Comparison of Iris L. morphogenesis in in vitro culture with seedling development in some highly specialized plants

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Abstract. A sharp change in the course of ontogenesis and transition to another type of nutrition (parasitic nutrition) is supposed to contribute to similarity of the morphogenetic processes in in vitro plants and seedlings of higher parasitic plants. Tracheary elements are formed in the shoots of Iris in in vitro culture as an adaptive response of the plant body to culture conditions. Underdevelopment of the vascular system for transporting nutrients from the agar medium to plant tissues results in the formation of this vascular system in the maternal shoots. Macronutrients and plant hormones are transported to the zones of meristematic activity via the vascular system. Parasitic plants obtain nutrients from their hosts through the newly formed vascular system. The elements of tracheary structures are unique for haustoria and play a vital role not only in deposition and transport of macronutrients, but also in the hydrostatic pressure regulation and protection against pathogens. The vascular system of parasitic plants is disordered similar to that of regenerated plants. This vascular system can be referred to as a distribution network, the structure capable of transforming a set of cells into the cellular transport system, which unites tissues and organs into a single growing organism.

1. Introduction
Plants grown in tissue culture differ from conventional plants in that they are formed from the existing buds and somatic cells during somatic embryogenesis or organogenesis, depending on the type of explants and culture conditions. Roots, stems, and leaves in in vitro culture are formed due to interaction between the external and internal factors. The structure and functioning of regenerated plants indicate experimental conditions in tissue culture. These signals and the technique to direct the "chain reaction" of the plant development are still poorly known. Changes during this development are the sequence of events in time and space, and therefore any interference in the signal transmission can lead to negative consequences. Furthermore, explant isolation not only modifies the existing internal gradients, but also disrupts the tissue sensitivity to external signals, including signals that induce stress. The stress results in anatomical and morphological pathological changes in tissues and organs, causing changes in the physiological state of plant tissues [1-4].

The mechanism of the tissue contact between parasites and host plants has been described in details in the scientific literature. Haustorium differentiation correlates with the parasitic way of life, stage of its development, and degree of its interaction with the host tissue. The morphology and functional anatomy of haustoria reflect the mechanism of piercing through the host tissues and connection to the xylem. The processes of vascular differentiation are regulated by the hormonal relationship between...
parasites and hosts. The elements of tracheary structures are unique for the haustorium and play a special role not only in deposition and transport of macronutrients, but also in the hydrostatic pressure regulation and protection against pathogens [5-12]. These processes best studied for Orobanche are termed as "out of seed stage of embryogenesis". The stage is characterized by the unipolar development from the basal embryo pole. The terms "protosoma", "procaulom", "protocorm", and some others were introduced to describe a specific organization of the plant body during this stage. The protosoma enables the search and consumption of nourishment from the host plant, as noted by Teryokhin [7]. In addition, the problem of vegetative reproduction is often solved through budding.

In vitro morphogenesis and plant regeneration are the basis for the cell and tissue culture methodology [13]. The development of meristem technologies, embryo culture, haploid technologies, cell selection, and genetic and cell engineering is purely based on the knowledge of in vitro biology of cells [14-16]. According to our data, Iris L. is a favorable model to study plant morphogenesis in tissue culture.

The research aimed to compare the sequence of events during the development of Iris shoots in in vitro culture with that of parasitic plant seedlings.

2. Materials and methods

Plant materials. The regenerated plants of Iris sibirica L., I. ensata Thunb. utilized in the research were supplied by Plant Biotechnology Department, Altai State University (Barnaul).

Culture media conditions. The research was based on the conventional methods used in plant biotechnology [17]. The standard MS [18] medium was used to study the morphogenetic potency of organs and tissues under in vitro conditions.

To control the processes of in vitro morphogenesis of Iris, the culture medium was supplemented with the following phytohormones: 6-benzylaminopurine (BAP), Sigma, the U.S., and α-naphthaleneacetic acid (NAA), Sigma, the U.S. The sucrose at a concentration of 30 g/l served as a major carbohydrate for culturing organs and tissues.

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

Histological studies. A number of sections were made for anatomical study of morphogenetic processes. The permanent preparations were made by conventional methods (Barykina et al. 2004) in our modification.

Tissues and organs were fixed in a 10% formalin solution at room temperature. The plant material was processed in the automatic system for the rapid processing of histological specimens TPC 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 paraffin-embedded specimens were attached to cassettes. The specimens were cut on a rotary microtome, the obtained sections were spread flat in warm water (50–55 °C), and then mounted onto the appropriately labelled glass slides.

