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REVIEW PAPER

Microclonal propagation of the species from the genus *Aristolochia*: a review

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ABSTRACT

Plants of the genus *Aristolochia* L. are included in the pharmacopoeias of different countries because they have a high potential as anti-inflammatory, bactericidal, wound healing agents, and antidotes and their spheres (fields) of medical application are diverse. Due to the depletion of natural populations, the renewal of the species by biotechnological methods has become relevant. *In vitro* reproduction or clonal micropropagation enables the researchers to massively duplicate plants with a definite genotype throughout the year. This review provides important information available today on the *in vitro* propagation methods and further successful regeneration for valuable plants from the genus *Aristolochia*. The generalized information is necessary to create a breeding technology for each of the species of the genus, which will save valuable medicinal resources.

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

РЕЗЮМЕ

Наконечная О.В., Волконская В.В. Микроклональное размножение видов рода *Aristolochia*: обзор. Растения рода *Aristolochia* L. внесены в фармакопеи разных стран поскольку обладают высоким потенциалом как противовоспалительные, бактерицидные и ранозаживляющие средства, в том числе как антидоты; сферы их медицинского применения разнообразны. Из-за истощенности природных популяций, возобновление видов биотехнологическими методами приобрело актуальность. Метод микроклонального размножения является привлекательным для исследователей из-за своего потенциала для массового дублирования растений с определенным генотипом в течение всего года. Этот обзор предоставляет важную информацию для лучшего понимания особенностей клонального размножения видов рода *Aristolochia*. Обобщенные сведения необходимы для создания технологии размножения каждого из видов рода, что позволит сохранить ценные лекарственные ресурсы.

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

Genus *Aristolochia* L. belongs to one of the ancient angiosperm families Aristolochiaceae Juss. (Chevallier 1996, Kelly & González 2003) and includes up to 400 species (Gonzalez & Stevenson 2002, Wagner et al. 2014), native to the tropical, subtropical, and temperate zones of the Northern and Southern hemispheres (Kharkevich 1987, Gonzalez & Stevenson 2002, Kelly & González 2003). The genus is represented by vines, shrubs and rhizome herbs (Endress 1990, 1994, Razzak et al. 1992). Some species of the genus are relics of the Paleogene flora (Tertiary relicts) (Kurentsova 1968, Adams et al. 2005, Gonzaloález et al. 2014) and local endemic to different regions, for example, *A. delavayi* Franch. (Yu et al. 2021), *A. indica* Linn., *A. saccata* Wall., and *A. cathcartii* Hook. f. (Sarma & Tanti 2015), *A. manshuriensis* Kom. (Kurentsova 1968). All members of the genus possess medicinal properties, and their fields of application are diverse (Zhou et al. 2011). Thus, *A. bracteolata* Linn. is used in Nigerian ethnomedicine, and its anticandidal activity has been shown (Gbadamosi & Egunyomi 2012). Roots of *A. elegans* Mast. are widely used in Mexican folk medicine as a remedy for scorpion venom (Osuna et al. 2007). Alcohol root extract of the *A. indica* L. possesses antitumor activity against the colon cancer cell line HT29 (Kangralkar & Kulkarni 2013). *A. longa* L. is used in Algerian folk medicine as an anti-cancer agent and for the treatment of fistulas, ulcers, boils, acne (Saidi et al. 2009).

Many species are the important components of biotopes, as relict butterfly species are fed on their leaves. Thus, *Parides ascanius* (Cramer, 1775) (Lepidoptera, Papilionidae) feeds on the leaves of *A. trilobata* L. (Grice et al. 2019), *A. contorta* Bunge lianas are necessary to Chinese imago sericinus, *Sericinus montela amurensis* (Staudinger, 1892) (Kurentsov 1961), the leaves of *A. macrophylla* Lam. serve as food for *Battus philenor* (Linnaeus, 1771) (Howe 1975), the imago *Battus philenor hirsuta* (Skinner, 1908) (Howe 1975) dwells on the *A. californica* Torr. lianas, and *Papilio alcinous* (Klug, 1836) feeds on the leaves of *A. manshuriensis*.

