Scientific framework for selecting explants of Iris L. genus for direct shoot regeneration

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Abstract. The explant type, its anatomical structure, and hormonal composition of culture media are crucial for selecting explants of Iris L. genus for direct shoot regeneration in tissue culture. In case of vegetative bud culture, antibiotics must be added to the agar medium with due regard to the antibiotic sensitivity of microflora. On MS medium (Murashige and Skoog 1962) supplemented with BA (6 µM) + NAA (5 µM) + L-glutamine and adenine sulfate (100 mg/l), the embryos of I. hybrida and I. sibirica developed into adventitious shoots and the multiplication coefficient of I. ensata increased in subsequent passages. In the budding phase, the rachis and perianth tube maintained meristematic tissues and exhibited morphogenetic capacity. The fragments of generative organs of I. ensata, I. hybrid and I. sibirica formed the shoots on MS media containing BA (4–8 µM) and NAA (3–5 µM). The highest percentage of the shoots (100%) formed from the perianth tube explants had the structure similar to perianth lobes instead of the first leaf primordia. Further culture resulted in the formation of the shoots typical of these species of iris.

1. Introduction
Irices are promising perennials. In Russia, the varieties with high decorative qualities and a wide range of flower shapes and colors, different flowering periods, high resistivity to biotic and abiotic factors of the environment have been developed for different climatic zones of the country. There is a rich collection of iris varieties and wild species. The official and folk medicine uses leaves, roots and rootstocks of Iris sibirica L. and Iris ensata Tnunb. to produce different herbal mixtures, herbal medicinal and cosmetic products. The alternative method of obtaining medicinal plants to produce essential oils using the callus culture of I. sibirica has been patented. The proposed method enhances the quality of essential oils, significantly increases the amount of the extracted irone, reduces the cost of the finished product, allows continuous culturing of tissue cultures regardless of the environmental conditions, and expands the plant material base. The difficulties encountered in the process of selection and maintenance of iris genotypes force the scientists to use the advanced methods of modern plant biotechnology. Most researchers used zygotic embryos to introduce irises of different species into in vitro culture. For this purpose, the apical meristem and somatic organs of both vegetative and generative shoots were occasionally used. To regenerate the shoots, only two methods were utilized: induction of somatic embryogenesis in callus culture and differentiation of buds in callus culture [1-9]. The present research aims to establish the mechanisms of the iris shoot regeneration in tissue culture with due regard to the explant type, its anatomical structure, and hormonal composition of culture media.
2. Materials and methods

Plant material. The prospective varieties of domestic and foreign selection and new elite hybrids of three species of iris (I. hybida, I. ensata and I. sibirica) from the collection of Siberian Research Institute of Horticulture (Barnaul) have been used in the current research.

The morphogenetic features of the development of various types of explants such as vegetative buds, embryos, and somatic tissues of reproductive organs in in vitro culture were thoroughly investigated. The regenerative capacity of the explants with due regard to the concentration of growth regulators was also considered. The fruits were harvested starting from 30–90 days after pollination, and the flowers gathered in the budding phase were the size of 20–30 mm.

For the vegetative buds of irises, disinfectants of various types were used separately or in combination in five different embodiments. Each experiment employed 20 or more explants. More than 300 explants were used for the three types of irises utilized in the experiments. All the tested methods of sterilization showed high vitality of the explants except for the vegetative buds of I. hybida subjected to chlorhexidine and a 70% ethanol solution with 3% hydrogen peroxide. In these experiments, the explants died very quickly due to the bud decay. Contamination of the explants of I. ensata and I. sibirica was insignificant during the first 7–10 days indicating that the surface sterilization was adequate, but it eventually reached high values, probably due to the internal contamination. Pre-sterilization treatment using a 0.3% fundazol solution for 16 h yielded no positive results. Increase in sterilization time for more than 20 min led to the explant death.

