Biotechnological aspects of multiplication of Iris sibirica L. on the basis of induced morphogenesis

Z Dolganova and L Tikhomirova
Altai State University, Lenina str., 61, 656049 Barnaul, Russia
Email: l-tichomirova@yandex.ru

Abstract. The article gives the results of research work in morphogenesis and regenerative capacity of specimen in vitro culture of floral axis and perianth of some cultivars of I. sibirica L. It is stressed that morphogenesis proceeds in accordance to hemmorhizogenesis and rhizogenesis, omitting callusogenesis. The spears formed de novo are solely endogenous. The spears close to the perianth carried floral elements at prophyllum primordium, and had typical monocotyledon constitution.

1. Introduction
Numerous species of Iridaceae family are being propagated in vitro culture since classical methods of vegetative reproduction with rhizomes and bulbs ceased to satisfy the needs in planting stock quantity [1]. Apart from commercial goals, micropropagation of some endemic species of Blueflag was conducted with the aim of its preservation. The study of morphogenetic peculiarities of some Liliaceae representatives in reproduction in vitro revealed the fact that morphogenesis proceeds in accordance to hemmorhizogenesis, omitting callusogenesis. During histologic research of explant tissue of flower organs polyads, combinations of meristematic cells were detached in various parenchymatous layers. Meristematic cells of polyads were slightly smaller in size in comparison to the rest parenchymatous cells, and were solely those which retained the ability for further division. In a while, some polyad cells gave rise to adventitious buds [2-7].

Rhizome and bulbous explants are considerably infectious since vegetative buds are located in soil and their sterilization is a matter of some difficulty. Embryoculture method enables to diminish the period of breeding material production considerably and to provide a sufficial quantity of blueflag in a short time. Unfortunately, the method is not suitable for breed multiplication. Given the above, flower organs were used as a primary explant [8, 9]. The study of explants of I. sibirica generative organs in vitro is of theoretical and practical interest. The major part of research work in this sphere is conducted on vegetative organs, while morphogenetic potential of generative organs remains comparatively unstudied.

2. Materials and methods
Plant materials. New elite hybrids of I. sibirica of the collection of Siberian Research Institute of Horticulture (Barnaul) were used in the study. The following explants were isolated from iris buds: transverse sections of rhachis (1–2mm thick), ovary fragments, perianth tube (fragments of 5×5 mm), anther filaments, anthers, style and stigma of pistil. An important aspect in the successful regeneration is the moment of the introduction of flowers into the tissue culture. The highest percentage of
regeneration was observed in the budding phase, when the flowers are tightly closed by the wrapping leaves. In this period the growth of the corolla petals is already observed, the anther filaments are shorter than the anthers, the carpel elongates, and the stigmas are formed. At the later stages of the flower development when the colored cone of the corolla is seen, single explants can be regenerated, and in case of full disclosure of I. sibirica lower there is no regeneration.

The material was sterilized in the laminar box. When working out the best way, 8 variants of the experiment were set. Only one of them provided a high sterility of the flower organs together with their high vitality. In this variant the material was sterilized in two stages. At the first stage the buds were soaked in 96% ethyl alcohol and burnt in the flame of a spirit lamp. At the second stage the material was immersed in a 0.1% sulfochlorantin solution for 30 minutes. The method provided a 95% sterility and 100% vitality of the material.

Culture media conditions. The research was based on the methods generally accepted in plant biotechnology [10]. In the study of morphogenetic potentials of organs and tissues in vitro, the standard culture medium MS [11] was used.

To control the process of in vitro morphogenesis of iris, the culture medium was supplemented with the following phytohormones: 6-benzylaminopurine (6-BA), Sigma, the USA, and α-naphthaleneacetic acid (NAA), Sigma, the USA. At the stage of introduction into the culture in vitro, the phytohormones were added at appropriate concentrations. 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. The series of sections were made for the anatomical study of morphogenetic processes. The permanent preparations were made by conventional methods (Barykina et al. 2004) in our modification. The tissues and organs were fixed in 10% formalin solution at room temperature. Then the vegetative material was processed in the automatic system ТРС 15 (Medite, Germany) according to the scheme:

- 10% formalin solution (1 container) – 1 h,
- Isopropyl Alcohol (9 containers) – 1 h. 05 min. in each,
- Paraffin (3 containers) – 1 h. 30 min. in each.

