

# Morphophysiological dormancy, germination, and cryopreservation in *Aristolochia contorta* seeds

Nina M. Voronkova<sup>1</sup>, Alla B. Kholina<sup>1,\*</sup>, Marina N. Koldaeva<sup>2</sup>,  
Olga V. Nakonechnaya<sup>1</sup> & Vitaliy A. Nechaev<sup>1</sup>

<sup>1</sup> Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, 159 Prospect 100 let Vladivostoku, Vladivostok, 690022, Russian Federation

<sup>2</sup> Botanical Garden-Institute, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Makovskii Str., 142, 690024, Russian Federation

\*Author for correspondence: [kholina@biosoil.ru](mailto:kholina@biosoil.ru)

**Background and aims** – *Aristolochia contorta* is a valuable medicinal plant, a relict of the Tertiary flora. Little is known about the germination biology of *Aristolochia*. The specific objectives of the present study were to (1) determine the type of dormancy in seeds of *A. contorta*, (2) describe the embryo development, and (3) explore the influence of deep freezing of the seeds in liquid nitrogen on their germinability.

**Methods** – Seeds were germinated in Petri dishes in sand previously sterilised at high temperature; germination experiments were carried out at  $27\pm 2^\circ\text{C}$  under natural light. All measurements of seeds and embryos were done using light microscopy (LM). For cryopreservation, fresh seeds were placed in aluminium foil bags, immersed into liquid nitrogen ( $-196^\circ\text{C}$ ), and stored for twelve months.

**Key results** – The seeds of *Aristolochia contorta* have non-deep simple morphophysiological dormancy. A variety of embryo forms were revealed for *Aristolochia* species for the first time. Two cases of polyembryony were noted in *A. contorta*. The seeds of *A. contorta* are resistant to cryopreservation in liquid nitrogen.

**Conclusions** – High variability in dormancy depth and the extended germination period of *A. contorta* seeds can be considered as adaptive strategies for survival in unfavourable conditions and renewal of germination under optimal conditions. Cryopreservation helped maintain the viability of *A. contorta* seeds but did not lead to the breaking of the dormancy; hence, for successful germination, it is necessary to use methods of breaking dormancy after freezing.

**Key words** – *Aristolochia contorta*, germination, seed dormancy, seed cryopreservation, embryo form, embryo development.

## INTRODUCTION

The presence of relict species, which are rare and endangered, is typical for the southern portion of the Russian Far East (Kurentsova 1968, Artyukova et al. 2012). Unlike many other regions at the same latitude, most of this area was not glaciated during the Pleistocene. Rare and endangered species are restricted to certain habitats, or their northern distribution limits are located in the region. Many of them have already become disappearing as a result of increasing anthropogenic pressure (Artyukova et al. 2012). The extinction of rare plants from their native habitats has led to the disruption of ecosystems and has impoverished the biodiversity of the region. The study of the reproduction systems of relict plants is necessary for the preservation of their gene pools and for reintroduction or cultivation. Medicinally valuable

plants are of particular interest. One of these plants is *Aristolochia contorta* Bunge (Aristolochiaceae), which is a relict vine of Tertiary flora (Kharkevich 1987, Nakonechnaya et al. 2014). This plant survives in refugia in the southern portion of the region. The rhizomes and fruits of *A. contorta* are used both in Chinese medicine to treat bronchial asthma, cough, and inflammation of the vocal cords and in herbal combinations to treat thyrotoxicosis (Akulova & Alexandrova 1996). The fruits are used to lower blood pressure, clean the liver and lungs, stop intestinal bleeding, and remove oedema during inflammation of the lungs (Zhou et al. 2011). Some compounds isolated from *A. contorta* also have strong cytotoxic activity (Zhang et al. 2005).

The northern boundary range of *A. contorta* is situated in Primorsky Krai, or Primorye, which is the south-eastern-

most region of Russia, and located between the 42° and 48° north latitude and 130° and 139° east longitude. In this portion of the range, small populations of *A. contorta* are suppressed, and their numbers are diminishing due to forest fires and uncontrolled plant harvest. This species requires specific habitats in sparse floodplain forests along river banks in the coastal zone up to 100 m (Nakonechnaya et al. 2010). In Primorye, *A. contorta* localities are isolated from the main range, which is situated on the Korean Peninsula, on the Japanese Islands and in China (Ohwi 1965, Kharkevich 1987, Oh & Pak 2001, Hwang et al. 2003). The species is recorded in the 'Red Book of the Primorsky Krai' and has a status of 'vulnerable' (Nesterova 2008). *Aristolochia contorta* is a unique component of ecosystems because it is the only nutrient for the relict butterfly *Sericinus montela* Gray, 1852 (Beljaev & Chistyakov 2005). The study of the initial stages of seed reproduction is critical to the research of regressing Tertiary relicts. The examination of the conditions of long-term storage of seeds is also very important. The identification of the type of seed dormancy, the definition of seed lifespan, and the possibility of seed cryopreservation are all unexplored issues with respect to *A. contorta*.

