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Preliminary results on the *in vitro* propagation by leaf explants and axillary buds of *Iris aphylla* L.

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Abstract In order to develop an *in vitro* micropropagation protocol of the rare, endangered and ornamental plant *Iris aphylla* L., leaf explants and explants of rhizome buds cultivated on normal- strenght Murashige and Skoog (MS) basal media, supplemented with growth regulators. Explant types were disinfected by standard methods. The higher percent of contamination was recorded for the rhizomes explants with axillary buds (86%). The potency of cytokine-like thidiazuron (TDZ) combined with auxin alpha naphtyl acetic acid (NAA) as plant growth regulators in evoking morphogenic responses from leaf explants and explants of rhizome buds in *Iris aphilla* L. was evaluated. Maximum percentage of callus induction was obtained from the leaf explants of *Iris aphylla* L. cultured on MS basal medium augmented with 2.0 mg l^{-1} TDZ and 3.0 mg l^{-1} NAA.

Key words

leaf and rhizomes explants, axillary buds, disinfection, culture medium, TDZ

The *Iris* genus belongs to the Iridaceae family, which includes over 300 species, originating from Europe, North America, Asia and Africa [3].

Being considered extremely rare and endangered in many European red data books and lists *Iris aphylla* L. is an attractive rhizomatous perennial herbaceous [8], characterized by their intense green foliage, formed directly from rhizome and entomophilous dark purple flowers (1–3, rarely 4–5). The stems are 20–70 cm tall, slender, branched below its mid-point and often from its base. Firstly flowering period is from mid-May through the early July, but sometimes is also blossoming in August-September.

Iris aphylla L. is an euriterm species, which vegetate through meadows and thickets on sunny and well drained rocky, on rocky sunny in the subalpine area. Also, this species presents an excellent natural resistance to could winter [7].

According to Taylor (2000) *I. aphylla* L. is a tetraploid form with 48 somatic chromosomes. On the other hand, Dostl (189) reports that number of chromosomes in *Iris aphylla* L. species is variable (2n=24, 40, 48).

In vitro micropropatation of flower and ornamental plants was initially developed in England and France in the 1960s. *In vitro* clonal multiplication, has caused great impact on commercial seedling production of flowers and ornamental plants [2].

Interest for the study of *in vitro* micropropagation potential of geophytes, including *I. aphylla* L. species, is usually determined by their importance in the fragrance industry, the pharmaceutical industry, outstanding their ornamental value. A major consequence of the economic importance is a great loss in species diversity, through bulbs collection from natural habitats (in present, bulbs collecting from the natural habitats is prohibited). Thus, *in vitro* micropropagation is a successful alternative method for the plant mass-propagation.

The objective of this study was to successfully place the explants of *Iris aphylla* L. into aseptic culture by avoiding contamination and then to provide an *in vitro* conditions that promotes stable shoot production.

Material and Methods

Plant material. *Iris aphylla* L. plants were collected from Ghițu Mountains, and were maintained in the original substrate area, in the glasshouse conditions. The mature rhizomes and axillary buds were directly taken from green house and therefore (Fig. 1), needed to be surface sterilized to eliminate the microorganisms present on the surface of the explants.

Surface disinfection procedure. The mature rhizomes and well developed axillary buds were detaches from the healthy plants and were used as explants sources. Rhizome fragments and axillary buds were thoroughly washed under running tap water to remove the dust particles.

Fig. 1. *Iris aphylla* L. plants.

The rhizomes fragments were soaked in water, scarified and washed carefully. Each rhizome was sliced into small pieces (0.5 to 1 cm) with buds on them. For surface sterilization, the rhizomes pieces and axillary buds were agitated for a few minutes successively in tap water supplemented with Dettol antibacterial soap and three drops of chloride based disinfectant, respectively. Immediately after that, were immersed successively in 70˚ethyl alchool for 2 minutes and 6% calcium hypochlorite for 10 minutes. The explants sources were subsequently rinsed with autoclaved distilled water 4 to 5 times. Also, sampling explants was kept in sterilized autoclaving distilled water under sterile conditions in a laminar air flow chamber (Fig. 2).

Fig. 2. Leaf and rhizomes fragments used as explant sources.

Culture medium. For the initiation of *in vitro* culture, rhizomes explants with axillary buds, excised from the rhizomes with approximately 0.2-0.3 cm of tissue and leaf explants of 0.3-0.4 cm in size (separated from the axillary buds of 1 cm in size) were placed on MS culture medium [6], supplemented with dextrose at 40 g l^{-1} and agar at 7.5 g l^{-1} concentration. The pH was adjusted to 5.8.

For each type of explants, four treatments with different combinations of *N*-phenyl-*N*-1,2,3-thiadiazol-5-yl urea (TDZ) and alpha naphtyl acetic acid (NAA) as growth regulators (Table 1) were used in order to find an adequate culture medium composition for inducing callus proliferation and shoots regeneration, respectively. Five ml media was poured into each test tube. The test tubes containing MS medium supplemented with different combination and concentration of growth regulators were autoclaved and after that was stored in sterile condition for subsequent

use. Sterilized explants were inoculated in test tubes containing the media, in the laminar air flow chamber.
Cultures conditions. The cultures have been incubated in a growth chamber at the temperature of 24-26°C, in the dark for two weeks and then has been transffered to a photoperiod of 14 hours light/8 hours darkness, and a light intensity of about 40 μmol $m^{-2}s^{-1}$ (Fig 4).

Ten explants were used for each treatment and the experiment was repeated tree times. Data were recorded after 1 month of culture. The data were recorded as percentage of infected explants and mean numebr of explants which formes callus.