Total time of processing: 15 h. 11 min.

After processing, the samples impregnated with paraffin were embedded in paraffin blocks and glued onto cassettes. The sample was cut on the rotary microtome, the resulting sections were straightened in warm water of 50–55 °C and placed on the glass slide, which was also labeled.

The basic method of the histological preparations coloring implied the use of the machine TST-44 (Medite, Germany), with simultaneous application of hematoxylin and eosin. Total time of coloring: 18 min. The sections were embedded in polystyrene and covered with glasses. The ready preparations were examined with the direct universal research microscope Axio Imager. Z1 (manufactured by Carl Zeiss). The photos were made by the digital camera AxioCam MRc 5.

3. Results and discussion

Culturing in vitro the floral axis fragments of I. sibirica cultivars revealed that the type of morphogenetic reaction depends on the amount and proportion of exogenous phytohormones. The 4mkM BAP, 4 mkM NAA growth medium (1:1) and 4mkM BAP, 5 mkM NAA growth medium (1:1.25) generated rhizogenesis. The medium types with cytokinine content exceeding auxine 1.2 times generated the floral axis fragments formed buds (from 6 mkM BAP and on).

The speed of regeneration process in an explant depended on the hormones concentration in the growth medium and on the genetic complex of the origin. On the 8 mkM BAP and 3 mkM NAA growth medium the I. sibirica explants (breed Sterkh) the first indications of hemmogenesis were registered on the 15th day of cultivation, while the 6mkM BAP and 5 mkM NAA growth medium this
period averaged 25 days. The maximum time was 35 days, later on discernible indications were not registered (figure 1 a, b, c).

Figure 1. Shoot and root development on floral axis explant of I. sibirica, the 30th day (4 mkM BAP 4 mkM NAA).

The held research of I. sibirica floral axis anatomy (VI-VII stage of organogenesis) on a transverse section revealed the following. A generative burgeon is covered with epidermis, formed by one layer of cells with cuticle. Minor magnification (x100) shows a cell annulus, in a form of a multiserial pericycle. In adolescent organs pericycle is represented as a primary lateral meristem, which body cells further on lose the ability for division and become completely differential as sclerenchyma fiber. A pericycle is a periblast of a stele. On its inner side among parenchymal cells of the core small collateral bundles are situated. The tissue peripheral to the stele appertain to the cortex which is twice smaller in width than the stele and is represented primarily with chlorenchyma. The cells of the cortex have thin cellulose walls, some of which contain pigment as well as the epidermis cells. Between the cortex parenchyma cells, closer to the pericycle, centres of division can be noticed, for at this stage of organogenesis the growth of the generative sprout is not over yet. High magnification (x400) reveals that the whole space of the cortex is filled with ground tissue, where sheltered conducting bundles are disseminated. The parenchyma cells are of an isodiametric form. Conducting bundles are situated in a chaotic way. At the periphery they are numerous but small, in the centre of the sprout they are solitary but bigger in size, which is typical for monocotyledonous plants. The location of the conducting bundles is sparsi-fascicular. Various stages of conducting bundles maturity can be noticed as well.

Floral axis and receptacle tissue are morphologically the organs of axile nature, which haven’t finished their embryonic development and haven’t lost their morphogenetic abilities (Batygina, 1983). In breeding of such geophytes as Allium ampeloprasum, Dichelostemma multiflorum, Eucrosia radiate, Gladiolus grandiflorus, Haemanthus coccineus, Hyacinthus orientalis, Narcissus tazetta, Nerine sariniensis, Ornithogalum dubium, Iris ensata, I. setosa, I. sanguinea [12-15], somatic tissue and flower organs (anthophore specifically) are often being used as explants of high regenerative capacity, instead of formative tissue.