The fact that the *Aristolochia* species are confined to certain biotopes, the peculiarities of the pollination process and the active use of these plants as medicinal raw materials have led to the significant population decrease of many species in natural habitats. They have become rare (*A. assamica* D. Borah & T.V. Do (Borah & Sarma 2022); *A. tagala* Cham. (Murugan et al. 2006); *A. contorta* (Nesterova 2008); *A. tongbiguanensis*

J.Y. Shen, Q.B. Gong & Landrein (Gong et al. 2018); *A. tomentosa* Sims (Ward et al. 2003), etc.) or endangered (*A. manshuriensis* (Nesterova 2008); *A. delavayi* (Yu et al. 2021)). Vegetative and seed propagation is often difficult. Therefore, the reproduction of the species using modern technologies has become particularly relevant. One of these approaches is the method of clonal micropropagation, which allows to obtain genetically identical and pathogen-free plants quickly, continuously and massively throughout the year. This review provides important information available today on the *in vitro* propagation methods and further successful regeneration for valuable plants from the genus *Aristolochia*. The generalized information is necessary to create a breeding technology for each of the species of the genus.

SPECIES OF THE GENUS *ARISTOLOCHIA IN VITRO*

Aristolochia bracteata Retz.

Conditions for reproduction of *A. bracteata* were selected by Sathish et al. (2011), who cultivated internodal segments as explants on MS medium (Murashige & Skoog 1962) with the addition of various concentrations and combinations of plant growth regulators for direct and indirect regeneration. The lowest percentage of explant infection (4 %) was obtained after sterilization with 0.1 % mercury chloride for 2 minutes. The highest rate of shoot proliferation (61.5±0.43) was observed on MS medium with the addition of 1.0 mg/L of BAP (6-benzylaminopurine) in combination with 1.0 mg/L NAA (α-naphthaleneacetic acid). The MS medium supplemented with 1.0 mg/L BAP in combination with 0.5 mg/L NAA provided the highest percentage (73.2±0.43) of shoots proliferation from internodes obtained from callus. The highest number of roots per shoot and their average lengths were observed on the half-strength MS medium supplemented with 1.0 mg/L IBA (Indole-3-butyric acid) (Sathish et al. 2011).

Aristolochia bracteolata Lam.

An effective regeneration protocol for *A. bracteolata* was developed by a direct organogenesis *in vitro* (Sebastinraj & Sidique 2011). Nodal segments and shoot tips were used as explants. High-frequency organogenesis and multiple shoot regeneration were induced on MS medium augmented with 1.0 mg/L BAP in combination with 1.0 mg/L NAA. The microshoots were further transplanted from the *in vitro* proliferated conglomerate on MS medium with the addition of 0.3 mg/L IBA for root formation. The obtained microplants were adapted to the conditions of protected soil. A culture medium supplemented with BAP 4.0 mg/L and NAA 0.5 mg/L proved to be suitable for producing more shoots (8.9). Both shoot bud induction and shoot reproduction were significantly higher in the nodal segments compared with the shoot tip and axillary bud (Sathish et al. 2011).

Aristolochia elegans Mast.

In order to preserve, and cultivate the plants of *A. elegans* and standardize their morphological and pharmacological properties, a micropropagation protocol was developed by Osuna et al. (2007). Leaves with axillary buds were taken from six-week-old aseptic plants grown from disinfected seeds and were applied for introduction to culture conditions. Stem explants (1 cm) were used for root induction. Nutrient MS medium with BAP (10 μM), pH 5.8, was used for reproduction and provided the activation of the largest number of buds (on average 3.1) in both types of explants. Addition of IBA (1.5 μM) to MS medium caused the highest root index (11.8) per explant. The obtained microplants were successfully adapted to soil. This protocol was later repeated by Izquierdo et al. (2010).

Aristolochia fimbriata Cham.

The micropropagation method for *A. fimbriata* was first described by Bravo et al. (1997). The MS medium contained salts, Gamborg vitamins and sucrose. Stem segments with one node were cultured on a nutrient medium with the addition of 0.1 mg/L GA3 (gibberellic acid), 2.5 mg/L BAP and IBA at various concentrations. A 5-fold increase in the number of developing shoots was shown after 14 days on the medium with 0.25 mg/L IBA. The resulting shoots were cut and transferred to a nutrient medium with BAP (1.0 mg/L), where an 8-fold increase in the number of shoots was obtained. For root formation, the microshoots were cultured on MS medium with half macrosalts supplemented with IBA or NAA. The addition of IBA resulted in root formation in all tested concentrations, while the addition of NAA resulted only in callus formation.

Later, Bliss et al. (2009) reported that *A. fimbriata* plants were propagated *in vitro* and rooted with 100 % efficiency. Leaves, petioles, and internodes (stem explants) were taken as explants, and the highest regeneraton rate (97 %) was observed for stem explants. Regenerated and rooted shoots were acclimated to greenhouse conditions and bloomed within 4 weeks after transplanting.

