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RESEARCH PAPER

In vitro propagation of herbaceous species of the *Aristolochia* genus

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ABSTRACT

This report provides the results of microclonal propagation of nine herbaceous species of the genus *Aristolochia* from to the sections *Diplolobus* and *Gymnolobus*. Each of the species has medicinal properties. For the cultivation, Murashige and Skoog medium was used, a hormone-free nutrient medium with a half concentration of macro- and micronutrients and iron chelate. *Aristolochia clematitis* was characterized by the highest growth rate, and *A. baetica* by the lowest rate. Explants of *A. rotunda* showed the highest potential for propagation (with the propagation coefficient equal to 7.5), and *A. gigantea* had the lowest potential (3.32), which was associated with the growth characteristics of the species under study. The obtained results of successful propagation allow simplification of the existing protocols. The use of the nutrient medium of modified composition is expected to reduce material and labor costs in implementation of programs for conservation of valuable medicinal plant resources.

Keywords: microcloning, reproduction, rare species, conservation, cultivation conditions, *Aristolochia*, *in vitro*

РЕЗЮМЕ

Наконечная О.В., Юсупова Е.П., Волконская В.В., Гафицкая И.В. Размножение *in vitro* травянистых видов рода *Aristolochia*. Представлены результаты по микроклональному размножению 9 травянистых видов рода *Aristolochia*, относящихся к секциям *Diplolobus* и *Gymnolobus*. Каждый из видов обладает лекарственными свойствами. Для культивирования использовали безгормональную питательную среду с половинной концентрацией макро- и микроэлементов и хелата железа по Мурасиге и Скугу. Показано, что наибольшая скорость роста характерна для *Aristolochia clematitis*, наименьшая для *A. baetica*. Наибольшим потенциалом к размножению обладали эксплланты *A. rotunda* (коэффициент размножения равен 7,5 шт.), наименьшим – *A. gigantea* (3,32 шт.), что связано с особенностями роста каждого исследованного вида. Полученные результаты успешного размножения позволили упростить существовавшие протоколы. Применение разработанного состава питательной среды позволит сократить материальные и трудовые затраты при реализации программ по сохранению ценных лекарственных ресурсов.

Ключевые слова: микроклонирование, размножение, редкий вид, сохранение, условия культивирования, *Aristolochia*, *in vitro*

Species of the genus *Aristolochia* L. (Aristolochiaceae Juss.) are vines, shrubs, and rhizomatous herbs (Endress 1990, 1994, Razzak et al. 1992) growing in the tropical, subtropical and temperate zones of both hemispheres (Kharkevich 1987, González & Stevenson 2002, Kelly & González 2003). All these plants have medicinal properties (Zhou et al. 2011). Their confinement to a narrow ecological niche, the degradation of their natural habitats, the difficulties in seed propagation due to the specifics of pollination, and the complicated vegetative reproduction have led to a decrease in their abundance in natural populations (Borah & Sarma 2022, Murugan et al. 2006, Nesterova 2008, Gong et al. 2018, Ward et al. 2003, Yu et al. 2021).

Modern technologies allow mass propagation of plants whose natural populations are declining. One of the most promising is the microclonal propagation method. Previously, this approach was successfully applied to propagate some of *Aristolochia* species (Manjula et al. 1997, Siddique et al. 2006, Biswas et al. 2007, Osuna et al. 2007, Veluchamy & Rajappan 2008, Saidi et al. 2009, Sathish et al. 2011, Remya et al. 2013, Sarma & Tanti 2017, Molkanova et al. 2018, Nakonechnaya et al. 2024). Nevertheless, optimization of protocols (simplification and selection of conditions for rapid cultivation) is still an urgent issue to address. For this reason, in the present study, we aimed to optimize the methods for *in vitro* propagation of herbaceous species of the genus *Aristolochia*.

MATERIAL AND METHODS

The studies were conducted at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences (FSCEATB FEB RAS, Vladivostok), in the sector of microclonal propagation of forest, agricultural and ornamental plants in 2023–2024.