The basic method of staining histological specimens implied the use of the programmable continuous slide stainer TST-44 (Medite, Germany), with simultaneous application of hematoxylin and eosin. The total staining time was 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 images were obtained using a digital camera AxioCam MRe 5.
3. Results
Stage of micropropagation. The anatomical sections of I. sibirica shoots, grown on the MS medium containing 1.0 µM BAP, revealed tracheid-like cells, most of which were found in the pericycle region between the central cylinder and primary cortex. Strands extended deep into the central cylinder (figure 10). The formation of adventitious shoots and roots in the regenerated plants occurred in the central cylinder, namely the pericycle region. Perhaps due to this, the majority of tracheary elements were concentrated in this region.

The stage of actual micropropagation revealed the relationship between the cytokinin (BAP) concentration in culture media, intensity of the tracheary system formation, and shoot formation activity in I. ensata.

The multiplication coefficient was equal to 2.5 ± 0.1 on the medium containing 2.5–5.0 µM BAP (figure 1a). Vascular bundles in the central cylinder were surrounded by hydrocytes. It should be noted that the number of tracheid-like cells in the basal part of the shoot is greater than that in the apical part. Few adventitious shoot primordia could be observed, and adventitious roots were clearly seen as well. A massive accumulation of tracheary elements occurred in the pericycle region, in the root initiation area, and along the root central cylinder. These cells are conducting elements, and we can assume that this contributes to the relationship between the maternal shoot and the root. The sections exhibited tracheid-like cells, which accompanied the root from the central cylinder of the maternal shoot through the primary cortex tissues and further. These cells wrapped the root vascular bundle from the outside. Probably, the vascular system of a young root formed in vitro is not well developed, and hence tracheary elements transport nutrients and water to the roots (figure 1b).

In this embodiment, the multiplication coefficient on the medium containing 7.5 µM BAP varied from 1.0 to 3.0 and it was equal to 2.0 ± 0.1. The analysis of the rhizome anatomy revealed a denser accumulation of vascular bundles in the central cylinder due to the increased layer of the tracheid-like cells surrounding the bundle. The vascular bundle was surrounded by a ring of hydrocytes, and, according to our observations, the diameter of the bundle decreased (figure 1c). The formation of a higher number of hydrocytes may be associated with meristematic activity of the cells in the pericycle region. Since no roots occurred in the shoots, the forming zones of meristematic activity were primordial shoots, which needed more nutrients for development.

![Figure 1a](image1a.png)  ![Figure 1b](image1b.png)  ![Figure 1c](image1c.png)

**Figure 1a.** The appearance of shoots of I. ensata grown on the culture medium containing 5.0 µM BAP, 1b. Shoots of I. ensata grown on the medium containing 5.0 µM BAP (×100), 1c. A cross-section of the vascular bundle (× 400).

The increase in BAP concentration up to 10.0 µM led to massive shoot initiation, however primordial shoots did not develop or were very short. A cross-section revealed insignificant accumulation of vascular bundles closer to the apical part of the shoot. This is a younger part of the shoot that did not manifest a great number of hydrocytes around the bundles. A massive accumulation...
of zones of shoot formation activity was evident. The parenchyma cells of the central cylinder were intensely colored; large nuclei and inclusions, probably starch grains, were clearly observed. The multiplication coefficient in this embodiment was equal to 3.0±0.1.

The anatomical sections revealed a higher density of vascular bundles in the apical part of the shoot and the formation of a dense network of hydrocytes in the basal part of the shoot. At the same time, the rhizome exhibited a high degree of meristematic activity.

The shoots of *I. ensata* looked oppressed on the medium containing 15.0 µM BAP and their forms were often ugly and leaves were necrotic. The multiplication coefficient ranged within 1.4 ± 0.1, and the shoot height was equal to 68.0±11.4 mm. The anatomical sections revealed weak meristematic activity. Vascular bundles were surrounded by a mass of hydrocytes even in the apical part of the shoot. The cells of the central cylinder were mostly dead and had thickened walls.

The shoots of *I. ensata* growing on the culture media containing 17.5–20.0 µM BAP died after the second passage. The multiplication coefficient was equal to 1.0. The anatomical sections revealed massive development of the hydrocyte system. Tracheid-like cells ran through the shoot central cylinder from the basal part to the apical part. High concentrations of cytokinins caused variations in shoot morphology of *I. ensata*. In addition, the histological analysis revealed significant changes in rhizome tissues. The central cylinder with a hypertrophied conducting tissue occupied the main part of the rhizome cross-section. The toxic effect of high hormone concentrations might lead to proliferation of tracheary elements around the vascular bundles, which caused the bundle oppression and death of the parenchyma cells of the central cylinder. In these shoots, the rhizome section manifested the primary cortex as a friable cell layer.

In the shoots of grown on artificial media, the tracheid-like cells had pitted thickenings. These tracheary elements accompanied vascular bundles throughout the shoot as well as from the area where the adventitious root and maternal shoot merged. (figure 2).