Aristolochia indica L.

The apical parts of shoots and nodal segments of *A. indica* were used for propagation by Manjula et al. (1997). Cultivation was performed on MS medium supplemented with 0.54 μM of NAA and 13.31 μM BAP. This medium composition contributed to the development of the maximum number of shoots (45–50) from the apical and axillary buds. The same authors obtained a callus culture from the leaves and internodal segments cultured on MS medium with NAA or 2.4-D (2,4-dichlorophenoxyacetic acid) and BAP or Kin (kinetin). The accumulation of phenols in the callus was controlled by adding 1.0 mg/L of PG (phloroglucinol) to the medium. For shoot regeneration from the callus, the MS medium with the addition of 2.69 μM of NAA, 13.31 μM of BAP and 1.0 mg/L of PG was pointed as the best. The direct *de novo* shoot development from leaf segments was achieved by using 13.31 μM BAP together with 50 mg/L activated charcoal. The microshoots were rooted on White's medium with the addition of 2.46 μM IBA. Adaptation to the soil was high (85%).

Soniya & Sujitha (2006) cultivated apical and axillary buds of *A. indica* on MS medium with 1–6 mg/L 2-iP (2-Isopentenyladenine) or 1–4 mg/L BAP and obtained multiple shoot formation. The maximum number of

shoots was observed on the medium with 5 mg/L 2-iP (about 12–14 shoots). Shoot differentiation occurred directly from the leaf bases and internodes cultivated on a medium containing 1–4 mg/L BAP and 0.8–2 mg/L NAA. Callus formation was observed on MS medium containing 0.6–4 mg/L NAA in combination with 0.8–3 mg/L BAP. When the callus was transferred to a medium with 1–6 mg/L BAP the regeneration of microshoots was observed. The elongated microshoots were rooted on MS medium containing 1 mg/L IBA. They were then transferred to soil after gradual acclimatization.

A method for establishing the *A. indica* callus culture was also published by Siddique et al. (2006). The highest percentage of callus induction (95) was achieved on MS medium supplemented with 2.0 mg/L Kin and 1.0 mg/L BAP. The development of adventitious shoots occurred during callus subcultivation on the same medium or in combination of BAP with NAA and IAA (indole acetic acid) or NAA, IAA and BAP in combination with Kin. The highest percentage of shoot regeneration (95) was obtained on MS medium enriched with 2.5 mg/L Kin and 1.0 mg/L BAP. The elongated shoots rooted on a medium containing 1 mg/L Kin.

Veluchamy & Rajappan (2008) tested the MS medium supplemented with various plant growth regulators: Kin, GA3, BAP, and AdS (adenine sulfate). The hormones were used separately and in combinations (Kin+GA3, Kin+BA and Kin+AdS) with 2.46 μM IBA. Stem sections with a leaf node were used for the experiment. Shoot proliferation was only promoted by 27.1 μM AdS alone or in combination with other plant growth regulators. The use of Kin (23.25 μm) and AdS (13.5 μm) strongly activated bud development. Addition of IBA increased root formation, while the inclusion of IAA induced rooting of shoots with intermediate callus formation at the basal end. The application of this protocol allowed to obtain 10–12 microplants from a single explant.

At the same period, Prabha et al. (2008) developed a protocol for *A. indica* micropropagation using apical and nodal explants. The synergistic effect of BAP at a concentration of 1 mg/L have led to the induction of a greater number of adventitious shoots from the nodal explants, compared to other medium variations. The maximum number of roots was induced on a medium with IBA (1 mg/L). The grown microplants were successfully transferred to the greenhouse where they showed a high survival rate (65 %).

In another research, the maximum percentage of *A. indica* microshoots regeneration was obtained by Siddique et al. 2010) on MS medium supplemented with 1.0 mg/L BAP and 2.5 mg/L NAA. The highest rooting rate for microshoots was observed on MS medium with 2.5 mg/L Kin and 2.0 mg/L IBA. Callus formation was observed on MS medium with the addition of 1.0 mg/L IAA and 1.0 mg/L BAP.