In the study, we used nine species from two sections: *Diplolobus* (*A. baetica* L., *A. clematitis* L., *A. contorta* Bunge, *A. rotunda* L., *A. sempervirens* L., and *A. tuberosa* C.F. Liang & S.M. Hwang) and *Gymnolobus* (*A. gigantea* Mart. & Zucc., *A. fimbriata* Cham., and *A. maxima* Jacq.). We took a microcutting with a bud as explant. The experiment was based on 20 explants of each species preliminarily introduced to *in vitro* conditions. For

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cultivation, we used Murashige and Skoog medium, a hormone-free nutrient medium with a half concentration of macro- and microelements and iron chelate (½ MS, Murashige & Skoog 1962). To identify the dynamics of development, we measured microplant height, counted number of leaf nodes, roots, and estimated propagation coefficient and proportion of explants with callus after eight weeks of cultivation. The number of microcuttings obtained from one microshoot per passage was defined as a propagation coefficient. The data obtained was processed using the Microsoft Office Excel software package. The experiment was carried out in triplicate. The values of the growth parameters in Fig. 1 and Table 1 are expressed as arithmetic mean ± standard error (n = 30).

RESULTS

According to the results we obtained, the explants of most species of the genus, except *A. maxima* and *A. contorta*, successfully grew on the hormone-free nutrient medium with a half concentration of micro- and macro-salts.

An analysis of the results showed that the initiation of bud growth in most species occurred at the second week of cultivation, and the root formation began after three weeks of cultivation. Of particular note was the observed monopodial growth of the apical meristem.

By the end of the cultivation period, microplants of *A. clematitis* were characterized by the maximum height, greater than 55 mm (Fig. 1A). Slightly lower values of this parameter were recorded for *A. fimbriata* and *A. rotunda*. These species showed the maximum values of the "number of leaf nodes" parameter (Fig. 1B). Microplants of the other species grew more slowly. The lowest microplants with minimum numbers of leaf nodes were *A. gigantea*, *A. baetica*, and *A. tuberosa*.

The number of leaf nodes in the species of the genus determined the number of micro-cuttings and, consequently, the propagation coefficient. Since the maximum values of the parameter were recorded from *A. rotunda* and *A. clematitis* (Fig. 1B), the propagation coefficient was the highest (Table 1). Consequently, microplants of *A. rotunda* showed the greatest propagation potential, while those of *A. gigantea* had the lowest potential.

The timing of callus formation on the wound surface varied between the species. Callus often began to form a week after passage and was represented by a few cells. Explants of *A. fimbriata* showed a higher tendency to callus formation, and their callus cells did not prevent both the transport of nutrients from the medium through conductive tissues to the bud and the root formation. The callus formation was least frequently observed in *A. sempervirens* (Table 1). The root formation often began about a week after the callus formation. In all the explants that had formed roots, microshoots were also developed, which suggested obtaining of fully viable microplants (Fig. 1C, Fig. 2). The root formation also varied between the species. The highest percentage of microplants was observed in *A. tuberosa* (Fig. 1C). Explants of *A. baetica* showed a lower tendency to form roots than others. In terms of number of roots per explants, the highest values were recorded from *A. clematitis*, and the lowest values from *A. baetica* (Table 1).

Table 1. Morphometric characteristics of seven *Aristolochia* species after eight weeks of cultivation

***Aristolochia* species**	**Propagation coefficient**	**Proportion of explants with callus**	**Number of roots, pcs.**
A. baetica	4.54	45.6±3.06	2.14±0.40
A. clematitis	6.48	53.77±18.48	5.94±0.72
A. fimbriata	5.07	93.5±6.5	4.15±0.33
A. gigantea	3.32	76.78±2.97	4.73±0.42
A. rotunda	7.5	64.58±10.28	3.28±0.29
A. sempervirens	5.19	41.43±11.43	3.43±0.54
A. tuberosa	4.18	86.31±6.21	4.91±0.33

DISCUSSION

As was reported earlier, a nodal segment culture of *A. gigantea* (Lucio et al. 2009) cultivated on Murashige and Skoog (MS) medium supplemented with indole-3-acetic acid (IAA, 0.52 μM) and 2.32 μM kinetin (Kin). The best results of the microshoot elongation were obtained with the use of an indole acetic acid (IAA) concentration of 0.52 μM (3.2 cm). The highest rooting percentage (80 %) and the number of roots (2.4 roots/explant) were promoted by exposure to 0.52 μM IAA and 0.49 μM indole-3-byturic acid (IBA) (Lucio et al. 2009).

