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RESEARCH PAPER

# Leaf mesostructure of *Aristolochia contorta* Bunge (Aristolochiaceae) grown *in vitro* and *in situ*

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## ABSTRACT

A comparative mesostructural analysis of leaves was performed for micro-clones and individuals of *Aristolochia contorta*, a rare medicinal plant, in juvenile and generative states. Micro-plants showed a cultural phenotype characterized by clones with poorly developed or absent root systems and relatively thick leaves with a thin epidermis and no epicuticular wax, along with high cell and plastid filling. Age-related mesostructural variation occurred in both juvenile and reproductive groups. With maximum cell and plastid filling, micro-clones fit the species' physiological optimum better than juvenile plants, representing compensatory adaptations to *in vitro* conditions. However, leaf blade vulnerability, unprotected epidermis, and weak roots were major causes of mortality during adaptation. Despite differences among the three variants, internal leaf structure remained conserved (dorsoventral mesophyll, equal palisade and spongy layers, etc.). Mesostructural analysis can reliably evaluate micro-clone quality in medicinal plants, particularly functional maturity.

**Keywords:** *Aristolochia contorta*, micro-cloning, reproduction, rare species, conservation, cultivation conditions, *in vitro*

## РЕЗЮМЕ

**Хроленко Ю.А., Юсупова Е.П., Наконечная О.В. Мезоструктура листа *Aristolochia contorta* Bunge (Aristolochiaceae) в условиях *in vitro* и *in situ*.** Проведён сравнительный мезоструктурный анализ листьев микроклонов и особей *Aristolochia contorta* – редкого лекарственного растения – в ювенильном и генеративном состояниях. У микрорастений выявлен культуральный фенотип, характеризующийся формированием клонов с недоразвитой или отсутствующей корневой системой и относительно толстыми листьями с тонким слоем эпидермиса и без эпикутикулярного воска, а также высокой клеточной и пластидной наполненностью листа. Возрастные изменения мезоструктурных признаков отмечены в обеих группах (ювенильной и репродуктивной). При максимальной клеточной и пластидной наполненности листа микроклоны *A. contorta* в большей степени соответствуют понятию физиологического оптимума для вида по сравнению с ювенильными растениями, что представляет собой компенсаторные адаптации к условиям *in vitro*. Однако уязвимость листовой пластинки, незащищённый эпидермис и слабая корневая система являются основными причинами гибели растений в период адаптации. Несмотря на выявленные различия между тремя изученными вариантами, внутреннее строение листа остается достаточно консервативным (дорсовентральный тип мезофилла, равное число слоёв палисадной и губчатой мезофилла и др.). Мезоструктурный анализ может использоваться как надёжный инструмент оценки качества микроклонов лекарственных растений, в частности для подтверждения их функциональной зрелости.

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

Viability of plants largely depends on their photosynthetic function. In most plants, the leaf is the main photosynthetic organ that has been formed in a specific way during evolution. The observed differences in the leaf anatomy are a manifestation of the ecological and climatic conditions under which the species has evolved (Kultiasov 1963). Vasilevskaya (1950) and Tyurina (1982) proposed studying the leaf ontogeny in order to identify the most important features that characterize the degree of specialization of the plant to environmental conditions. Previously, Tselniker (1978) showed that the photosynthetic function is positively correlated with such characteristics as the number of cells and chloroplasts per unit area of leaf. The structure of the assimilation tissue is shaped by the specifics of leaf ontogeny and is chosen as a diagnostic feature.

It has been found (Voronkova et al. 2008, Burkovskaya 2011, Markovskaya et al. 2015) that the quantitative parameters of the stomatal and assimilation apparatuses of the leaf vary between different habitat conditions. The structure of leaf mesophyll shows the greatest plasticity in ecological terms. Thus, the types of the mesophyll structure reflect the growing conditions of the species: the homogeneous

 

type is more commonly found in shade-requiring plants (sciophytes); the dorsoventral type is characteristic of meadow mesophytes; and the isolateral type is characteristic of xerophytes (Esau 1969, Gamaley 1988, Vasilevskaya & Butnik 1982).