Dey et al. (2021) used Schenck and Hildebrandt media (SH) enriched with (BAP) (2.0 mg/L)+put (putrescine) (0.5 mM) and in another experiment BAP (2.0 mg/L) + SPD (spermidine) (1 mM) and achieved the best results with 41 and 39.2 axillary shoots per nodal explant, respectively. After 4–6 weeks of incubation the explants were propagated on SH medium with the addition of various combinations and concentrations of BAP and Kin (0.5, 1, 1.5 and 2 mg/L), SPD (0.5 and 1 mM). The SH medium supplemented with BAP (2.0 mg/L) + SPD (0.5 mM) showed the best regeneration response with an average number of 47.5 base shoots obtained from callus. After 6 weeks of cultivation on a SH medium + 1.0 mg/L IAA + 0.5 mM SPD, the maximum average number of roots per shoot (7) was observed on explants.

Pattar & Jayara (2012) used leaf and nodular explants to initiate callus formation. The authors cultivated the explants on MS medium with the addition of 0.8 mg/L BAP. The nodular explants provided better regenerative response (95 %), compared to the leaf explants (85 %). Shoots were induced from callus on MS medium + 0.8 mg/L BAP + 0.5 mg/L NAA. To obtain roots on microshoots, MS media with 0.8 mg/L NAA was used. The microplants were further acclimatized and successfully transferred to the field.

Shah et al. (2013) obtained aseptic cultures by growing nodal segments (from 1 to 1.5 cm) on MS medium containing 5.0 μM BAP. Five nutrient media were used in the experiments: MS, woody plants medium (WP), Gamborg medium (B5), Nitsch and Nitsch medium (NN) and SH medium with the addition of various cytokinins and auxins at a concentration of 10 μM. The MS medium + 5 μM BA turned out to be optimal for shoots propagation *in vitro*. The experiments resulted in 100 % number of shoots per explant after 15 days and 61.9 % after 30 days on MS medium, 65.2 % number of nodes per shoot after 15 days and 196.2 % after 30 days on WP medium, and 147.5 and 366.6 % number of nodes per explant 30 days after planting on MS medium.

For *A. indica* callus cultures, the addition of 5 mg/L BAP to the MS medium resulted in the highest callus induction. The MS medium enriched with 4.0 mg/L BAP + 1.0 mg/L NAA provided the highest shoot regeneration rate (Thandar & Tun 2014). It was also shown that elongated shoots rooted on MS medium without growth regulators. The rooted plants were transferred to a mixture of soil and compost. They were gradually acclimatized and successfully transferred to field conditions.

In 2017, seven media were tested *in vitro* to induce callus from the stem segments, leaf bases, and cotyledons of *A. indica*, five of which were based on MS medium, one on B5 medium, and one on White medium (Nehra et al. 2017). The highest efficiency of callus formation was observed on MS medium with 5 mg/L 2.4-D in combination with 1.6 mg/L BAP. This variant of the medium also contributed to multiple shoots induction from the callus. The half-salts MS medium with 1.2 mg/L BAP and 0.6 mg/L IBA was the best for root regeneration. Darkness promoted rapid root regeneration. The rooted microplants were successfully transplanted into pots and were transferred to a greenhouse after 25 days for further acclimatization in natural conditions. Survival rate was 63 % (Nehra et al. 2017).

The same authors evaluated different types of explants (cotyledons, stem segment, and leaf base) for their effectiveness in inducing callus on different media. The leaf base was shown to be the best explant type in terms of the percentage of callus formation and the average callus fresh

mass accumulation. Cotyledons were the least effective explants. The MS medium with the addition of 5 mg/L 2,4-D and 1.6 mg/L BAP demonstrated high efficiency in shoot regeneration, which may be due to high concentrations of the hormones which are considered to be highly effective in initiating multiple shoots from callus (Nehra et al. 2022).

Dey et al. (2021) conducted an *in vitro* study of *A. indica* to isolate aristolochic acid and analyzed its endogenous level in shoots and roots. The nodal explants and apical shoots were cultured in SH medium enriched with various concentrations of polyamines (0.5–1 mM) such as putrescine, spermidine, spermine, as well as plant growth regulators auxins (IAA – 1.5 mg/L, IBA – 1 mg/L) and cytokinins (Kin – 2 mg/L, BAP – 1.5 mg/L) for callus induction, direct shoot organogenesis and formation of multiple axillary shoots. As a result, a high-performance liquid chromatography (HPLC) study of the plant extracts revealed that the content of aristolochic acid in roots in vitro was higher than in plants grown in the natural field. This confirms the key role of polyamines together with phytohormones in increasing the concentration of aristolochic acid (Nath et al. 2022).

Aristolochia longa L.

