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Partial research concerning the behavior of the *Hepatica transsilvanica* in the process of *in vitro* micropropagation

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Abstract In order to develop an *in vitro* micropropagation protocol of the rare, endagered and ornamental plant *Hepatica transsilvanica* Fuss different types of explants cultivated on normal-strenght Murashige and Skoog (MS) macronutrients and micronutrients supplemented with BAP, alone or in combination with NAA were tested. For the *in vitro* establishment of *Hepatica transsilvanica* Fuss axillary buds, leaf and petiol explants were disinfected by standard methods, varying the time action of disifectant agents. The best results were obtained when axillary buds from rhizomes were cultivated on MS medium (modified) supplemented with 0.5 mg/l BAP, 78.9% of explants regenerating shoots.

Key words

endemic, *in vitro*, nutritive media, initiation

Genus *Hepatica* Mill is represented by the herbaceous perennial plants, native to Central and North East Asia, and North America. The genus consists of ten species with several varieties of perennial plants, all with lobed leaves and blue flowers. The growth in scattered through shady areas, forests and thickets within the cold temperate zones of the northern hemisphere [4].

In Europe, the genus *Hepatica* is represented by three species, two of which are found only in Romania: *Hepatica nobilis* Schreb and *Hepatica transsilvanica* Fuss. In our country is found also the hybrid known as *Hepatica x media* Simonka [7].

Hepatica transsilvanica Fuss (Fig. 1) is an endemic specie, with relatively limited geographical coverage being dependent on a limestone habitat [1]. Is a Pleistocene relict, vernal species and protheranthous plant. It occurs sporadically in Romania, being more common in the Eastern and Southern Carpathians [6]. It prefers rocky wet limestone areas in shady areas that are covert with forests and shrubs vegetation [3].

Differential species is *Asplenio trichomanis* - *Cystopteridetum Oberd fragilis*. (1939) 1949 *campanuletosum carpaticae* [10]. Coldea 1992, subassociation identified in the Dâmboviţa Complex Keys [8].

Plants with highly decorative potential provide an important source of germplasm that enrich the range of species used in landscape architecture and floriculture [2, 5]. *Hepatica transsilvanica* Fuss has a very high ecological value and has a decorative quality that is effective in parks and gardens.

Because the *Hepatica transsilvanica* Fuss has been declared an endemic species, protected by law, development of an *in vitro* micropropagation protocol is necessary. The goal of the present paper is to study the in vitro behavior of *Hepatica transsilvanica* Fuss and to establish a routine protocol for its micropropagation.

Fig 1. Hepatica Transsilvanica in the Dambovița Complex Gorges (original).

Materials and Methods

Hepatica transsilvanica Fuss plants were collected from Dâmboviţa Gorges (an area which is part of the National Park Piatra Craiului) and were maintained in the original substrate area, in the glasshouse conditions. The explants were excised from the healthy plants and were represented by the axillary buds (from rhizomes), leaf and petiole fragments respectively (Fig. 2).

All explants were washed vigorously under running tap water to remove the dust particles. For surface sterilization, the explants were agitated (few minutes) in tap water supplemented with three drops of chloride based disinfectant and after that were immersed successively in 96˚ethyl alchool for 7 minutes and 6% calcium hypochlorite for 5 minutes.

The explants were subsequently rinsed with sterile distilled water. Excess sampling explants was kept in sterilized autoclaving distilled water (Fig. 3).

Axillary buds were inoculated on basal medium consisted from normal concentration (n) of macronutrients and micronutrients Murashige-Skoog (1962), normal concentration (n) of vitamins White (1937), supplemented with 6-benzylaminopurine (BAP) and jellified with 7.5 g/l agar (Table1).

For the initiation phase of the *in vitro* culture starting from leaf and petiole explants the basal medium MS (modified) have been supplemented with auxine naphtylacetic acid (NAA) and cytokinine BAP (Table 1).

Explant type	Basal culture medium	Carbon source	Growth regulators (mg/l)
Axilary buds	Macronutrients MS (n)	Dextrose 40 g/l	BAP 0.5 mg/l
	Micronutrients MS (n)		
	Vitamins White (n)		
	Vitamins MS		
	Agar 7.5 g/l		
	NaFeEDTA 32 mg/l		
Leaf and petiole explants	Macronutrients MS (n)	Dextrose 50 g/l	BAP 0.4 mg/l + NAA 0.1 mg/l
	Micronutrients MS (n)		
	Vitamins MS (n)		
	Agar 7.5 g/l		
	NaFeEDTA 32 mg/l		

Table 1

Culture media composition used for initiation phase

Fig. 2. Stock plants from which the explants were taken

Fig. 3. Disinfection and sterilization of biological material

The cultures have been incubated in a growth chamber at the temperature of 24-26°C, with a photoperiod of 14 hours light/8 hours darkness, and a light intensity of about 40 μmol $m^{-2}s^{-1}$ (Fig 4).

Each experiments consisted of minimum three replicates and data represents the mean of three experiments. The data were recorded as percentage of explants infected and percentage of shoots regerated in the initiation phase of *in vitro* culture.

