Botanica Pacifica. A journal of plant science and conservation. 2025.
DOI: 10.17581/bp.2025.14111

RESEARCH PAPER

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

Olga V. Nakonechnaya*, Elizabeth P. Yusupova, Vlada V. Volkonskaya & Irina V. Gafitskaya

Olga V. Nakonechnaya *
e-mail: markelova@biosoil.ru

Elizabeth P. Yusupova
e-mail: ve.foxhole@gmail.com

Vlada V. Volkonskaya
e-mail: vladvol2609@gmail.ru

Irina V. Gafitskaya
e-mail: gafitskaya@biosoil.ru

Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS, Vladivostok, Russia

* corresponding author

Manuscript received: 25.12.2024
Review completed: 01.03.2025
Accepted for publication: 09.03.2025
Published online: 11.03.2025

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

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

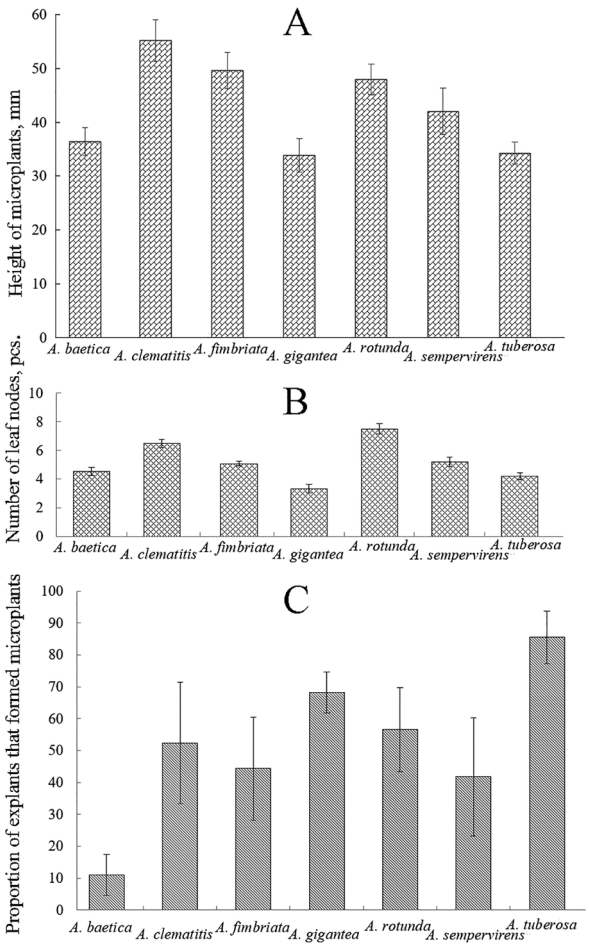

Figure 1 Morphometric characteristics of *Aristolochia* species after eight weeks of cultivation. A – height of microplants; B – number of leaf nodes in microplants; C – proportion of explants in different *Aristolochia* species that formed microplants

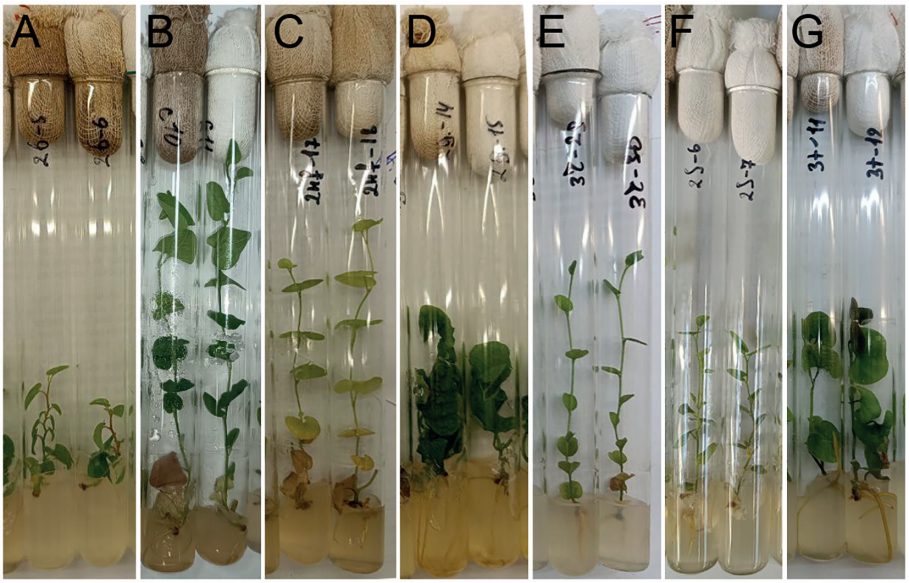

Figure 2 Microplants of *Aristolochia* species after eight weeks of cultivation: A – *A. baetica*; B – *A. clematitis*; C – *A. fimbriata*; D – *A. gigantea*; E – *A. rotunda*; F – *A. sempervirens*; G – *A. tuberosa*

(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.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Svetlana Nesterova (Botanical Garden-Institute FEB RAS, Vladivostok, Russia) for providing seeds of the studied species. This work was carried out within the state assignment of the Ministry of Science and Higher Education of the Russian Federation (theme no. 124012200181-4). The explants studies were done using the plants from the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS resource collection #USU-2797657.