Saidi et al. (2009) showed for the Algerian species *A. longa* that sterilization with 0.6 g/L $HgCl_2$ for 10 minutes was more effective than that with calcium hypochloride. The authors noted that cultivation on MS medium with the addition of 1.5 mg/L BAP and 0.5 mg/L NAA is the best for shoot induction. The shoots elongated more intensively on the MS medium with 0.5 and 1.5 mg/L GA3 A high rooting percentage was observed on a medium with 1 mg/L NAA. During acclimatization, 100 % of the microplants survived in peat after 2 months of growth.

Aristolochia manshuriensis Kom.

In the first studies on the microclonal propagation of *A. manshuriensis* (Svensson 1999), MS medium with the addition of BAP (0.4 mg/L) was used. The explants were placed in the dark during the first 1–7 days. This provided an increase in the percentage of rooted shoots. It was also useful to separate the shoot base before rooting. During the reproduction, illumination with the following levels of photosynthetic photon flux (PPF) was tested: 20, 40, 60, and 80 μmol/m^2·s. The effect of PFF levels on reproduction stabilized after three passages (84 days); a PPF level of 20 μmol/m^2·s resulted in the lowest reproduction rate (RR) per month (3.0), and 80 μmol/m^2*s provided the highest RR per month (4.2). The level of PPF during reproduction also affected subsequent rooting. According to the results, the maximum percentage of rooted shoots was achieved under 80 μmol/m^2·s. A prolonged cultivation period on the propagation medium before rooting (6 weeks instead of 4 weeks) significantly increased the multiplication rate, but negatively affected subsequent rooting, which decreased from 41 to 12 % (Svensson 1999).

The method of *A. manshuriensis* clonal micropropagation was patented in 2016 by Molkanova et al. (2018b). It includes isolation of apical and lateral buds from immature and virginal plants, sterilization with sodium hypochlorite (exposure for 7–10 minutes), planting on MS medium with 0.8 mg/L BAP and 0.05 mg/L IAA, propagation of microshoots, their subsequent rooting on half-strength MS medium containing 20 mg sucrose and adaptation of regenerated plants to *ex vitro* conditions by planting on a mixture of sand, peat and soil at a ratio of 1:1:1, preliminarily sterilized at 85–90°C for 1–2 hours. The invention allows to increase the yield of regenerating plants to more than 150 thousand specimens per year (Molkanova et al. 2018b). Molkanova & Egorova (2017) noted that the maximum reproduction coefficient (14.84 ± 0.8) was obtained on MS medium with 0.8 mg/L BAP and 0.05 mg/L IAA. The optimal medium for rooting was pointed as half-strength MS medium with 3.0 mg/L IBA. The same authors obtained seedlings from buds, microshoots, and microcuttings via organogenesis and somatic embryogenesis at a temperature of 24 ± 1°C and a light intensity 2–3 klux. The authors showed that storage of the viable explants at 4–8°C and light intensity 0.2–0.3 klux inhibited their growth. The optimal explants for a long-term storage were buds, microshoots, and microcuttings. RAPD-analysis (randomly amplified polymorphic DNA) was performed for model species to control the genetic stability of explants preserved *in vitro* (Molkanova et al. 2018c). In another study, Molkanova et al. (2018a) used stem explants. The optimal nutrient medium for rooting was half-strength MS medium containing 3.0 mg/L IBA. It was found that explants from young (no older than 4-6 years) plants of *A. manshuriensis* were characterized by a higher ability for shoot proliferation compared to plants that reached the age of 12 years. The explants obtained from 2-3-year-old plants were characterized by a higher morphogenetic capacity (Molkanova et al. 2018a).

Aristolochia rotunda L.

A micropropagation protocol for *A. rotunda* was developed by (Gatti & Vecchi 2017). The cytokinin BA was shown to affect the formation of new shoots, but did not affect the length of shoots. The addition of IBA negatively affected the length of shoots, but provided the best rooting percentage and root length at concentration of 1.5 μM.

Aristolochia saccata Wall. and *A. cathcartii* Hook.

