day old *in vitro* propagated plants were used. Overproduction of MDA is signature for lipid peroxidation in plant cells which is a result of high concentrations of reactive oxygen species (ROS) in the plant organism caused by different stress factors.

Hydrogen peroxide (H_2O_2) is commonly produced in plants during normal physiological processes and in response to stress situations [19]. The quantitative determination of hydrogen peroxide is important in numerous studies since H_2O_2 is involved in oxidative cellular damages as well as in signaling processes [20, 21].

The results showed that plants growing in *ex vitro* conditions have higher concentrations of MDA and H2O2 compared to the *in vitro* cultivated plants. These results could be explained with the high levels of stress during the *ex vitro* acclimatization process in plants.

Conclusion

The successful initiation of *in vitro* and *ex vitro* plant cultures is an alternative biotechnological approach for preservation of *A. thracica* and would allow further analysis of metabolite profile.

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Table. 1. Morphometrical characteristics of *Achillea thracica* plants cultivated on MSB5 medium supplied with IBA in different concentrations, where K-MSB5 medium without growth regulators, 0.1 IBA-MSB5 medium supplied with 0.1 mg/L IBA, 0.5 IBA- MSB5 medium supplied with 0.5 mg/L IBA, 1 IBA- MSB5 medium supplied with 1.0 mg/L IBA.

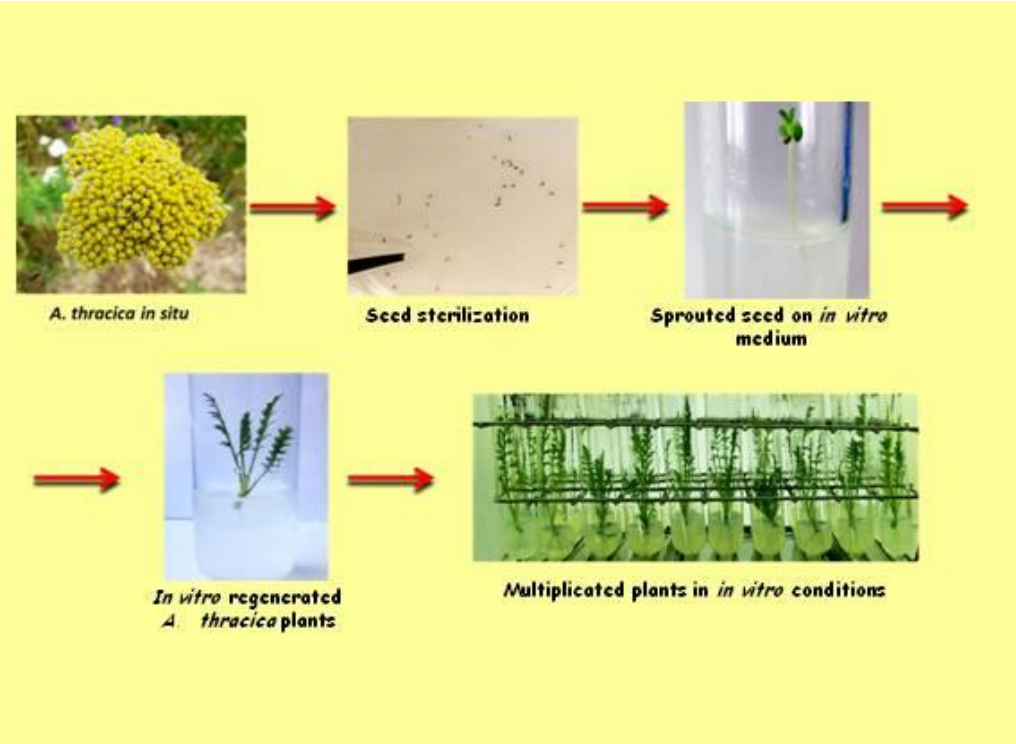
Variants	**Length of shoots (cm)**	**Number of shoots**	**Root formation**	**Root number**	**Root length (cm)**	**callus formation**
K	5,81±0,92	2.88±0.4	+	10,1±1.22	4,53±0,92	-
0.1 IBA	5,75±2,56	2±0.22	+	7,38±2,2	3,77±1,03	+[a]
0.5 IBA	7,25±1,56	1,25±0.41	+	9,375±1,69	3,78±1,15	+[a]
1 IBA	6,38±0,88	1,5±0.3	+	11,38±3,58	3,67±1,73	+[b]

a Week callus formation.

b Significant callus formation.

Figures Caption

Fig. 1. Micropropagation stages on Achillea thracica ex situ conservation: A) Initiation of in vitro culture;



B) Stages of ex vitro adaption.