Changes in the assimilation apparatus structure occur during *in vitro* cultivation, when micro-clones are grown under controlled conditions on a nutrient medium. Identification of the characteristics of micro-clones responsible for successful adaptation to the conditions of closed soil is a relevant issue, resolution of which will provide production of high-quality planting material, especially when dealing with rare species. One of such species is the northern pipevine, *Aristolochia contorta* Bunge, a herbaceous medicinal vine listed on the Red Data Book of Primorsky Krai (Nesterova 2008) where its status is categorized as "rare".

The present study aimed to identify species-specific features of the leaf mesostructure in *A. contorta* plants in the juvenile and generative ontogenetic states and in their micro-clones.

## MATERIAL AND METHODS

The studies were conducted at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences (FSCEATB FEB RAS, Vladivostok), in the sector of microclonal propagation of forest, agricultural, and ornamental plants in 2025–2026.

We studied the parameters of the leaf assimilation apparatus in micro-plants grown *in vitro*, juvenile plants, and reproductive individuals. An analysis of published data on the microclonal propagation of *Aristolochia*, presented earlier (Nakonechnaya & Volkonskaya 2023), and a selection of nutrient media and component concentrations for successful propagation of species of this genus (Nakonechnaya et al. 2024, 2025) showed stable proliferation of micro-shoots of *A. contorta* using the MS (Murashige & Skoog 1962) nutrient medium supplemented 6-benzylaminopurine (BAP) at a concentration of 0.5 mg/L. The media were autoclaved for 20 min at a pressure of 0.8 atm. Stem samples with one leaf node and two axillary buds were used as explants. Exposure to diocide (0.1 % solution) for 2–4 min was used for explant sterilization (Butenko 1964).

Juvenile plants were obtained from seeds after a long-term (24 months) stratification according to Nakonechnaya et al. (2018) and grown in closed-soil conditions. Micro-plants and juvenile plants were cultivated at 24 ± 1°C, with a 70 % air humidity, and a 16 h photoperiod under fluorescent lamps with an illumination intensity of 4 000 lux.

Leaves of generative plants of *A. contorta* were collected from a natural population in the valley of the middle Shkotovka River, Shkotovsky District, Primorsky Krai. There the plants grew in a floodplain forest (within an area of 100 × 25 m) and in areas of a broadleaf forest along a road (a plot of 200 × 40 m). The population consisted of approximately 70 individuals, of which three were in the generative state (coordinates 43°32.40'N 132°40.09'E).

We carried out anatomical studies using light and scanning electron microscopy. The analysis method was in accordance with the recommendations from the Ural State University (Borzenkova & Khramtsova 2006, Mokronosov & Borzenkova 1978). We made cuts of a specified size (15 discs per sample, diameter 3 mm) from the middle part of the leaf blade (between the midrib and the edge), fixed them in a 3.5 % glutaraldehyde solution in phosphate buffer (pH 7.0), and then prepared macerated tissues by heating them for a short time in a 50 % KOH solution. We counted numbers of cells of the palisade and spongy parenchyma in 90 squares of the Goryaev chamber grid (20 replicates). To count chloroplasts per cell (50 replicates), the fixed leaf discs from micro-plants were treated with 0.2 % Cellulysine (Cellulase Cellulysine from Trichoderma viride, Calbiochem) for 40 min at room temperature, followed by tissue maceration. Chloroplast count per cell was determined using a cell suspension prepared by macerating fixed native leaf discs in a 5 % chromium trioxide ($CrO_3$) solution in 1 M hydrochloric acid HCl (50 replicates). Hand-cut cross-sections of fixed leaves were examined under a Zeiss Axioskop-40 light microscope. Images were taken using a Zeiss AxioCam (HRs) digital camera with AxioVision 4.8.3 software. Leaf thickness and chloroplast dimensions (length and width) were then measured in the photomicrographs, using at least 16 replicates.

We processed the data using Statistica version 13 (StatSoft Inc., USA) and presented the results as means ± standard errors of the means (SEM). Group differences were evaluated by one-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test. Differences were considered statistically significant at $p < 0.05$.

## RESULTS

The mesostructural characteristics of leaves of *in vitro* grown micro-plants and plants in the juvenile and generative states are provided in Table 1. The analysis of plants in the generative state showed that their leaves were hypostomatic, with the dorsoventral mesophyll type.