In vitro reproduction of *A. saccata* and *A. cathcartii* was performed by Sarma & Tanti (2017) using nodal explants. These explants in both species demonstrated direct somatic embryogenesis when cultured on MS medium with different concentrations of BAP (1–4) and 2-iP (1–4) separately or in combination with low concentrations (0.5 and 1.0 mg/L) of auxin (NAA). A combination of BAP and NAA was more effective for shoot induction than the hormones used separately. The best combination for *A. saccata* explants proliferation (96 %) during 28 days was 3.0 BAP and 1.0 mg/l NAA. For *A. cathcartii*, the best result (88.3 % of explants) was obtained using 4.0 mg/L BAP with 0.5 mg/L NAA. The range of concentrations (0.1, 0.5, 0.8, and 1.0 mg/L) of NAA and IBA was tested for rooting of *in vitro*-grown shoots. The best results in rooting were obtained with half strength with IBA (0.5 mg/l).

Aristolochia tagala Cham.

An effective protocol for *A. tagala* regeneration by a direct organogenesis *in vitro* was developed by Biswas et al. (2007) using nodal segments as explants. Multiple buds were induced directly from nodal explants cultured on the MS medium with the addition of 2.0 mg/L BAP and 0.5 mg/L NAA. The multiplication coefficient (number of shoots per explant) was equal to 6. The best result in microshoots rooting was observed on a half-strength MS medium containing 0.5 mg/L IBA. The rooted microplants were transferred to the natural environment after acclimatization.

Later, Remya et al. (2013) developed the protocol for plant propagation *in vitro* from somatic tissues and production of artificial seeds by node encapsulation. Leaves were used as explants for callus induction. The maximum number of adventitious shoots was regenerated from the leaf callus on MS medium containing BAP 2 μM, NAA 0.5 μM and 10 μM PG (phloroglucinol). Multiple shoots were successfully regenerated from nodes cultured on MS medium with 3 μM BAP and 0.5 μM Kin. The regenerated shoots were successfully rooted and acclimated to greenhouse conditions. A protocol for root regeneration from *A. tagala* callus was also developed. The maximum root length was obtained by growing the callus on MS medium with the addition 1 μM Kin, 0.5 μM IAA, 0.1 μM NAA, and 10 μM PG. The biochemical parameters of calli grown on the medium with and without PG were studied to find out a correlation between these parameters and shoot morphogenesis.

Remya et al. (2016) cloned the apical bud explants. The addition of activated carbon to a medium helped to solve the problem of polyphenol exudation from explants into the medium, which blocked the regeneration of adventitious shoots. The most effective medium composition was MS with 3 μM BAP, 0.5 μM Kin, and activated carbon (0.1 %). The maximum number of shoots (12.6) was obtained from apical bud explants after 25 days of inoculation. Well-developed shoots were rooted on MS medium with the addition of 1.5 μM IAA, 1.5 μM Kin, and 0.5 μM BAP. Regenerating shoots from apical buds were successfully rooted and acclimated to greenhouse conditions.

Rajanna & Shailaja (2015) developed an effective protocol for axillary bud proliferation and direct organogenesis in *A. tagala* using *in vitro* propagation method. The shoot tips and nodal segments (1–1.5 cm) of these plants were used as explants. They were washed with running tap water, then treated with a few drops of Twin-20, then 0.3 % bavistin, and then thoroughly washed with sterile distilled water. MS medium with various concentrations of PGRs was used for cultivation. The results showed that the medium enriched 1 mg/L with BAP, and half-strength MS medium with 2 mg/L IBA were the best for inducing shoots and roots, respectively.

CONCLUSIONS

Medicinal properties of the plants from the genus *Aristolochia* have determined an increased interest in each species of the genus for several centuries. An intensive use of plants as an officinal raw material contributed to the depletion of natural populations. The application of the microclonal propagation method to the plants of the genus *Aristolochia* can be a reliable alternative to seed propagation. The regenerated plants can be used for further reintroduction, as well as to create nurseries for growing plants for medical application. Reaction of the plants of different species on the introduction into culture vary considerably, and depend on genotype, tissue or organ, stage of development, maturity and seasonal variations, the protocols of microclonal reproduction are different for each species. By the present time, the protocols have been developed for only 11 species out of 400 (2.8 %). Revealing the features of microclonal reproduction for the distinct *Aristolochia* species and generalization of the previously obtained data are the initial and necessary steps to preserve and restore the population of the valuable medicinal resources.

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LITERATURE CITED

Adams, C.A., J.M. Baskin & C.C. Baskin 2005. Trait stasis versus adaptation in disjunct relict species: evolutionary changes in seed dormancy-breaking and germination requirements in a subclade of *Aristolochia* subgenus *Siphisia* (Piperales). *Seed Science Research* 15(2):161–173.