The following explants were isolated from the iris buds: transverse sections of rhachis (1–2 mm thick), ovary fragments, perianth tube fragments (5×5 mm), filaments, anthers, style, and stigma. The stage of flower development at the time of introduction into tissue culture was crucial for successful regeneration. The highest percentage of regeneration was observed in the budding phase. In this period, the growth of the corolla petals is evident, the filaments are shorter than the anthers, the carpel elongates, and the stigmas are formed. At the later stages of flower development, single explants are regenerated when the colored cone of the corolla is plainly visible, and no regeneration is observed when the flower of I. ensata is fully open.

Sterilization was performed in a laminar box. Eight experiments were to be carried out to find the efficient sterilization method. Only one of the eight methods ensured successful sterilization of the floral organs and their high vitality. The method included two stages of sterilization. At the first stage, the buds soaked in 96% ethanol were burnt in the flame of a spirit lamp. At the second stage, the material was immersed in a 0.1% sulfochlorantin solution for 30 min. The considered method ensured 95% of the material sterility and 100% of its vitality.

Culture media conditions. The research was based on the conventional methods used in plant biotechnology. The standard MS medium was used to study the morphogenetic potency of organs and tissues under in vitro conditions.

To control the process of in vitro morphology of iris, the culture medium was supplemented with the following phytohormones: 6-benzylaminopurine (BA), Sigma, the USA, and α-naphthaleneacetic acid (NAA), Sigma, the USA. The non-hormonal growth regulators L-glutamine and adenine sulfate were used at a concentration of 100 mg/l. The sucrose at a concentration of 30 g/l served as the main carbohydrate for culturing organs and tissues.

The explants were grown in the culture chamber at the temperature of 20–30 °C, 16-h photoperiod, light intensity of 2000–4000 lx and 70% relative humidity.

Histologic examination. A number of sections were made for the anatomical study of morphogenetic processes. The permanent preparations were made by conventional methods [10] in our modification.

The finished preparations were examined with the direct universal research microscope Axio Imager. Z1 (manufactured by Carl Zeiss). The images were obtained using a digital camera AxioCam MRe 5.
3. Results and discussion
Vegetative buds. The explants of rhizomatous and bulbous origin have a high degree of contamination due to difficulties in sterilization of the vegetative buds which are in the soil [11]. Even in case of successful surface sterilization, the signs of latent contamination are manifested throughout all the stages of micropropagation, when the vegetative buds of irises (I. hybridia, I. ensata and I. sibirica) are introduced into in vitro culture. To eliminate the latent contamination, it is necessary to conduct microbiological control of the explants introduced into the culture and to determine antibiotic sensitivity of the isolated microorganisms.

Microorganisms isolated from the contaminated explants of cultivars of I. hybridia, I. ensata and I. sibirica were examined and compared with those of the rhizome tissues of intact plants of these iris species. The bacterium Erwinia spp. was mostly found in I. hybridia. The presence of the bacterium in rhizome tissues of intact plants and the period of their manifestation in in vitro culture (after 7–10 days) indicate that this contamination is internal and, therefore, the most stringent sterilization methods are inefficient. The internal fungal contamination occurred in I. ensata and I. sibirica.

The antibiotics were selected based on the microflora sensitivity to various antimicrobial agents. From the given list of antibiotics, Erwinia spp. was resistant only to ampicillin, and Pseudomonas spp. showed resistance to doxycycline, tetracycline, cefotaxime, and ceftriaxone. These bacteria exhibited high sensitivity to the majority of antibiotics. This allowed selection of less toxic antibiotics which did not inhibit the growth of explants of the iris vegetative buds.

In case of introduction of I. hybridia into in vitro culture (hybrids 5-282-98 and 8-282-98), chloramphenicol (100 mg/l) was added to the culture media. The medium without any antibiotics was very useful as control. The percentage of contaminated explants of I. hybridia dropped to 20 on the media containing antibiotics. Probably, the presence of bacterial flora is typical of this iris species. For the explants of I. ensata (hybrid 1-103-97) and I. sibirica (hybrid 5-282-98), the percentage of contamination was at the control level indicating the prevalence of fungal contamination. To inhibit the contamination, terbinafine (100 mg/l) was added to the culture media.