Table 1

The combinations and concentration of growth regulators added to MS medium used for initiation phase of *in vitro* culture of *Iris aphylla* L.

Type of explants	Basal culture medium	Growth regulators	
		TDZ	NAA
Axillary buds, Leaf explants	Murashige and Skoog (1962)	2.0 mg l^{-1}	-
		2.0 mg l^{-1}	1.0 mg l^{-1}
		2.0 mg l^{-1}	2.0 mg l^{-1}
		2.0 mg l^{-1}	3.0 mg l^{-1}

Results and Discussions

After one month from the *in vitro* culture initiation the contamination percentage varied with a range of 20% to 86%, depending on the type of explants. In this work, the percent of contamination was 86% for the rhizomes explants with axillary buds, but 80% contaminant free cultures were recorded for leaf explants (Fig. 1).

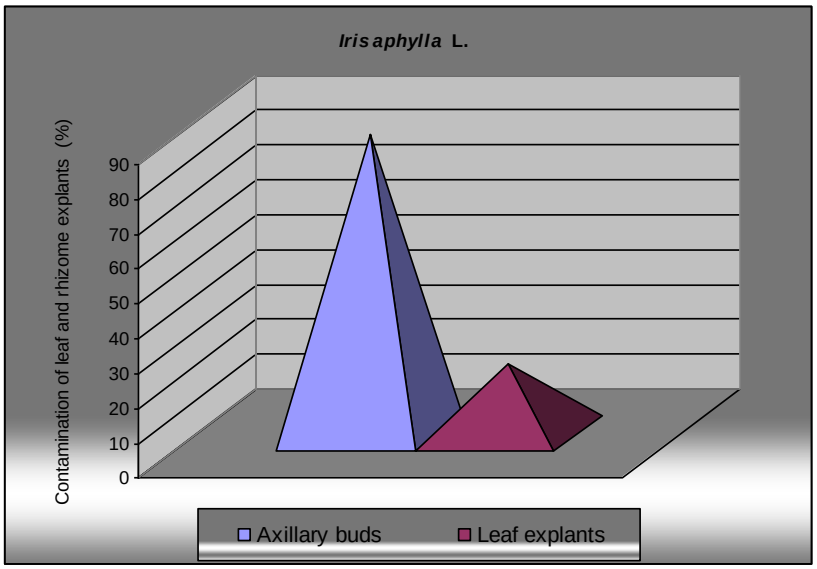

Fig. 1. The comparison of the contamination percentages of rhizomes explants with axillary buds and leaf explants on *Iris aphylla* L. cultivated on MS medium, irrespective of the combination and concentration of growth regulators.

Disinfection of initial explants is a major problem in micropropagating species where underground rhizomes are the only source of axillary buds. Perhaps, the great percent of contaminated cultures in this work is due to the absence of mercuric chloride ($HgCl_2$), which is knew as very effective disinfectant in plant tissue culture explant pretreatment protocols [1]. But, heavy metals, like mercury, are known for immunotoxic and neurotoxin properties and their use is not recommended.

According to Jevremović et al. (2012) somatic embryogenesis and/or organogenesis are most suitable methods for *in vitro* plant regeneration in *Iris* species from different type of explants. But, *in vitro* regeneration of shoots via callus, irrespectively of the type of explants is highly dependent of culture medium composition, with respect to the basal culture medium

and combination and concentration of growth regulators. In previous experiments that we attempts to develop tissue culure propagation were unsuccessful as *Iris aphylla* L. was viewed as recalcitrant species (data not shown). In this work, the protocol of callus induction involved morphogenic control by using a combined auxin, as NAA, and cytokine, as TDZ, in the basal culture media. Thidiazuron is a phenyl urea that has gained importance as a potent plant regulator for *in vitro* propagation systems of various plant species

In our works, buds from rhizomes inoculated on culture media were unable to response to the MS media, irrespectively of combination and concentration of growth regulators and died in 22 days. In comparison, leaf explants cultured on MS medium supplemented with 2 mg l^{-1} TDZ in combination with 3 mg l^{-1}ANA formed callus after 28 days from culture initiation, but failed to regenerated shoots. Leaf explants cultured on MS medium supplemented with lower concentrations of growth regulators was unable to form callus after 21 days from culture initiation and died or got contamination.

Callus derived leaf explants failed to developed shoots and after 45 days turned brown and died. It is reported that TDZ induces shoot regeneration in many plant species. TDZ fraction plays very important role in morphogenesis like, lower concentration induces axillary shoot proliferation, whereas higher fraction causes adventitious shoot development. In this work, that the incapacity of callus to form shoots can be a consequence of too long period that it was kept on callus induction media and/or of the high concentration of growth regulators, especially TDZ. Mulanda et al. (2012), showed that for *Melia volkensii*, the callus fresh mass produced at 0.05 mg l^{-1} was double or up to three times larger than the callus masses obtained in the absence of TDZ or at concentrations greater than 0.05 mg l^{-1}. There are no previous reports on the effect of TDZ on leaf explants capacity to form callus in the genus *Iris*.

Beacuse only one combination growth regulators induced callus formation (2 mg l^{-1} TDZ in combination with 3 mg l^{-1} NAA added to MS medium) indicate the important role of exogenous plant growth regulators on callus formation in *Iris aphylla* L.

Conclusions

In the present study, the percent of *in vitro* culture contamination was 86% for the rhizomes explants with axillary buds, but 80% contaminant free cultures were recorded for leaf explants.

Higher concentration of cytokine-like (TDZ) combined with auxin NAA, added to the MS basal medium induced higher percentage of callus derived from leaf explants.

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