Bliss, B.J., L. Landherr, C.W. DePamphilis, H. Ma, Y. Hu & S.N. Maximova 2009. Regeneration and plantlet development from somatic tissues of *Aristolochia fimbriata*. *Plant Cell, Tissue and Organ Culture* 98(1):105–114.

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.

Bravo, C., G. Yormann & B. Llorente 1997. Micropropagation of *Aristolochia fimbriata* Cham. *Acta Horticulturae* 502:339–346.

Chevallier, A. 1996. *The encyclopedia of medicinal plants*. Dorling Kindersley Chronica Botanica Co., Waltham, Massachusetts, 336 pp.

Dey, A., P. Nongdam, S. Nandy, S. Mukherjee, A. Mukherjee, L. Tikendra, A.K. Hazra & D.K. Pandey 2021. Polyamine elicited aristolochic acid production in *in vitro* clonally fidel *Aristolochia indica* L.: An ISSR and RAPD markers and HPTLC based study. *South African Journal of Botany* 140:326–335.

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* 151(4):581–583.

Gbadamosi, I.T. & A. Egunyomi 2012. *In vitro* propagation and antimycotic potential of extracts and essential oil of roots of *Aristolochia bracteolata* Linn. (Aristolochiaceae).

African Journal of Traditional, Complementary and Alternative Medicines 9(1):50–55.

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.

González F., S.T. Wagner, K. Salomo, L. Symmank, M.S. Samain, S. Isnard, N.P. Rowe, Ch. Neinhuis & S. Wanke 2014. Present trans-Pacific disjunct distribution of *Aristolochia* subgenus *Isotrema* (Aristolochiaceae) was shaped by dispersal, vicariance and extinction. *Journal of Biogeography* 41(2):380–391.

Grice, H., A.V.L. Freitas, A. Rosa, O. Marini-Filho, N. Mega, F.M.S. Dias, O. Mielke & M. Casagrande 2019. *Parides ascanius* (amended version of 2018 assessment). In: *The IUCN Red List of Threatened Species* 2019: e.T16239A145165808. https://doi.org/10.2305/IUCN.UK.2018-2.RLTS.T16239A145165808.en. Last accessed 19 June 2023.

Izquierdo, A.M., E.V. Zapata, J.E. Jiménez-Ferrer, C.B. Muñoz, A.J. Aparicio, K.B. Torres, & L.O. Torres 2010. Scorpion antivenom effect of micropropagated *Aristolochia elegans*. *Pharmaceutical biology* 48(8):891–896.

Kangralkar, V.A. & A.R. Kulkarni 2013. *In vitro* cytotoxic activity of alcoholic extract of *Aristolochia indica*. *Research Journal of Pharmacy and Technology* 6(11):1240–1241.

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].

Kurentsov, A.I. 1961. *In the refuge of the Ussuri relics*. Primorskoe knizhnoe izdatel'stvo, Vladivostok, 184 pp. (in Russian). [Куренцов А.И. 1961. В убежищах уссурийских реликтов. Владивосток: Приморское кн. изд-во. 184 с.].

Kurentsova, G.E. 1968. *Relict plants of Primorye*. Nauka, Leningrad, 72 pp. (in Russian). [Куренцова Г.Э. 1968. Реликтовые растения Приморья. Л.: Наука. 72 с.].

Manjula, S., A. Thomas, B. Daniel & 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., Y.N. Gorbunov & D.A. Egorova 2018a. Conservation and clonal micropropagation of rare endemic species *Aristolochia manshuriensis*. *Acta Horticulturae* 1324:19–26.

Molkanova, O.I., E.I. Lyubimova, L.N. Konovalova & D.A. Egorova 2018b. *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. 2018b. Способ клонального микроразмножения кирказона маньчжурского (*Aristolochia manshuriensis* Kom.). Патент R U266 26 82 C2 от 21.12.2016].

Molkanova, O., I. Shirnina & I. Mitrofanova 2018c. Conservation and micropropagation of rare and endemic species in genepool collections of the Russian Federation. *Journal of Biotechnology* 280:S83–84.

Molkanova, O.I. & D.A. Egorova 2017. Some aspects of *Aristolochia manshuriensis* Kom. *in vitro* cultivation. *Vestnik Udmurtskogo universiteta. Seriya "Biologiya. Nauki o Zemle"* 27(2):151–157 (in Russian with English summary). [Молканова О.И., Егорова Д.А. 2017. Некоторые аспекты культивирования *in vitro Aristolochia manshuriensis* Kom. // Вестник Удмуртского университета. Серия «Биология. Науки о Земле». Т. 27, № 2. С. 151–157].