**Table 1.** Mesostructural characteristics of *Aristolochia contorta* leaves

| Growth state | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | Leaf thickness, μm | Chloroplast volume, μm³ | Number of chloroplasts in | | Number of cells per 1 cm² leaf, $10^5$/cm² | Number of chloroplasts per 1 cm² of leaf, million |
| | | | palisade cells | spongy cells | | |
| Micro-plants | 133.36 ± 8.57 | 47.38 ± 5.09 | 14.54 ± 0.40 | 14.64 ± 0.46 | 10.09 ± 0.59 | 14.79±0.59 |
| Plants in the juvenile state | 78.42 ± 3.18 | 103.59 ± 9.62 | 14.00 ± 0.36 | 16.03 ± 0.84 | 4.36 ± 0.30 | 6.66±0.37 |
| Plants in the generative state | 184.50±9.04 | 42.20 ± 4.62 | 29.67 ± 0.90 | 22.57 ± 1.03 | 7.58±0.43 | 19.51±0.90 |

Quantification of *Aristolochia contorta* leaf characteristics is presented in Fig. 1 (such as variations in cell filling of leaf, see Fig. 1a) and anatomical characteristics of *A. contorta* leaves in Fig. 2. Of the three variants studied, the leaves of generative plants were characterized by the highest thickness values (Table 1) and were differentiated into palisade and spongy tissues. The palisade tissue was represented by a single layer of cells; the spongy tissue, by 2–3 layers. A similar differentiation into tissue types with the same numbers of layers was observed in juvenile plants and micro-plants. Leaf thickness differed significantly among the groups (Table 1).

The ratio of palisade to spongy tissue varied across the experimental groups (Fig. 1a). In the sequence of micro-plants, juvenile plants, and generative plants, the index changed to 0.46±0.03, 0.31±0.04, and 0.86±0.03, respectively.

A significant effect of growth conditions on chloroplast volume was found between the groups ($F(2, 45) = 24.87$, $p < 0.001$). Leaf chloroplast volume in micro-plants was 2.3-fold lower than in juvenile plants (Table 1), but did not differ significantly from that of plants in the generative state according to Tukey's test ($p = 0.854$).

Growth conditions significantly affected the number of spongy mesophyll chloroplasts ($F(2, 129) = 27.97$, $p < 0.001$). However, the two variants did not differ significantly from each other ($p = 0.474$, Tukey's test), and both exhibited lower values for this parameter than plants in the generative state (Table 1). The number of chloroplasts in palisade mesophyll cells differed significantly between the groups ($F(2, 126) = 192.09$, $p < 0.001$). Significant differences were observed between the groups in the number of cells per unit leaf area ($F(2, 77) = 74.07$, $p < 0.001$). The total plastid filling per unit leaf area in micro-plants was 2.2-fold greater than that in juvenile plants and, simultaneously, 1.3-fold lower than in generative plants (Table 1, Fig. 1b). The values of this characteristic differed significantly among the three groups ($F(2, 115) = 78.59$, $p < 0.001$).

## DISCUSSION

The analysis of the results showed a significant difference between the groups in the parameter "leaf thickness". The leaves in micro-plants were 1.7-fold thicker than those in juvenile plants (Table 1). The thicker leaves of micro-plants, compared to juvenile plants, were formed through an increase in cell size, since the number of mesophyll layers did not change. The leaves in generative plants also contained a single layer of palisade and 2–3 layers of spongy mesophyll cells, but the palisade cells were much longer and were represented by typical columnar cells that had the shape of a cylinder with rounded ends (Fig. 2). Under *in situ* conditions, the cells continued to grow in length for a long time until reaching their definitive size. Due to this, the contribution of the palisade parenchyma to the leaf mesophyll in generative plants exceeded that in the other two variants (Fig. 1a). Similar trends were reported in a study on *Filipendula camtschatica* grown *in vitro* (Khrolenko et al. 2015).

The analysis of the ratio of palisade to spongy tissue in a sequence such as micro-plants, juvenile plants, and generative plants, showed that this index varied across the experimental groups. Under the exposure to the hormone BAP, the leaf mesophyll in micro-plants proliferated, resulting in a lower palisade-to-spongy tissue ratio compared to that observed in generative plants. As the cells became rounded, the palisade layer looked more like a spongy tissue, and the clear boundary between them disappeared. Similar results were reported for other species in previous studies (Krasinskaya et al. 2010).