LITERATURE CITED

Biswas, A., M.A. Bari, M. Roy & S.K. Bhadra 2007. *In vitro* regeneration of *Aristolochia tagala* Champ. a rare medicinal plant of Chittagong hill tracts. *Journal of Bio-Science* 15:63–67.

Borah, P.J. & R. Sarma 2022. GC-MS analysis and qualitative phytochemical screening of *Aristolochia assamica*, a newly discovered rare medicinal plant species of India. *Indian Journal of Natural Products and Resources* 13(4):552–558.

Butenko, R.G. 1964. *Culture of isolated tissues and physiology of plant morphogenesis*. Nauka, Moscow, 272 pp. (in Russian). [Бутенко Р.Г. 1964. Культура изолированных тканей и физиология морфогенеза растений. М.: Наука. 272 с.].

Davidyuk, L.K. 1974. Life cycle of the birthwort in floodplain oak forests of the Gorky region. In: *Biological basis of increasing the productivity and protection of forest, meadow and aquatic plant communities of the Gorky Volga Region, vol. 2*, pp. 76–81, Gor'kovskii Gosudarstvennyi Universitet, Gorky (in Russian). [Давидюк Л.К. 1974. Жизненный цикл кирказона обыкновенного в пойменных дубравах Горьковской области // Биологические основы повышения продуктивности и охраны лесных, луговых и водных фитоценозов Горьковского Поволжья. Горький: Изд-во Горьковского ун-та. Вып. 2. С. 76–81].

Endress, P.K. 1990. Evolution of reproductive structures and functions in primitive angiosperms (Magnoliidae). *Memoirs of the New York Botanical Garden* 55:5–34.

Endress, P.K. 1994. Floral structure and evolution of primitive angiosperms: recent advances. *Plant Systematics and Evolution* 192:79–97.

Gatti, E. & M. Vecchi 2017. Micropropagation of *Aristolochia rotunda* L. *Plant Biosystems – An International Journal Dealing with all Aspects of Plant Biology* 151(4):581–583.

Gong, Q.B., S. Landrein, H.C. Xi, X.D. Ma, Z.H. Yang, K.W. He & J.Y. Shen 2018. *Aristolochia tongbiguanensis*, a new species of Aristolochiaceae from Yunnan, China. *Taiwania* 63(3):183–187.

González, F.A. & D.W. Stevenson 2002. A phylogenetic analysis of the subfamily Aristolochioideae (Aristolochiaceae). *Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales* 26:25–60.

Kelly, L.M. & C.F. González 2003. Phylogenetic relationships in Aristolochiaceae. *Systematic Botany* 28:236–249.

Kharkevich, S.S. 1987. Family Aristolochiaceae. In: *Vascular plants of the soviet Far East, vol. 2* (S.S. Kharkevich, ed.), pp. 19–21, Nauka, Leningrad (in Russian). [Харкевич С.С. 1987. Сем. Aristolochiaceae // Сосудистые растения советского Дальнего Востока / отв. ред. С.С. Харкевич. Л.: Наука. Т. 2. С. 19–21].

Lloyd, G. & B. McCown 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Combined Proceedings International Plant Propagators' Society* 30:421–427.

Lucio K.A., M.A. Esqibel, A. Sato & C.L.S. Lage 2009. Micropropagação or *A. gigantea* Mart et Zucc. (Aristolochiaceae) through nodal segment culture. *Plant Cell Culture & Micropropagation* 5:129–134.

Manjula, S., A. Thomas, Daniel, B. & G.M. Nair 1997. *In vitro* plant regeneration of *Aristolochia indica* through axillary shoot multiplication and organogenesis. *Plant Cell, Tissue and Organ Culture* 51(2):145–148.

