Features of Microclonal Reproduction and Organogenesis of the Far Eastern Species *Aristolochia Manshuriensis Kom.* as a Biological Resource

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Abstract. Plants of Manchurian birthwort (*Aristolochia manshuriensis Kom*), obtained under in vitro conditions, are characterized by a high potential for accelerated reproduction by weakening the action of their growth hormones during a certain period of phenological development and as a result of a response to the presence of 1mg/l IMK in the nutrient medium. Thus, the microplants can adapt to the conditions of the closed soil, while the total percentage of survival is 49.12%.

Keywords: Birthwort Manchurian · Nutrient medium · Rizogenesis · Organogenesis

1. Introduction

The preservation of biological resources is due to the need for an alternative search for herbal medicines. Most of the plants preserved in the Far East flora of the Russian Federation in the process of evolution managed to maintain a combination of poisonous and medicinal properties. First of all, plants' toxic properties are necessary for the survival of relics in current conditions. The adaptation of pathogenic flora and fauna to modern means and methods of combating it is continually increasing. However, ancient plants' life processes' chemical composition is a deterrent to most pathogenic microorganisms during their growth and development. At the time of the death of individual parts of the plant, this factor is lost.

Genus Birthwort (*Aristolochia L.*) – represented by more than 450 species of tropical and subtropical plants, and only a few species are distributed to temperate latitudes. In the Russian Far East, only two species of birthwort have survived – Manchurian (*A. manshuriensis Kom.*) and twisted (*Aristolochia contorta Bunge*), lomonosovid (*Aristolochia clematitis L.*) [1, 2].

Birthwort Manchurian – a woody liana, in Russia, it is represented by several small populations in the south of Primorsky Krai [9]. This species is listed in the Red Book of Primorsky Krai with endangered (EN) [4].

Natural plant reserves *Aristolochia manshuriensis Kom.* not enough to conduct in-depth pharmacological and chemical research. Stocks of cultivated, there are practically no individuals. Only individual specimens grow in several botanical gardens of the Russian Federation.

Studies by the number of employees of the Federal Research Center for the Conservation of Biodiversity of the Terrestrial Biota of East Asia BPI DVO RAS [8] proved the feasibility of preserving this population species.
Being a unique natural source of many chemicals (aristolochiam and other acids (p-cumaric, ferulic, p-hydroxybenzoic, etc.), alkaloids, and steroids are essential oils, antioxidants, etc.), the nature of which remains completely unexplored, birthwort deserves special attention from practical pharmacology. Also, having several medicinal properties (immunostimulating, antifungal, antitumor, antibacterial, etc.), birthwort can use analog to artificially obtained drugs [8].

Study of the features of microtional reproduction and organogenesis of the Far Eastern species Aristolochia manshuriensis Kom. as a biological resource – the present study's purpose.

2. Materials and Methods
The object of the study is a species of the genus birthwort: Aristolochia manshuriensis Kom. (Birthwort Manchurian) (figure 1). These are plants of 7–8 years of age growing in artificial plantations of an arboretum belonging to the Branch of the Federal State Budgetary Institution “Federal Scientific Center for Terrestrial Biota of East Asia” of the Far Eastern Branch of the Russian Academy of Sciences “Gornotaezhnaya Station named after V. L. Komarov.”

Figure 1. Lianas Aristolochia manshuriensis. Source: Compiled by the authors.

Experiments on clonal micro division were carried out in the growing period 2018–2019 in the biotechnology laboratory of FSBOU VO Primorskaya GSHA.

The work was carried out in 3 stages. Each stage consisted of 9 variants of nutrient media and three repetitions for each variant.

