Features of microclonal reproduction of species of the genus Aristolochia L. in various nutrient environments

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Abstract. The paper presents a comparative analysis of the development of microshoots in primary explants of the species belonging to the genus Aristolochia, using various nutrient media. This allows selecting the most optimal methods for mass production of the sterile regenerated plant cultures in a shorter time for further introduction, reintroduction, and creation of industrial plantations.

1. Introduction

The problems of plant biodiversity conservation in the context of the development of biotechnology and sustainable use of biological resources were first considered in the International Convention on Biological Diversity [2]. The use of biotechnological methods offers fundamentally new opportunities for the preservation and reproduction of the gene pool of plants. When creating genetic banks in vitro plant species, the primary explant is preferred as a seed material [3], [5].

Domestic research in the field of microclonal reproduction of the genus Kirkazon confirms that for liana, such as Aristolochia manshuriensis Kom., reproduction is carried out by activating the development of existing meristem [4]. It has also been established that the presence of Cytodef and Dropp preparations at a concentration of 0.01 mg/l in the composition of the nutrient medium stimulates the process of induction of the formation of adventitious buds, the formation of microshoots, and increases the multiplication factor [7], [8].

A comparative analysis of the development of microshoots in primary explants of species of the genus Aristolochia using various nutrient media is the purpose of the present study.

2. Materials and Methods

The following species of the genus Kirkazon are the objects of research for introduction into culture in vitro: A. manshuriensis Kom., A. macrophylla Lam., A. contorta Bunge, A. fimbriata Cham. & Schltdl, and A. clematitits L.

Experiments on microclonal reproduction were carried out in the period 2017-2018, in the Laboratory of Biotechnology of the Primorskaya State Agricultural Academy. After sterilization using the Butenko method [6], the cuttings of the studied types of Kirkazon were placed vertically on three variants of nutrient media.

The I nutrient medium is made on the basis of macro- and micro-salts according to MS-Murashige [10], with addition of casein hydrolyzate (80 mg/l), meso-inositol (50 mg/l), thiamine (0.5 mg/l), pyridoxine (0.5 mg/l), ascorbic acid (0.5 mg/l), kinetin (0.2 mg/l), and Indole-3-acetic acid (1.0 mg/l), pH is 4.49;
The II nutrient medium is made on the basis of macro and micro salts according to WPM [9], with the addition of nicotinic acid (0.5 mg/l), pyridoxine (0.5 mg/l), glycine (1.0 mg/l), 2-isopentenyladenine (8 mg/l), and Indole-3-acetic acid (4 mg/l); pH is 4.51;

The III nutrient medium is made on the basis of macro- and micro-salts according to MS (half content), with the addition of thiamine (0.5 mg/l), pyridoxine (0.5 mg/l), and ascorbic acid (0.5 mg/l); pH is 4.52.

At the first stage of the experiment, in connection with a small amount of starting material, the microcurrents of the following species A. trilobata L., A. sempervirens L., A. tomentosa Lam., A. maxima Jacq., A. littoralis Parodi. and A. gigantea Mart. & Zucc. were planted in the variant III of the nutrient medium, with the purpose of obtaining the required number of explants.

After obtaining the required number of micro-cages, in the second stage, the explants were transplanted to the (IV) and (V) variants of modified nutrient media containing macrosols according to the MS and WPM micro-salts (Woody Plant Medium). The staff of the Microclonal Reproduction Sector of Rare, Ornamental, and Agricultural Crops (A. V. Mikheeva and I. V. Gafitskaya) developed the basic medium composition [1] for the cultivation in vitro of rare and decorative rhododendron species and other woody and herbaceous plants.

In the IV variant of the medium, we used the Murashige macro-salts and WPM micro-salts with the addition of nicotinic acid (0.5 mg/l), pyridoxine (0.5 mg/l), glycine (1.0 mg/l), Indole-butyric acid (1.0 mg/l), sucrose (20 g/l); pH is 5.58.

The V nutrient medium is made on the basis of macrosols according to MS and micro salts according to WPM, with the addition of nicotinic acid (0.5 mg/l), pyridoxine (0.5 mg/l), glycine (1.0 mg/l), 2-isopentenyladenine (4 mg/l), and Indole-3-acetic acid (2 mg/l); sucrose (10 g/l); pH is 5.53.

The main stages of research are presented in Figure 1.

Figure 1. Diagram of the microclonal reproduction of species of the genus Aristolochia: (1) selecting the initial explant; (2) obtaining a sterile culture; (3) reproduction of microshoots (grafting) and transplantation of explants for IV and V variants of media; (4) the growth of the kidneys and the formation of micro shoots; (5) conversion of plants to greenhouse conditions.