Introduction of antibiotics and fungicides into the culture media results in obtaining a sterile culture, but antimicrobial agents manifest adverse effect. The low concentration of antibiotics in culture media only inhibits the growth of pathogens, but does not lead to the death of microorganisms. Due to this, microorganisms become antibiotic-resistant in subsequent passages, and further introduction of the antibiotic used in the treatment is inefficient. If the plant is not strongly depressed, the antibiotic can be replaced by the analog to obtain a sterile culture. The propagation is not ensured though.

In exceptional cases, when a small fragment of the iris rhizome with a few buds is available for a biotechnologist, and the species is in urgent need of propagation, the vegetative buds can be used as explants.

Iris embryo culture. When embryos were used as explants, the period of introduction into in vitro culture was from 50 to 80 days for irises I. hybridia, I. ensata and I. sibirica. Irises are characterized by the slow development and differentiation of the embryo [12]. On the 30th day after pollination, the iris embryos were scarcely visible. Due to this, the attempts to isolate them from the seed failed. It was found that the optimal period for the embryo isolation was from 50 to 80 days after pollination, when the majority of seeds reached the size of 2–3 mm. After 80 days of the development, the endosperm became very dense and complicated the embryo isolation.

The anatomical sections showed that the embryo was small and straight, consisted of elongated polygonal cells, and occupied a central position in the endosperm (figure 1a). A large mature endosperm of distinct radial structure occupied the bulk of the seed volume (figure 1b). According to F. Netolitzky [13], endosperm cells contain oils and aleurone grains with crystalloids without globoids.

The embryo seems to be unsegmented, but the longitudinal section reveals the boundary between the axis and terminal cotyledon of the embryo. The radicle faces the micropyle and transitions into a straight hypocotyl terminating at a shoot apex. The latter is notable for its small cells observed on the
section. Cotyledon is cylindrical, its lower part represents the sheath which surrounds and covers the apex. The development of iris embryos in in vitro culture is mostly similar to the seed germination, but it has its own peculiarities. In germination, the enhanced nutrition triggers the growth of all the organs (figure 1c).

Figure 1. I. sibirica seed: a) longitudinal section, b) anatomical section (50×magnification), c) I. hybrida embryo on the 30th day of culture, medium supplemented with BA (6 µM) + NAA (5 µM) + L-glutamine and adenine sulfate (100 mg/l).

The embryos on artificial medium absorb nutrients through the entire surface, and apparently there is no need to develop a long petiole. On the 4th day of the embryo culture, the cotyledonal petiole began developing, but ultimately remained in germ, and the degree of development of other embryo organs depended on the culture period and hormonal composition of the medium.

When transferred onto the artificial culture medium, the embryo mostly develops into one plant. However, the formation of axillary meristems can be directly induced in the embryo on some culture media. This results in increase of the multiplication coefficient by several times and forces the propagation of a hybrid plant. At the stage of introduction into the culture, the components of the culture medium are of great importance for propagation. The impact of these components can be observed in iris up to 4 months of culturing the embryos.

The regenerated embryos of the three iris species exhibited well-developed leaves and sometimes roots on all the tested culture media. On the medium containing BA (6 µM) and NAA (5 µM) supplemented with L-glutamine and adenine sulfate, the embryos of I. hybrida and I. sibirica developed into adventitious shoots, and the multiplication coefficient of I. ensata increased in subsequent passages.

A year after planting out, regenerated plants had 1–3 shoots and all blossomed. The height of vegetative shoots was 40–60 cm, and the height of stems was 60–80 cm. There were 3–4 flowers per stem. Two-year seedlings had 1–3 vegetative and 0–1 generative shoots.