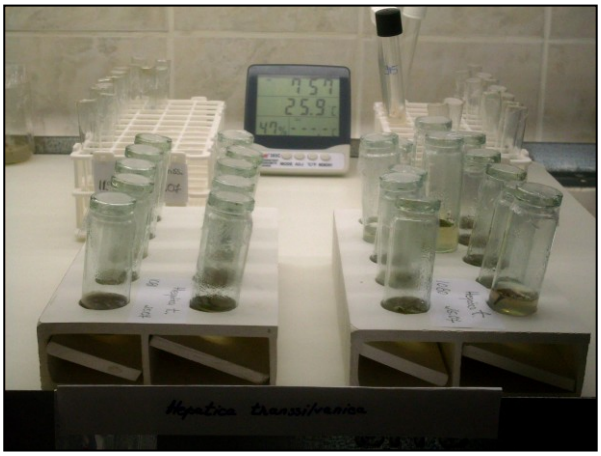

Fig. 4. *In vitro* cultures of *Hepatica transsilvanica* Fuss in the growth chamber

Results and Discussions

An important stage in developing protocols is the establishment of aseptic plant tissue to culture. Due to the fact that we work with both structure, buds from rhizome (this structure permits the isolation of aseptic shoot tips) and leaf and petiole explants, respectively, a disinfection solution was necessary for surface sterilization of plant tissue, by exposing them to the septic environment. As compared with leaf and petiole explants, the disifenction protocol used was more efficient in the case of buds from rhizomes. Thus, the percentage of aseptic culture was 31.5% of inoculated leaf and petiole fragmentsand 52.3% of inoculated buds from rhizomes (Fig. 5).

For *Hepatica transsilvanica* Fuss, the occurence of first shoots was noted at about 20 days after the culture initation (Fig. 6).

In terms of the number of explants which regenerated shoots the buds from rhizomes gave a better response (78.9%) then leaf and petiole fragments. Moreover, the leaf and petiole explants failed to form callus in MS medium supplimented with 0.4 mg/l BAP and 0.1 mg/l NAA and died after 12 days (Fig. 5). Similar results have been obtained in *Muscari mirum*, when leaf explants were used as an explant sources, no callus formation were obtained on MS medium, supplemented with different kind and concentration of plant growth regulators [6].

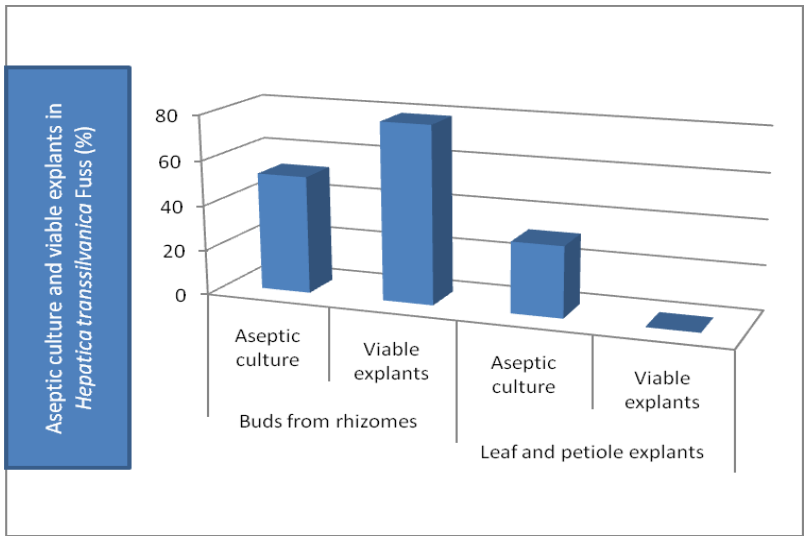

Fig. 5. Procents of aseptic culture and viable explants in *Hepatica trasnssilvanica* Fuss.

The better response of axillary buds to *in vitro* culture could be the consequence of the presence of meristem tissue. This type of tissue contain actively dividing cells that are responsable for lenght extension of the plant body and therefore have a greater capacity for regeneration.

This study showed that basal culture medium and plant growth regulators types, concentrations and combinations are key factors regulating callus induction and shoot organogenesis. Thus, MS supplimented with BAP 0.4 mg/l and NAA 0.1 mg/l was ineffective in callus induction and shoot regeneration in *Hepatica transsilvanica* Fuss.

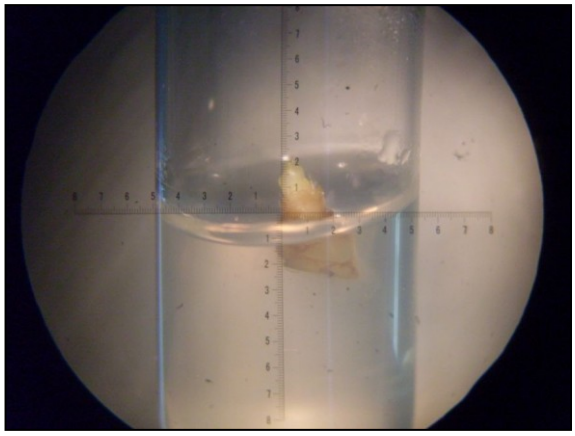

Fig. 5. Axillary bud after 20 days of inoculation on macronutrients and micronutrients MS (n), vitamins White (n), fortified with BAP.

Conclusions

The disinfection protocol, 7 minutes in 96˚etilic alcohol and 5 minutes in 6% calcium hypochlorite does not control contaminating microorganisms present on the explants.

Explant type represented by the rhizome bud had the best response during *in vitro* initiation culture. Basal medium supplemented with 0.5mg/l BAP resulted in 78.9% neoplantlet regeneration in the initiation phase.

Further studies are necessary to establish optimal conditions for *in vitro* micropropagation of *Hepatica transsilvanica* Fuss.

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