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.

Nath, S., N. Ghosh, T.A. Ansari, A. Mundhra, M.T. Patil, A. Mane, A.V. Gopalakrishnan, Md.H. Rahman, M. Kumar, Radha, M. Ghorai, S. Paul & A. Dey 2022. Genetic diversity assessment and biotechnological aspects in *Aristolochia* spp. *Applied Microbiology and Biotechnology* 106:6397–6412.

Nehra, A., D.K. Shrivastava & R.C. Jeeterwal 2017. Response of different culture medium on *in vitro* plant regeneration through different explants in Ishwarmul (*Aristolochia indica* Linn). *Journal of Pharmacognosy and Phytochemistry* 6(4):1576–1578.

Nehra, A., J. Singh & R.C. Jeeterwal 2022. Effect on shoot and root regeneration of various culture media in medicinal plant of *Aristolochia indica*. *The Pharma Innovation Journal* 11(2):526–528.

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: Апельсин. 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.

Pattar, P.V. & M. Jayaraj 2012. *In vitro* regeneration of plantlets from leaf and nodal explants of *Aristolochia indica* L. an important threatened medicinal plant. *Asian Pacific Journal of Tropical Biomedicine* 2(2):S488–S493.

Prabha, A., R. Subbu & K. Shrishail 2008. Micropropagation of *Aristolochia indica* L. (Aristolochiaceae). *Ecology, Environment and Conservation* 14:409–411.

Rajanna, L. & G.S. Shailaja Sharma 2015. *In vitro* axillary bud proliferation and direct organogenesis of *Aristolochia tagala* Cham: a rare medicinal plant. *Research & Reviews: Journal of Botanical Sciences* 4:21–28.

Razzak, M.A., T. Ali & S.I. Ali 1992. The pollination biology of *Arislohchia bracteolata* 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.

Remya, M., V. Narmatha Bai, S. Murugesan & V. Mutharaian 2016. Changes in bioactive components of *Aristolochia tagala* Cham., a rare species of medicinal importance during its *in vitro* development through direct regeneration. *bioRxiv* 037028.

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 2015. Karyomorphology of three species of *Aristolochia* – rare and endemic medicinal plants of Assam, India. *Caryologia* 68(2):154–158.

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.

Sebastinraj, J. & K.I. Sidique 2011. *In vitro* rapid clonal propagation of *Aristolochia bracteolata* Lam. (Aristolochiaceae) – a valuable medicinal plant. *World Journal of Agricultural Sciences* 7(6):653–658.

Shah S.N., A.M. Husaini & F. Shirin 2013. Micropropagation of the indian birthwort *Arsitolochia indica* L. *International Journal of Biotechnology and Molecular Biology Research* 4(6):86–92.

Siddique, N.A., M.A. Bari, M.M. Pervin, N. Nahar, L.A. Banu, K.K. Paul, M.H. Kabir, A.K.M.N. Huda, Md.K.B.F. Khandaker & M.J. Hossin 2006. Plant regeneration from axillary shoots derived callus in *Aristolochia indica* Linn. an endangered medicinal plant in Bangladesh. *Pakistan Journal of Biological Sciences* 9(7): 1320–1323.

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.

Soniya, E.V. & M. Sujitha 2006. An efficient *in vitro* propagation of *Aristolochia indica*. *Biologia Plantarum* 50(2):272–274.

Svensson, M. 1999. Effect of irradiance level during *in vitro* propagation of *Aristolochia manchuriensis*. *Acta Horticulturae* 530:403–410.

Thandar, S. & O.M. Tun 2014. *In vitro* callus induction and shoot regeneration of myanmar medicinal plant, *Aristolochia indica* Linn. In: *Fifth International Conference on Science and Engineering*. Yangon, Myanmar. 1–4 pp.

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.

Wagner, S.T., L. Hesse, S. Isnard, M.S. Samain, J. Bolin, E. Maass, Ch. Neinhuis, N.P. Rowe & S. Wanke 2014. Major trends in stem anatomy and growth forms in the perianth-bearing Piperales, with special focus on *Aristolochia*. *Annals of Botany* 113(7):1139–1154.

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*. V. I – 557 pp.; V. II – 525 pp.; V. III – 669 pp.; V. IV – 636 pp.; V. V – 601 pp.; V. VI – 730 pp. Springer-Verlag, Berlin-Heidelberg.