3. Results
Our research (which took 4 weeks) allowed us to conduct a comparative analysis of the development of microshoots in primary explants of species of the genus Kirkazon (Table 1).
Table 1. Development of microshoots in primary explants of species of the genus *Aristolochia*.

| Species of the genus *Aristolochia* | Environment option |  |  |  |  |  |  |  |  |
|-----------------------------------|-------------------|---|---|---|---|---|---|---|---|
|                                   | I                | II | III | I    | II | III | I    | II | III |
|                                   | Microshoots age, weeks | Microshoots height, cm | Number of shoots, % | Microshoots age, weeks | Microshoots height, cm | Number of shoots, % | Microshoots age, weeks | Microshoots height, cm | Number of shoots, % |
| *A. manshuriensis Kom.*           | 2-3 to 0.9       | 10% | – | 2-3 to 0.5 | 25% |
| *A. macrophylla Lam.*             | 4 to 1.0         | 6% | – | 2-3 to 0.8 | 5% |
| *A. contorta Bunge*               | 2-3 to 0.5       | 20% | 2-3 to 0.6 | 45% | 2-3 to 0.6 | 36% |
| *A. fimbriata Cham. & Schltdl.*  | 3-4 to 0.5       | 40% | 5 to 0.5 | 50% | 2 to 0.5 | 50% |
| *A. clematitis L.*               | 2 to 2.5         | 83% | 2 to 0.3 | 17% | 2 to 3.0 | 83% |

For grassy non-climbing species *A. fimbriata Cham. & Schltdl.* and *A. clematitis L.*, the highest number of shoots in all variants was observed, especially high rates were found in the mediums No. I and III. At the same time, for woody *A. manshuriensis Kom.*, *A. macrophylla Lam.*, the number of shoots is small, and in the II medium variant, the shoots did not develop. Interestingly, *A. contorta Bunge*, being a grassy liana, occupies an intermediate position in terms of lignescent vines and grassy non-climbing species on I and III medium variants; at the same time, the largest number of shoots of a species is recorded on medium II. The maximum height of the shoots (2.5–3.0 cm) was noted in *A. clematitis L.* on I and III variants of nutrient media. In the remaining cultivated species of *Aristolochia* L., this figure ranged from 0.3 to 0.9 cm (Fig. 2).

Figure 2. Micro shoots of species of the genus *Aristolochia* L.: on the right is *A. Manshuriensis Kom.*; on the left is *A. clematitis L.*

After practicing the method of introducing 4 species of the genus *Aristolochia* L. into culture, the experiment was supplemented with 6 more species in 2018: *A. trilobata* L., *A. sempervirens* L., *A. littoralis* Parodi, *A. maxima* Jacq., *A. tomentosa* Sims and *A. gigantea* Mart. & Zucc. In 2018, the cultivation period of the above-mentioned species averaged two months. The main results on the cultivation of species are presented in Table 2.

In newly introduced species, the formation of vegetative shoots bearing leaves at the base was recorded after 3 weeks. The maximum height of microshoots was noted in *A. sempervirens* L. (4.6 cm.). The minimum was for in *A. trilobata* L. (0.5 cm). In most species, elongation of micro-shoots is observed only by the end of the 6th week, disclosure of leaf blades occurs very slowly, their deformation (blackening of edges, yellowing of the entire surface) is observed in some cases. In *A. sempervirens* L., in comparison with the other studied species of the genus *Kirkazon*, a more intensive development of microshoots was noted. Preliminary results showed that the species such as *A.*
trilobata L. and A. littoralis Parodi, the formation of aerial roots is typical already at the end of the first and beginning of the second week of cultivation in 6-10% of cases.

In explants of A. maxima Jacq., during the entire cultivation period, the callus developed at the base, but the differentiation of the roots did not occur (Fig. 3).

![Figure 3. Microcurrents Aristolochia maxima Jacq.](image)

Cultivation of explants on a hormone-free nutrient medium in vitro contributed to a change in morphology. This was manifested in an increase in the internal tissues, i.e., a change in their structure (gelatinous mass of dark color) and a rupture of the outer integumentary tissues.