As previously documented (Borzenkova & Mokronosov 1976), plant hormones drive cell growth, upsetting the balance between cytoplasmic volume and chloroplast count, which signals the organelles to replicate. The plant hormones then trigger cytoplasmic ribosomes to promote chlorophyll synthesis and direct intra-chloroplast growth.

While the cytokinin BAP stimulates cell division to form new plant tissue, auxins promote growth by driving cell elongation (Butenko 1984, Kataeva & Butenko 1983, Kieber & Schaller 2014). BAP at a concentration of 0.5 mg/L induced cell division, and the leaves in micro-plants became significantly different in the number of cells per unit leaf surface area from the leaves in juvenile plants (Table 1). This value in micro-plants was 2.3-fold higher than that in juvenile individuals, which compensated for and exceeded

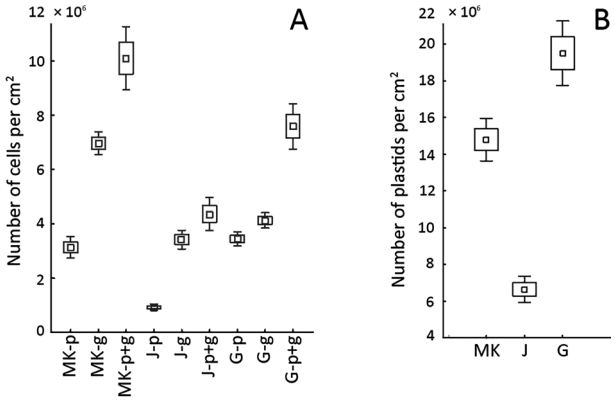


**Figure 1** Boxplot with *Aristolochia contorta* leaf characteristics. A – variation in cell filling of leaf: p – palisade cells; g – sponge cells; p+g – total; B – variation in plastid filling of leaf. MK – micro-plants; J – juvenile plants; G – generative plants

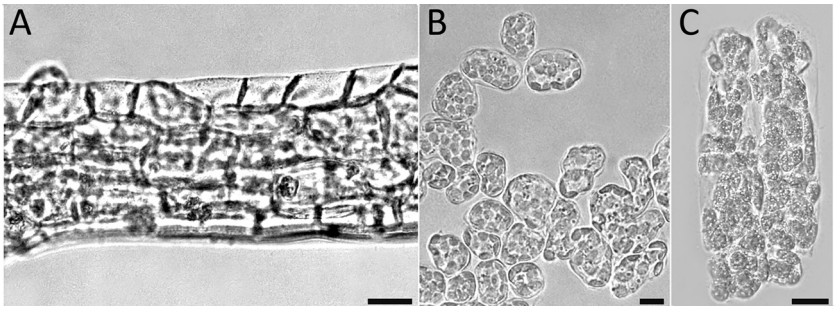


**Figure 2** Anatomical characteristics of *Aristolochia contorta* leaves: (A) transverse section through micro-plant leaf; (B) spongy micro-plant cells; (C) two palisade cells of generative plants. Scale bars: a – 20 μm; b, c –10 μm

the value of plastid filling of leaf by 1 $cm^2$, because the first two experimental variants did not significantly differ in the number of chloroplasts per cell (Table). According to Mokronosov (1978), such characteristic as the number of chloroplasts per cell is stable, genetically fixed, and its phenotypic variation within a single genotype can be 1.5–2-fold.

Application of 0.5 mg/L BAP did not change leaf chloroplast volume in micro-plants compared to the generative state (Table 1). The effects of various cytokinins (6-benzylaminopurine riboside [BAR], thidiazuron [TDZ], and metatopolin [TOP]) on the chloroplast ultrastructure, leaf anatomy, and photosynthetic pigment content of *in vitro* shoot cultures of two apple cultivars (HR and McI) were previously investigated by Máthéné Szigeti et al. (2026). BAR is a synthetic cytokinin plant growth hormone related to BAP. The results demonstrated that 4.5 μM BAR exerted moderate, cultivar-specific effects on the plastid apparatus. In the McI cultivar, BAR application increased grana diameter and repeat distance, whereas it showed no such effects in the HR cultivar. 4.5 μM TDZ induced oxidative stress, damaged chloroplasts, and lowered photosynthetic pigment content. Ultrastructural responses to various cytokinins and photosynthetic pigment content exhibited distinct cultivar-specific variations (Máthéné Szigeti et al. 2026).