As a result of the study of explant tissue it was marked that the generative sprout anatomy of I. sibirica (Berlin Ruffles breed) on the VI-VII stages of its organogenesis is typical for a plump body of monocotyledonous plants. At the same time, the tissue systems had a number of differences from those in a mature flower stalk. According to G. I. Rodionenko [16], cortex is cut off the stele by an annulus of pericycle sclerenchyma. We have established the fact that at this stage of floral axis differentiation lignification of pericycle cells and their metamorphosis into sclerenchyma haven’t taken place yet, and they have not lost the ability for division. On this account the first local sections of meristem have been noticed exactly in this tissue. According to the data presented by G. I. Rodionenko [16], flower stalks of the majority of blueflag breeds grow most intensively (up to 10-15 mm a day) in preanthesis period. Moreover, the intercalary growth of the generative sprout prevails,
that is the growth of body internodes in length due to the long lasting meristematic cell activity in their base (intercalary meristem). In our research work during the first 10 days of cultivation the size of explants was growing not only due to the cell distension, but to the extension of cortex parenphyma layer from 12 to 18 cellular rows. The explant diameter at this stage practically corresponded to the diameter of a flower stalk of intact plants of the same growth period. Centers of callus tissue were not visualized, neither outer, nor inner ones.

It is well known that the major part in a plump body of monocotyledonous plants is filled with a medulla. At this stage of explant evolution (VI-VII stages of organogenesis) the stele occupied 2/3 of the mutual volume, while the cortex occupied one third, respectively. As the floral shoot tissue of I. sibirica mature, the cells of the ground tissue become hollow, and at the same time a considerable quantity of stele cells completely fail, leading to the formation of a hollow floral shoot [16]. At the time of explantation the body of the floral shoot was plump.

The meristematic activity of I. sibirica explants (Berlin Ruffles breed) was being registered from the 7th day of cultivation, and was confined by the pericycle area. The first cellular septums were periclinally oriented. Under the integument of the initial cell a group of small cells appeared with dense cytoplasmic contents and a well-defined nucleus – a polyad (figure 2a). Having produced by its division some daughter cell, the cell regained its shape and size and remained invariably meristematic. Daughter cells, generated through the initial cells’ division, retained for a while meristematic abilities and propagated themselves. In this way centres of division arose in the explant. By the 10th day, the wave of cytocinesis extended along the boundary between the cortex and the stele, forming a continuous zone. At this stage the mass polyads and primordial sprouts rejuvenescence could be observed practically throughout the floral axis fragment’s perimeter (figure 2b).

**Figure 2a.** Polyads (x400), **2b.** Anatomical structure of the I. sibirica rachis explants 10th day (x100).

On the 14-17th day the evolving explants did not increase their size, yet some active processes were registered in all their tissue. The number of cortex parenchyma layers decreased from 18 to 12 ones, due to the involving of inner layers into building-up of meristematic centers. Polyads originated in bulk quantity from inner layers of cortex parenchyma cells, between the large division centers already in existence. At this time shoots appeared on the surface of the explant. System of tissue of the shoots of I. sibirica (Berlin Ruffles breed) in the floral axis’ explant is likely to be developing out of pericycle cells and cortex parenchyma cells both. The emergence of first signs of rhizogenesis and hemmogenesis on the explant’s surface was registered on the 20th day of cultivation. In 20 days of development, on the longitudinal section of the explant one could clearly see, that the newly formed due to division rootlet possess its own meristem and a spathella. The boundaries between the generated roots’ tissue systems were very distinct. The medial part of it was formed by stele with tightly attached to each other conductive elements, separated from the cortex by endodermis and surrounded by a clearly visible layer – pericycle. The growing primordium furrowed up its way
through the cortex of the maternal explant and moved outwards. When using a perianth fragments as an explant, we registered that only its adaxial side had a regenerative ability. During the whole period of cultivation signs of regeneration were not registered while the explant was put on the medium surface with its abaxial side.

Histologic research allowed to distinguish different level of regenerative activity of the inner and outer sides of the perianth of all the studied cultivars of I. sibirica.