In the cultivation of *A. rotunda* (Gatti & Vecchi 2017), Schenk & Hildebrandt (SH) medium (Shenk & Hildebrandt 1972) was used for development initiation, with subsequent transfer to the MS nutrient medium supplemented with 6-Benzylaminopurine (BAP, at 0, 4.4, 8.8, 17.6, and 26.4 μM) and IBA

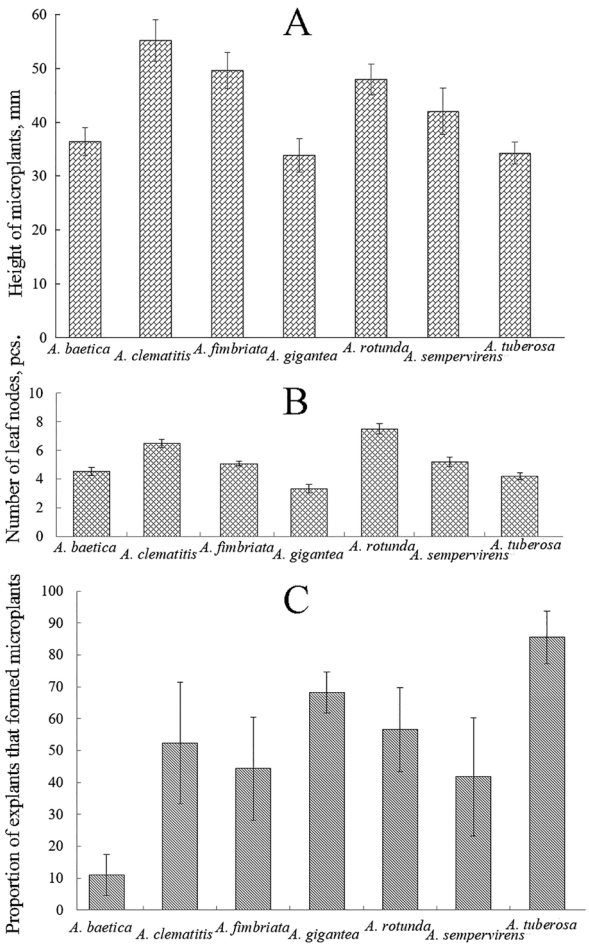

Figure 1 Morphometric characteristics of *Aristolochia* species after eight weeks of cultivation. A – height of microplants; B – number of leaf nodes in microplants; C – proportion of explants in different *Aristolochia* species that formed microplants