In each repetition were examined for ten explants. In total, 270 explants were investigated at each of the stages of the experiment. Microchips were prepared as follows:

In the first decades of June, July, and August, non-wood shoots 1.5 cm long, and 0.3 cm in diameter with one (two) axillary buds were cut off from birthwort plants. Mercury (II) chloride at a concentration of 0.2% (5 min) was used as a sterilizing agent. Then the microchips were washed three times with autoclaved distilled water. After decontamination, the primary explants (cuttings) were vertically placed on three nutrient media variants. Each medium had three modifications:

Ia – based on macro- and microsalts on MS [7] with addition of a hydrolyzate of casein – 80 mg/l, a meso-inositol – 50 mg/l, thiamine – 0.5 mg/l, a pyridoxine – 0.5 mg/l, ascorbic acid – 0.5 mg/l, a kinetina – 0.2 mg/l and 3-indolylacetic acid – 0.5 mg/l, environment 4.49 pH;

Ib – 3-indolylacetic acid – 1.0 mg/l;

Ic – 3-indolylacetic acid – 1.5 mg/l. [5] with addition of nicotinic acid – 0.5 mg/l, pyridoxine – 0.5 mg/l, glycine – 1.0 mg/l, 2-isopentenyladenine – 8 mg/l and 3-indolylacetic acid – 4 mg/l; pH media 4.51;

IIa – 2-isopentenyladenine – 4 mg/l and 3-indolylacetic acid – 8 mg/l;

IIb – 2-isopentenyladenine – 4 mg/l and 3-indolylacetic acid – 4 mg/l.

IIIa – WPM macro- and microsoles [5] with addition of nicotinic acid – 1.0 mg/l, pyridoxine – 1.0 mg/l, glycine – 1.0 mg/l, indolyl butyric acid – 0.5 mg/l; pH media 4.53;
ill butyric acid – 1.0 mg/l; 
IIIc – indolyl butyric acid – 1.5 mg/l.

The nutrients were poured into tubes with a 10 mm diameter and a height of 15 cm and sterilized by autoclaving at 1.2 atm for 12 minutes. Explants at all stages of the experiment were cultured at a temperature of +24 °C, a 16-hour photoperiod (16/8), illumination with white fluorescent lamps with an intensity of 6.5 thousand lux. and 60% relative air humidity.

3. Results and Discussion

The work on the clonal microdivision of the species is not quite perfect due to its biological characteristics. Molkanova O. Yi, et al. [6] indicates that it is most useful to use apical and lateral kidneys isolated from plants of immature and virgin age states. Optimal 1/2 MS culture medium containing 3 mg/l indolyl butyric acid (hereinafter referred to as IMC). With this method, it takes an average of 4 weeks to obtain micro-plants and 3–4 weeks to adapt them to soil conditions, while the first three weeks are the most critical [6].

According to the research of T. T. Doan [3], it was found that for the Birthwort liana of Manchurian, reproduction is carried out by activating the development of existing meristems. Cytodef and Dropp preparations in the nutrient medium at a concentration of 0.01 mg/l stimulate the formation of adventitious kidneys, the shape of microbugs, and increases the reproduction coefficient.

At the stage of introducing cuttings into the culture, observation of the development of sinus kidneys was carried out daily. Daily passages were performed for two weeks to prevent phenols' accumulation secreted from explants in the medium. Cuttings with visible signs of manifested infection were subjected to secondary sterilization in the same sequence. During this period, the development of sinus kidneys was not recorded. The appearance of the first shoots was noted at 3–4 weeks of cultivation. The maximum number of nodes was formed in July in version III of the medium containing IMC at a concentration of 1 mg/l (Table 1). Similar figures for the previous month and the following month were relatively lower.

The presence of indolyl acetic acid (hereinafter referred to as IAC) in the media at concentrations of 1 and 4 mg/L did not stimulate the formation of histogenesis at all stages of the experiment. The combination of IUK and 2-isopentyl adenine (starting now – 2ip) in all versions enabled an increase in callus tissue volume at the lower base of the cuttings. The IMC content of 1 mg/L was optimal for root formation in most birthwort micro-plants (figure 2).

![Figure 2](image.png)  
Figure 2. Sinus escape, enlargement of callus tissue at the base of the flight, risogenesis (left to right). Source: Compiled by the authors.
The results of in vitro culture of birthwort microferenks are shown in table 1.