Molkanova, O.I., E.I. Lyubimova, L.N. Konovalova & D.A. Egorova 2018. *Method for micropropagation of manchurian birthwort* (*Aristolochia manshuriensis* Kom.). Patent R U266 26 82 C2 from 21.12.2016 (in Russian with English summary). [Молканова О.И., Любимова Е.И., Коновалова Л.Н., Егорова Д.А. 2018. Способ клонального микроразмножения кирказона маньчжурского (*Aristolochia manshuriensis* Kom.). Патент R U266 26 82 C2 от 21.12.2016].

Murashige, T. & F. Skoog 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* 15(3):473–497.

Murugan, R., K.R. Shivanna & R.R. Rao 2006. Pollination biology of *Aristolochia tagala*, a rare species of medicinal importance. *Current Science* 795–798.

Nakonechnaya, O.V., I.V. Gafitskaya & E.P. Yusupova 2024. *Aristolochia* of the *Siphisia* section *in vitro*. *Biology Bulletin* 51(6):1671–1678.

Nakonechnaya, O.V., S.V. Nesterova, N.M. Voronkova & A.B. Kholina 2015. Ontomorphogenesis of *Aristolochia fimbriata* Cham. (Aristolochiaceae) in protected ground. *Vestnik DVO RAN* 1:50–57 (in Russian). [Наконечная О.В., Нестерова С.В., Воронкова Н.М., Холина А.В. 2015. Онтоморфогенез *Aristolochia fimbriata* Cham. (Aristolochiaceae) в условиях защищенного грунта // Вестник ДВО РАН. № 1. С. 50–57].

Nesterova, S.V. 2008. *Aristolochia contorta*. In: *Red Data Book of Primorsky Krai: Plants* (A.E. Kozhevnikov, ed.), p. 65, Apel'sin, Vladivostok (in Russian). [Нестерова С.В. 2008. Кирказон скрученный // Красная книга Приморского края: Растения. Владивосток. С. 65].

Osuna, L.T., I.A. Mora, E.Z. Ventura, E.F. Jiménez, C.M. Bazaldúa & A.A. Jiménez 2007. Micropropagation of *Aristolochia elegans* (Mast.). *Journal of Crop Science and Biotechnology* 10(3):141–146.

Razzak, M.A., T. Ali & S.I. Ali 1992. The pollination biology of *Arislohchia bracteolate* Lamk (Aristolochiaceae). *Pakistan Journal of Botany* 24(1):79–87.

Remya, M., V. Narmatha Bai & V.N. Mutharaian 2013. *In vitro* regeneration of *Aristolochia tagala* and production of artificial seeds. *Biologia Plantarum* 57(2):210–218.

Saidi, F., H.S. Cherif, H. Metidji, A. Rouibia, C. Chaouia, M.S. Abdulhussain, R.M. Said & M.S. Hamaidi 2009. Studies on the *in vitro* multiplication by direct organogenesis of a medicinal plant: *Aristolochia longa* L. *Agricultura-Revistă de Scedilla tiintcedilla ă scedilla i Practică Agricolă* 3/4:53–65.

Sarma, B. & B. Tanti 2017. *In vitro* regeneration of plantlets from nodal explants of *Aristolochia saccata* and *Aristolochia cathcartii*. *European Journal of Biological Research* 7(3):191–201.

Sathish, S.S., N. Janakiraman & M. Johnson 2011. *In vitro* propagation of *Aristolochia bracteata* Retz. – a medicinally important plant. *Research in Biotechnology* 2(6):44–52.

Shenk B.U. & C. Hildebrandt 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* 50:199–204.

Siddique, N.A., M.H. Kabir & M.A. Bari 2010. Comparative *in vitro* study of plant regeneration from nodal segments derived callus in *Aristolochia indica* Linn. and *Hemidesmus indicus* (L.) R. Br. endangered medicinal plants in Bangladesh. *Journal of Plant Sciences* 5(2):146–158.

Yu, Y.L., H.C. Wang, Z.X. Yu, J. Schinnerl, R. Tang, Y.P. Geng & G. Chen 2021. Genetic diversity and structure of the endemic and endangered species *Aristolochia delavayi* growing along the Jinsha River. *Plant Diversity* 43(3):225–233.

Veluchamy, S. & A.P. Rajappan 2008. Stimulation of micropropagation of the medicinal plant *Aristolochia indica* L. through nodal explants by adenine sulphate. *Asian and Australasian Journal of Plant Science and Biotechnology* 2(1):39–41.

Ward, D.B., D.F. Austin & N.C. Coile 2003. Endangered and threatened plants of Florida, ranked in order of rarity. *Castanea* 68(2):160–174.

Zhou, J., G. Xie & X. Yan 2011. *Encyclopedia of traditional Chinese medicines: molecular structures, pharmacological activities, natural sources and applications, vol. 1–5*. Springer-Verlag, Berlin-Heidelberg.