As Vechernina et al. (2008) note, when cultivated on an artificial nutrient medium, plants are provided with mixotrophic nutrition: heterotrophic due to sucrose, and autotrophic through photosynthesis. However, since the tubes are sealed with plugs for sterility, the available $CO_2$ quickly runs out, which eventually inhibits the autotrophic growth. The $CO_2$ deficiency causes the adaptation of leaf assimilation tissue, changes in the functional activity of the photosynthesis apparatus, and in the state of the guard cells of stomata. Non-functioning stomata appear. However, the high (close to saturated) air humidity and the lack of a gradient of leaf water potential between the evaporating surface of the leaf and the atmosphere lead to inhibition of transpiration, which is the driving force of the process of liquid turning into vapor, and, consequently, the long-distance transport system. The authors (Vechernina et al. 2008) indicate that all of the above processes decrease the water retention capacity of regenerant leaves due to the lack of stomatal function, poorly developed cuticles, and reduced osmotic potential. With non-functioning stomata and decreased osmotic pressure, the roots become dehydrated on the agar medium and the water balance is disrupted. This causes *in vitro* grown plants to form a specific cultural phenotype, which is adaptive and differs from that of native plants in anatomical and physiological characteristics (Gigaloshvili et al. 1997).

The obvious difference in the leaf structure (in addition to the age-related variation between juvenile and adult individuals) may be associated with the difference in the mode of nutrition between plants grown *in vitro* and *ex vitro*. In the former case, regenerant plants grow on a nutrient medium under sterile and strictly controlled conditions of laboratory and, therefore, have the heterotrophic mode of nutrition. Juvenile and generative plants are characterized by the autotrophic mode of nutrition. The transition of the plant from one nutritional state to another is accompanied by its structural and functional restructuring under new conditions. Such a transition can influence the adaptation process and determine the survivability of micro-clones. Earlier, Chudetsky with co-authors (2022) noted that the high mortality rate of plants during the transition from *in vitro* to *ex vitro* is caused by the switch from the heterotrophic to autotrophic nutrition modes, the poor development of the root system, and the lack of waxy coating on leaves.

It is also worth mentioning that an analysis of the ecological optimum of the species has shown the maximum cell and plastid filling of the leaf. Micro-plants of *A. contorta* are more closely fit the concept of physiological optimum (maximum photosynthetic efficiency under the specified conditions) than juvenile plants. However, elevated cellular and plastidic density in micro-clones should be interpreted as a compensatory adaptation to *in vitro* stress rather than as a sign of a physiological optimum for the species. Besides, these plants are expected to better survive the period of adaptation to open-ground conditions after *in vitro* cultivation, which is the most challenging and risky stage of cultivation. For example, even after one-month *ex situ* cultivation, Saskatoon berry micro-plants retained the same number of mesophyll layers, though they exhibited development of the adaxial cuticle and a slight elongation of epidermis cells and spongy chlorenchyma (Raeva-Bogoslovskaya et al. 2023).

From a physiological point of view, the ecological optimum of the species causes the most developed leaf structure to form under favorable conditions. It is manifested as the maximum assimilation surface created inside the leaf, which provides maximum conductivity for $CO_2$ and maximum photosynthesis rate (Tselniker 1978, Ivanova & Pyankov 2002).

Thus, micro-plants are characterized by a high cell and plastid filling of the leaf, which hypothetically should provide greater photosynthetic activity of micro-clones compared to juvenile plants. Such micro-plants represent largely a cultural phenotype shaped by *in vitro* conditions, which is not found in natural habitats. Leaves of micro-clones contain a large number of small, densely packed cells that, in turn, include numerous chloroplasts. However, when plants switch to autotrophic nutrition, the thin epidermis without epicuticular wax cannot protect their leaves from drying out, damage, etc., which subsequently results in the death of clones. The decrease in the intensity of water evaporation by leaves, because they lose their capability of transpiration during the adaptation to test tube conditions, induces a number of physiological processes that slow down the entire long-distance transport of water and salts in the plant. A properly designed scheme for transferring plants from sterile test tube conditions to closed-soil conditions will help micro-clones gradually adapt. The leaf is a photosynthetic organ with all processes interrelated in the whole plant, and, as the main processes are activated, the assimilation system becomes integrated with them.

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