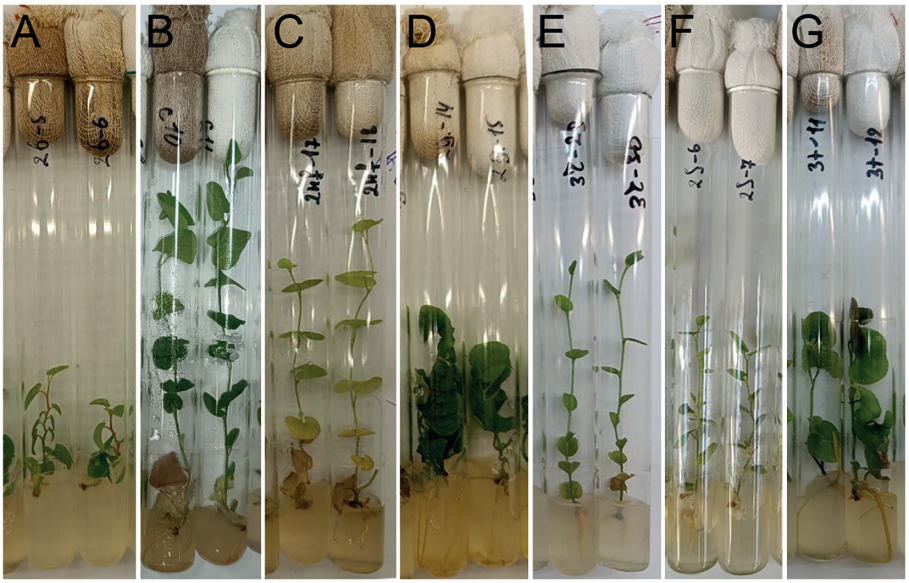

Figure 2 Microplants of *Aristolochia* species after eight weeks of cultivation: A – *A. baetica*; B – *A. clematitis*; C – *A. fimbriata*; D – *A. gigantea*; E – *A. rotunda*; F – *A. sempervirens*; G – *A. tuberosa*

(0.5 μM). The maximum values of microshoot height (16.33 ± 1.18 mm) were obtained with exposure to 4.4 μM BAP after 3 weeks of cultivation. The maximum values of microshoot length were obtained with 8.8 μM BAP (Gatti & Vecchi 2017).

In our study, explants of *A. gigantea* successfully grew on the ½MS hormone-free medium, formed up to five leaf nodes and more than four roots, and also successfully adapted to the closed soil conditions. Microshoots of *A. rotunda* reached 120 mm when cultivated on the ½MS hormone-free medium. A similar positive result of using this composition of nutrient medium was obtained in cultivation of other herbaceous representatives of *Aristolochia* under study (*A. baetica*, *A. clematitis*, *A. sempervirens*, *A. trilobata*, and *A. tuberosa*) that were introduced to *in vitro* culture for the first time.

It should be noted that plants of this genus do not always successfully grow on nutrient media with the same composition. As was previously shown, representatives of the section *Siphisia* (Nakonechnaya et al. 2024) did not grow on ½MS. For their growth, the MS medium supplemented with 0.5 mg/L BAP was required, i.e., an additional hormonal signal to activate bud development. Furthermore, a nutrient medium of ½WPM (Woody Plant Medium) (Lloyd & McCown 1980) supplemented with IBA was necessary for their rooting. In the present experiments, explants of *A. maxima* and *A. contorta* did not develop on the hormone-free nutrient medium, as well as most of the species we studied. They probably needed additional substances for growth, including, necessarily, plant hormones.

According to the analysis of our results, microplants of the species of this genus grew at different rates and, consequently, had significant height differences by the end of the experiments. Their growth may apparently depend on the life form. Thus, *A. clematitis* and *A. fimbriata* are herbaceous shrubs, while the life form of the rest of the species is vine. Furthermore, the genetic component cannot be ruled out as well.

It is likely that the ontogeny of the species may also have an effect on the rate of microshoot development on the explant. For example, *A. rotunda* is a herbaceous plant up to 30 cm tall with a life cycle of up to 3 yr. The plant enters the generative phase as early as at 2 months after being planted on closed soil (unpublished data). Other representatives of the sections *Diplolobus* and *Gymnolobus* exhibit longer life cycles (more than 10 yr) and extended ontogeny states compared to *A. rotunda* (Davidyuk 1974, Nakonechnaya et al. 2015).

Organogenesis processes may have a certain effect on the rate of explant development as well. Thus, by the end of the first month of cultivation, roots in the explants of the species from the section *Gymnolobus* were already formed, which contributed to enhanced nutrition of microshoots compared to those of the species from the section *Diplolobus* whose roots had not yet developed by that time.

Thus, development of plants *in vitro* is influenced by a multitude of factors (internal and external) each of which can become decisive.

CONCLUSION

As a result of the study, we have found that the use of a hormone-free nutrient medium with a half concentration of micro- and macrosalts for propagation and rooting allows obtaining well-formed microplants in seven out of the nine species considered here. A reduction in the stages of subcultivation on the nutrient medium with the selected low concentrations of substances leads to accelerated propagation, which, in turn, will reduce material and labor costs. Our study has provided a new protocol for microclonal propagation of *Aristolochia* to ensure further successful conservation of rare endemic species living in different parts of the globe. It will help to propagate *Aristolochia* plants for creating plantations of medicinal species as a source of biologically active substances. Such measures are expected to decrease the pressure on natural populations and, in turn, contribute to the protection of valuable species from extinction.

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