| Environment option | June | July | August |
|--------------------|------|------|--------|
| Ia                 | 10/10/10 | 5/2/7 | OK     |
| Ib                 | 10/10/10 | 4/6/9 | OK     |
| Iv                 | 10/10/10 | 5/5/6 | OP     |
| Ia                 | 10/10/10 | 4/0/7 | UK     |
| Ib                 | 10/10/10 | 3/2/5 | OK     |
| Iv                 | 10/10/10 | 2/0/2 | OP     |
| Ia                 | 10/10/10 | 7/5/2 | UK     |
| Ib                 | 10/10/10 | 8/1/6 | OK     |
| Iv                 | 10/10/10 | 1/3/2 | OP     |
| Ia                 | 10/10/10 | 4/5/2 | UK     |
| Ib                 | 10/10/10 | 1/1/6 | OK     |
| Ia                 | 10/10/10 | 9/10/9 | OP |
| Ib                 | 10/10/10 | 9/9/8 | UK     |
| Ia                 | 10/10/10 | 10/10/10 | OK |
| Ib                 | 10/10/10 | 10/10/10 | OK |
| Ia                 | 10/10/10 | 8/7/5 | UK     |
| Ib                 | 10/10/10 | 8/5/3 | OK     |
| Ia                 | 10/10/10 | 10/10/10 | OK |

Table data analysis is shown in the diagram (figure 3). In the research, we found that variant I of the medium is most optimal for the formation of shoots from the sinus kidneys of cuttings cut in the beginning and middle of the growing season.

The highest ability to rhizogenesis was noted in microchalks prepared for cultivation in the first decade of July. During this period, the percentage of rooting is 80–94%. The reasons for the lack of ability to root formation in microcherenks in other cases of our experiments were not revealed.

Analysis of the obtained data shows that at the beginning of the phase of the liana's active growth, to stimulate the growth processes of sinus kidneys in explants of Manchurianian birthwort, it is enough to add 0.5–1.5 mg/l to the IUK medium. Simultaneously, the enhancement of the action of IUK with hormone 2ip leads to a slight increase in the number of sinus shoots. And replacing the IUK with IMK dramatically increases the formation and growth of sinus shoots.

**Note:** OK – total number of explants in repetitions; OP – total number of viable escapes; CC – number of entrenched explants. **Source:** Compiled by the authors.

![Figure 3. The plot of sinus shoots formation under in vitro conditions](source: Compiled by the authors.)

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In the middle of the growing season, the effect of own hormones on plants is significantly reduced. During this period, the content in the medium of only IMC is insufficient. Simultaneously, the enhancement of the action of IUK with hormone 2ip leads to a slight increase in the number of sinus shoots. And replacing the IUK with IMK dramatically increases the formation and growth of sinus shoots.
However, at the end of the growing season, the effect of all the hormones used is practically equalized. Simultaneously, in the middle of the vegetation phase (July), the use of IMC in conjunction with the partial termination of its growth hormones in explants leads to the formation of rhizogenesis.

Adaptation of test plants to closed soil conditions was carried out in laboratory conditions of FSBOU VO Primorsky GSHA. The soil used was a mixture of soil and sand in a ratio of 3:1. The test plants were planted in plastic containers (figure 4), rinsed the soil surface with a pulverizer, and closed tightly with a lid.

Watering of micro plants was carried out by shaking water droplets from the lid's inner surface onto the soil inside the container. After 45 days, the plants were transplanted into individual reforestation containers.

Figure 4. Micro plants of birthwort when grown in a container. Source: Compiled by the authors.

During the period of research, we planted 57 micro-plants. The engraftability under conditions of the closed container was not more than 68.42%, of which 49.12% were transferred to individual refilling containers. After 5.5 months, the height of plants, on average, taking into account the root system, was 12.5–15.5 cm, excluding the root system – 5.5–8.5 cm (figure 5).

Figure 5. Birthwort plants at the age of 5.5–6 months. Source: Compiled by the authors.

The total average plant growth in the first month was from 2.5 to 3.7 cm. In subsequent months, it did not exceed 2.8 cm. The formed plants had the correct shape of a leaf of saturated green color, with darker veins. The stem had a yellowish tint in some places – up to brown. The root system with a pronounced rod root and asymmetric branches of pale yellow is almost white in areas.
4. Conclusion
Our studies have proved that the plants of Manchurian birthwort (*Aristolochia manshuriensis Kom*), obtained under in vitro conditions, are characterized by a high potential for accelerated reproduction by weakening the action of their growth hormones during a certain period of phenological development and as a result of a response to the presence of 1mg/l IMK in the nutrient medium. Thus, the microplants can adapt to the conditions of the closed soil, while the total percentage of survival is 49.12%.

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