![Figure 2](image-url)

**Figure 2.** Longitudinal section of *Iris sibirica* rhizome grown on medium with 6-BA (5.0 µM) (× 400): 1-tracheids, 2-vessels, 3-hydrocytes.

### 4. Discussion

Biennial shoots of *I. ensata* in in vitro culture have about 50 vascular bundles in the central cylinder. The bundle formation requires a certain time, but adventitious shoots are initiated at each passage (basis of micropropagation) and need nutrients and water for further development. In this case, the formation of tracheid-like cells is probably the most optimal for plants. As a result, regenerated plants have a complex vascular system of vascular bundles, containing sieve tubes and vessels, and hydrocytes. The tracheary elements of *I. ensata* intertwine with each other at nodes to form a fairly
dense tissue in the central cylinder parenchyma. The tracheid-like cells, living cells surrounded by living tissues of the shoot, are a powerful system to transport water and macronutrients, which enables the primordia of shoots and roots to quickly develop.

However, the process of hydrocyte propagation cannot be infinite. High levels of BAP result in the formation of excessive number of hydrocytes, which occupy the entire central cylinder, replacing parenchyma and inhibiting vascular bundles. The shoot undergoes water and nutrition stress; it looks flaccid and suffers a decline in tissue turgor. The plant no longer propagates and eventually dies. In case the shoots with a hypertrophic hydrocyte system are transferred onto the culture medium with low BAP concentration, or the culture scheme is changed, the anatomical structure of the shoot central cylinder becomes normal after several passages. The shoots begin to actively grow and propagate. The mass of adventitious shoots appear first, and then the active growth of the maternal specimen can be observed.

Assumption that the regenerated plants are similar to parasitic ones may contribute to the explanation of the occurrence of a mass of the tracheid-like cells. The literature investigated allowed us to make certain conclusions on the mechanism of the host-parasite relationship. Similar to the nutrition of the shoots formed in Iris explants, the nutrition of the developing parasites is completely heterotrophic. The nutrition is provided due to the contact between the haustorium and the host root vascular tissues as well as the development of the unusual vascular system in the tubercle, which structure is very similar to that in the studied species of different genera. Similar to that of explants, the system exhibits disorder: strands of the vascular elements in the central part of the tubercle, mainly short tracheids with reticulate and spiral thickenings of the wall, are randomly scattered in the main parenchyma. The researchers note that strands are arranged more or less independently and without a specific orientation, although all strands converge at the base of the tubercle and branch off towards the forming meristematic zones. In Orobanche hederae Duhy, the vascular tissue strands are not typical vascular bundles, they differ in size and shape, and represent short chains of tracheids (no vessels occur) and chains of phloem elements. It was hypothesized [19] that the tubercle vascular system exhibits this structure due to the fact that nutrients from the host plant are transported to numerous areas of active formation of the shoots and secondary haustoria. This is what can be observed in the explant with a network of irregular strands of tracheal elements (short tracheids), which development is induced by the formation of numerous meristematic regions, the initial zones of the shoot and root apexes. This vascular system can be referred to as a distribution network [20, 21], the structure capable of transforming a set of cells into the cellular transport system. Similar to the endoplasmic network, the structure is probably formed in the explants and callus-like tubercles as a transport system that unites cells into a single growing organism.

It should be noted that a sharp change in the course of ontogenesis and transition to another type of nutrition (parasitic nutrition) contribute to similarity of morphogenetic processes in in vitro plants and seedlings of highly parasitic plants. The formation of specialized organs and the nature of different tissues determine the potential of the plant survival. At the same time, parasites can exist only in the environment where modification of certain organ tissues is reasonable. If the conditions change, parasite plants die, and regenerated plants restore their normal anatomical structure after being planted in the soil.

5. Conclusions
Tracheal elements (hydrocytes) are formed in Iris shoots grown on the artificial culture medium as an adaptive response of the plant body to in vitro culture conditions. The vascular system is formed in the explants and shoots when the vascular system for transporting nutrients from the agar medium to plant tissues is underdeveloped. The system enables transport of macronutrients and hormonal growth regulators to the zones of meristematic activity. The developing parasites receive nutrition through the newly formed unusual vascular system. Similar to that of explants, the parasite system exhibits disorder: the strands of the conducting elements in the tubercle central part, mainly short tracheids with reticulate and spiral thickenings of the wall, are randomly scattered in the main parenchyma. The
similarity of morphogenetic processes in in vitro plants and in seedlings of higher parasitic plants may be due to a sharp change in the course of ontogenesis and transition to another type of nutrition (parasitic nutrition). The formation of specialized organs and the nature of different tissues contribute to the potential of plant survival. At the same time, parasites can exist only in the environment where modification of certain organ tissues is reasonable. Under changed conditions, parasites die, and the normal anatomic structure of the regenerated plants is restored after being planted in the soil.

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