According to the preliminary results of cultivation of this species, a repeat of the experiment is planned for a more detailed study of the response of the A. maxima Jacq explants on the composition of the nutrient medium and sending material for cytological examination. This will allow to identify exactly which plant tissues respond in this way to micropropagation in vitro. In some cases, there was a staining of the nutrient medium in brown or almost black color and the blackening of the microcurrents. In this case, the release of phenols by the plant into the nutrient medium contributes to this, which leads to a change in its basic properties and undesirable consequences for the explant itself.

| Number of microshoots, pcs; including rooted, pcs | Dimensions of microshoots, cm: (3 weeks) | Sizes of primary sheet, (3 weeks) | Sizes of primary sheet, (6 weeks) | Number of microshoots removed from the experiment, pcs |
|--------------------------------------------------|------------------------------------------|--------------------------------|--------------------------------|------------------------------------------------------|
| 50 (82% (6%))                                     | 0.5 1.1 0.4x0.4 0.8x1.1                   |                                |                                | 9 (8%)                                               |
| 40 (82.5%)                                        | 0.3 1.4 0.6x0.5 1.0x1.0                   |                                |                                | 7 (7.5%)                                             |
| 40 (82%)                                          | 0.5 1.7 0.6x0.5 1.0x0.7                   |                                |                                | 7                                                    |

Table 2. Cultivation results of species of the genus Aristolochia L.
As a result of cultivation of *A. tomentosa* Sims, elongation of microshoots was observed only in 7 out of 20 explants (Fig. 4). Some micro shoots had a yellowish tint. The remaining 13 shoots did not show significant changes after 6 weeks of cultivation, except for staining the nutrient medium in a dark yellowish-brown tint. *Aristolochia tomentosa* Sims and *A. maxima* Jacq. woody. Bud formation was recorded in the 1st explant in the second month of cultivation. This indicates that at the time of planting in a nutrient medium, the generative kidney was already laid in this explant.

Figure 4. Micro-shoots of *Aristolochia tomentosa* L. is on the right, and the bud formation is on the left.

To continue the experiment on microclonal propagation, sterile micro shoots after preliminary grafting were transplanted into two variants of the nutrient medium. The variant IV contains substances stimulating rhizogenesis in the *in vitro* micro-vegetative shoots. And the variant V contains those stimulants that cause an intensive growth of meristem tissue and the formation of several micro-shoots on the 1st explant. The results of this part of the experiment are shown in Table 3.

The total number of microplants planted in greenhouse conditions was 31.87% of the total number of secondary explants used in the experiment. 25.63% of them rooted on the IV variant of the nutrient medium. Higher rates of rhizogenesis in microchips according to the results of the experiment were recorded in *A. littoralis Parodi* (62.5% of those used in the experiment), low results were recorded in *A. maxima* Jacq. (5%). In the secondary explants of *A. sempervirens* L. and *A. tomentosa* Sims, rhizogenesis was not recorded. In *A. tomentosa* L., a more intensive release of phenols into nutrient media and darkening of microcurrents was observed. During the growing period from January 4 to March 4, 2018, the survival rate of microplants in *A. littoralis Parodi* and *A. trilobata* L. was 100% in greenhouse conditions. The experiment on microclonal reproduction of these species must be
repeated to clarify the causes of the death of microplants *A. maxima* Jacq. and *A. gigantea* Mart. & Zucc during acclimatization to greenhouse conditions.

### Table 3. The results of the cultivation of secondary explants of the genus *Aristolochia* L.

| Landing options | A number of explants on the medium variant | A total number of explants, pcs |
|------------------|------------------------------------------|---------------------------------|
|                  | IV                                       | V                              |
| *A. gigantea* Mart. & Zucc | 20                                       | 20                             | 40 |
| Closed ground    | 8                                        | -                              | 8  |
| *A. littoralis* Parodi | 20                                       | 20                             | 40 |
| Closed ground    | 18                                       | 7                              | 25 |
| *A. maxima* Jacq. | 20                                       | 20                             | 40 |
| Closed ground    | 2                                        | -                              | 2  |
| *A. trilobata* L. | 20                                       | 20                             | 40 |
| Closed ground    | 13                                       | 3                              | 16 |

3. Conclusion

Comparative analysis of the development of microshoots in primary explants of species of the genus *Aristolochia* using various nutrient media allows, in a shorter time, to select the most optimal methods for mass production of sterile regenerated plant cultures, with the aim of further introduction, reintroduction, and the creation of industrial plantations. We believe that this method of breeding of species of the genus *Aristolochia* L., including *A. manshuriensis* Kom., is the most effective, because the technology for producing a large number of plants allows to constantly update the *Kirkazon* plantings in greenhouses, plantations, and other conditions of the artificially restored populations.

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