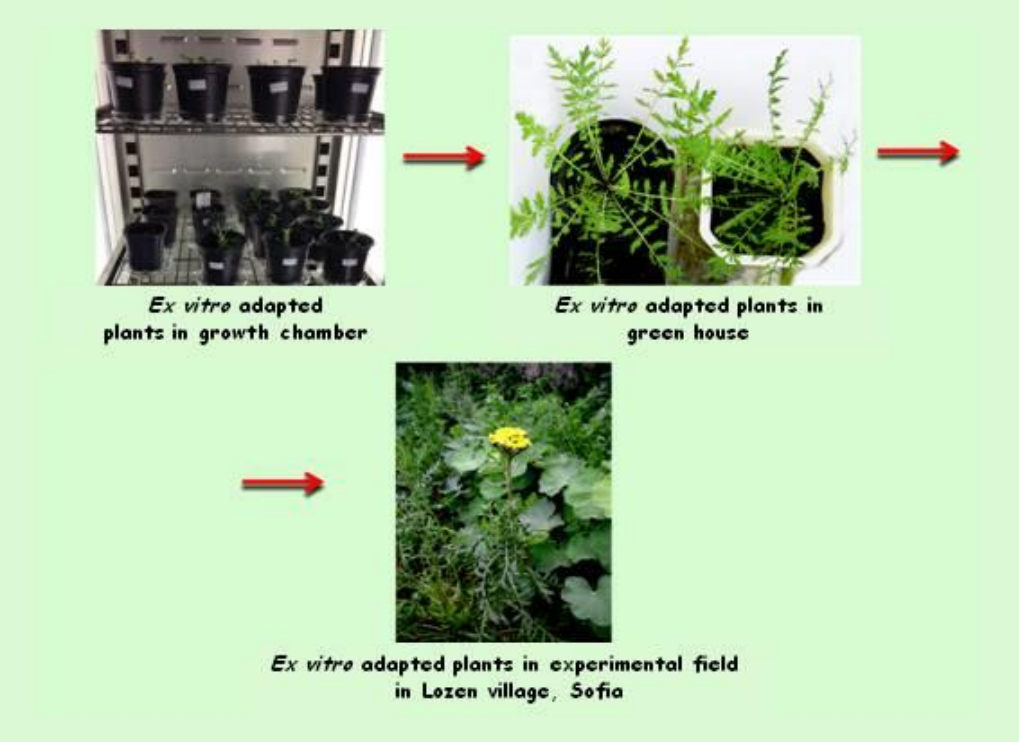


Fig. 2. Estimation of levels of lipid peroxidation by measuring of malondialdehyde concentration in *Achillea thracica* plant material. ** P≤0.001

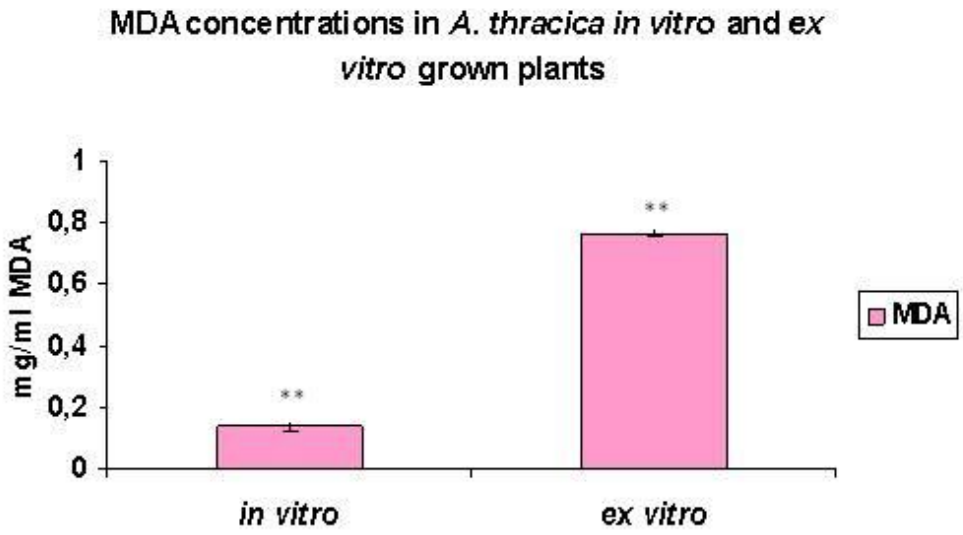


Fig. 3. Estimation of H_2O_2 levels in in vitro grown and ex vitro adapted A. thracica plants. ** P≤0.001