The anatomical study of intact plants’ perianth of Iris genus representatives at the VI-VII stage of organogenesis showed that the abaxial side is covered by epidermis, constituted with smooth-wall cells of medium size and protective cuticle. This side of the perianth mainly consists of thin-walled parenchyma cells, which contain insertions, most probably starch grains. Parenchyma cells are tightly closed, some of them contain pigments in their enchylema. The conduction system consists of one large and a system of fine strings. The conducting bundles are collateral, sheltered. The tracheal elements have annulate and acyclic thickening, which indicates that the organ is elongating. Tha adaxial side is also covered with epidermis of smooth-wall tightly closed cells, but they are of a smaller diameter, than the abaxial side’s ones. The subepidermal layer consists of microcells, with a comparatively large nucleus each, and dense cytoplasmic contents. This is especially true for the first to the third, subepidermal outer layers of the cells. Those cells haven’t apparently lost the ability for division at this stage. Some division centres are clearly visible on the preparations. The parenchyma cells stand out for high content of starch grains.

As K. Essau registered [17], a leaf axis thickens due to the activity of a band of cells which are situated under the adaxial protodermis, called the adaxial meristem. According to this research, a perianth’s adaxial side also contains an adaxial meristem. That must take place due to the activity which thickens the perianth, while cellular division in the centres of intercalary meristems provide the organ’s lengthening. From the very first days of cultivation the growth of explants was registered. As the cells of epidermis and the subepidermal layers of the perianth’s adaxial side haven’t lost their meristematic activity at this stage of the plant evolution, the explant growth took place not only due to the cellular stretching, but to their division in these layers as well. This can be visually observed, as the adaxial surface becomes more and more uneven.

The morphological research allowed to record that the meristematic activity was at first bounded within 1-3 outer layers of the adaxial side only. As a result of division, a compact mass of smaller meristematic cells formed under the initial cell’s cytoderm – a polyad. In a while the process extended into the explant. The polyads’ existence can be various: a) each of them differentiate into a shoot cone; b) only some of them take part in shoot apex generation, c) destruction and, most probably, lysis of their coating leads to the generation of a mass of meristematic cells (“proembryo”cellular complex), out of which many shoots differentiate later on. On the 25th day of cultivation 100 per cent of the formed shoots gain corollaceous structures – floral elements instead of leaves’ primordium. Their epidermal layer is covered with a cuticle, parenchyma cells are large, with light cytoplasm, pigment contents and a stomatal mechanism. The anatomy of the floral elements reveals that a typical texture of the petals is presented by large polygonal elongated cells with large vacuoles with pigmented cellular fluid. It proves their floral nature.

In a while, these structures obtained typical coloration for flowers of the breed. The appearance of the floral elements in generative organs’ explants in vitro was described by a number of authors [18]. The perianths’ explants of all the studied blueflag genotypes maintained the ability for reproduction de novo of floral elements, regardless the phytohormones’ concentration within the radius of experience. The registered phenomenon of cyclic reproduction of generative structures can be interpreted as a result of regulatory gene expression, which trigger certain morphogenetic processes. The checked variants of growth medium with the contents of 4-8 mkM BAP and 3-5mkM NAA, started hemmogenesis of all the studied breeds and hybrids of I. sibirica. The perianth explants formed shoots which had a typical constitution for innovation shoots of monocotyledons (figure 1). Further cultivation of perianth explants with the aim of active proliferating culture production demanded a change in hormonal composition of growth medium. The contents of cytokine appeared to
be defining. For generation of normal mature shoots of I. sibirica the contents of BAP must be within the limits of 5-7.5mkM.

4. Conclusions
The type of morphogenic reaction and the speed of regeneration process in explants depended upon hormones concentration in the growth medium and the proportion of cytokinines to auxines. The anatomy of a generative sprouts of I. sibirica at the VI-VII stages of organogenesis is typical for a plump body of monocotyledons. The meristematic activity (polyads’ appearance) was registered from the 7th day of cultivation, and was bounded with the pericycle zone and inner layers of the cortex cells. In I. sibirica perianth explant, on the growth medium with 4-8 mkM BAP and 3-5mkM NAA the morphogenesis proceeded in accordance to hemmogenesis, omitting callusogenesis. The anatomy of the adaxial side of the perianth at the VI-VII stages of organogenesis differs from that of the abaxial side. The meristematic activity (polyads’ appearance) in I. sibirica perianth explants was registered from the 7th day of cultivation, and was registered only on the adaxial side. The difference was the floral elements generation instead of prophyllum primordium. The newly formed sprouts of floral axis and perianth’s explants of I. sibirica were solely of endogenous nature.

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