The method of embryo culture is crucial to intensify the selection process of irises. Two-year regenerated plants are highly decorative that allows comprehensive assessment of the hybrid vitality and decorative features in earlier periods and simultaneous micropropagation of the selected plants. This speeds up the selection process for 3–4 years.

The propagation of either hybrids or wild species enabled the embryos utilization as explants. The shoots developed from the embryos had a multiplication coefficient which was rather high for irises. The embryo culture method was found unsuitable for species propagation. Due to this, the floral organs were used as initial explants.

Fragments of generative organs. The rachis and perianth tube had the greatest regeneration potential among the explants of floral organs regardless of plant species. On MS media supplemented
with BA (4–8 μM) and NAA (3–5 μM), the fragments of generative organs of I. ensata, I. hybrid and I. sibirica formed shoots with the structure typical of vegetative organs of these species.

The first cell divisions were observed in the explants of I. ensata and I. sibirica starting from the 7th day after transfer onto the culture medium. The explants of I. hybrid have greater regenerative activity, the first polyads (group of cells under a common wall) were found on the 4th day of culture, and the first visual signs of gemmogenesis were observed after 14 days of culture on these culture media (a few days earlier than for I. ensata and I. sibirica).

The comparison of the dynamics of regenerative processes in the perianth tube explants of cultivars of I. sibirica, I. ensata and I. hybrid revealed their similarity. Only adaxial side of the perianth tube was found to exhibit regenerative capacity. When the explant was placed with abaxial side down on the surface of the culture medium, the signs of regeneration were not observed within the entire period of culture. Histologic analysis revealed different levels of the tissue regeneration activity on the inner and outer surface of perianth tube in all the examined cultivars of I. hybrid, I. ensata and I. sibirica (figure 2a). The parenchymal cells maintained meristem activity in the subepidermal layers of the adaxial side, where multiple zones of division were observed and vegetative shoots were initiated (figure 2b). All the formed shoots developed the structures similar to the perianth lobes instead of the first leaf primordia. Afterwards, the structures got the coloring typical of the flowers of this species (figure 2c). Further culture resulted in the formation of the shoots typical of these species of iris.

Figure 2. A) anatomical section of the perianth tube of I. sibirica a) in the budding phase, b) on the 12th day of culturing the explants (100× magnification), c) Formation of the floral elements of the perianth tube explants of I. sibirica 'Sterh'.

According to our observations, I. sibirica 'Cambridge' formed an average of 20 vegetative shoots and 3 generative shoots after a year of growing the regenerated plants in the field. After two years of vegetation, these figures went up to 80 and 16, respectively (figure 3). The micropropagation technology allows mass production of irises within a short period of time not only to facilitate planting in urban areas, but also as an alternative way of drug production.

Figure 3. Regenerated plants of I. sibirica 'Cambridge' obtained from the floral fragments after planting out: a) regenerants at the moment of planting out and after one year of growing, b) after two years of growing in the field.
4. Conclusion
The establishment of the mechanisms of iris shoot regeneration in tissue culture enables the development of micropropagation technology for species of Iris L. with due regard to the explant type, its anatomical structure, and hormonal composition of the culture media. The iris explants of rhizomatous origin experience a high degree of contamination. Introduction of antibiotics into the agar medium makes it possible to maintain up to 80% of explants viable and sterile. The less toxic groups which ensure high antibiotic sensitivity of microflora are preferable. If used as explants, the embryos of I. hybridia, I. ensata and I. sibirica must be introduced into in vitro culture starting from 50–80 days after pollination. On MS medium supplemented with BA (6 µM) + NAA (5 µM) + L-glutamine and adenine sulfate (100 mg/l), the embryos of I. hybridia and I. sibirica developed into adventitious shoots and the multiplication coefficient of I. ensata increased in subsequent passages. In the budding phase, the rachis and perianth tube maintained meristematic tissues and exhibited morphogenetic capacity. The fragments of generative organs of I. ensata, I. hybrid and I. sibirica formed the shoots typical of these species on MS media containing BA (4–8 µM) and NAA (3–5 µM).

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