There are approximately 400 species of *Aristolochia* (González & Stevenson 2002). However, little is known about seed germination in this genus. In 1996, the germinability of *A. manshuriensis* Kom. was 85% after sowing in autumn (Voronkova et al. 1996). The seedling emergence percentage of *A. serpentaria* L. was 58% under common gardening conditions and 14% under field conditions (Dávalos et al. 2015). Adams et al. (2005a) published 'the first report about the seed dormancy and germination for the *Aristolochia* genus'. These authors studied the seed germination of four species in the laboratory: *A. californica* Torr., *A. macrophylla* Lam., *A. manshuriensis* Kom., and *A. tomentosa* Sims. In 2006, the seed germination data of *A. baetica* L. and *A. paucinervis* Pomel were presented (Berjano Pérez 2006). Since then, only six studies pertaining to *Aristolochia* seed germination have been published: *A. triangularis* Cham. (Scalon et al. 2007), *A. esperanzae* Kuntze (Maekawa et al. 2010), *A. galeata* Mart. (Alves-da-Silva et al. 2011), *A. fimbriata* Cham. (Bliss et al. 2013), *A. debilis* Siebold & Zucc. (Zhou et al. 2014), *A. gigantea* Mart. (Gois & Almeida 2016). The seeds of some species of *Aristolochia* are in a state of dormancy (Adams et al. 2005a, Alves-da-Silva et al. 2011). This state prevents the premature appearance of the seedlings, promotes the formation of a soil seed bank, and conserves the gene pool of the species. However, the delay of seed germination impedes the restoration of valuable, medicinal, and rare plant species; therefore, it is very important to study the conditions of seed germination and dormancy (Nikolaeva 2001, Baskin & Baskin 2004).

Furthermore, to prolong the period of seed viability for rare species, it is necessary to study the methods of long-term seed storage. The germinability of seeds quickly decreases if they are stored at room temperature and under conditions of high humidity, which are typical for the southern portion of the Far East due to the monsoon climate. Therefore, it is necessary to use another method to store *A. contorta* seeds. In particular, cryopreservation (deep freezing of seeds in liquid nitrogen at -196°C) is the most prospective method. The

positive results obtained thus far regarding the cryotolerance of far-eastern plant seeds, including rare plants (Kholina & Voronkova 2008, 2012, Voronkova & Kholina 2010), suggest that *A. contorta* seeds can resist ultralow temperatures.

We previously obtained data on the fruit structure and seed morphology of *A. contorta* (Nechaev & Nakonechnaya 2009), as well as on seedling morphology (Nakonechnaya et al. 2012), embryo structure, and seed anatomy (Nakonechnaya et al. 2013). Mature seeds of *A. contorta* contain a linear embryo that has a well-defined axis and two cotyledons (Nakonechnaya et al. 2013). Based on the examination of seeds from different populations of the Russian part of the *A. contorta* range, we concluded that the linear form of the embryo is not the only possibility.

Our goal was to address the following questions: (1) Do fresh, mature seeds of *A. contorta* experience dormancy? If so, what class, level, and type of seed dormancy occur? (2) How do the embryos elongate and develop during the seed germination process? and (3) Can the seeds tolerate ultralow temperatures during cryopreservation?

## MATERIALS AND METHODS

The birthwort *Aristolochia contorta* Bunge is a herbaceous perennial vine that belongs to the subsection *Euaristolochia*, section *Diplolobus* Ducharte, subgenus *Orthoaristolochia* Schmidt, genus *Aristolochia* L., tribe Aristolochieae Schmidt, subfamily Aristolochioideae Schmidt, family Aristolochiaceae Juss. (Schmidt 1935, González & Stevenson 2002). *Aristolochia contorta* blooms in July and August, the seeds ripen in September and the wing-shaped form of the seeds promotes dissemination via the wind. At the same time, some of the seeds fall into water, and river water carries them away, especially during abundant autumn rainfalls (Nechaev & Nakonechnaya 2009). We collected mature fruits of *A. contorta* by random sampling in the valley basins of the Razdolnaya, Shkotovka and Artemovka Rivers in the southern portion of the Russian Far East.

A batch of fifty seeds was subjected to moisture determination via oven drying at 105°C for 24 h until the seeds had attained a constant dry mass. The moisture content was obtained from three independent determinations and expressed as a mean percentage of the fresh weight.

To study the germination dynamics, three replicates each of fifty seeds were sown in Petri dishes containing sand previously sterilised at high temperature; the seeds were then watered daily with tap water. The tests were monitored every day, and germinating seeds (radicle extension  $\geq 2$  mm) were counted and removed. Because the optimal germination conditions for *A. contorta* have not yet been reported, we attempted to germinate the seeds at 23°C according to the recommendations of Nikolaeva et al. (1992), which suggested using a temperature of 18°C or greater for the germination of seeds of thermophilous plants. First, the seeds were germinated at a constant temperature of 23°C for 7 months. In the remaining experiments, the seeds were germinated at 27±2°C under natural light. To check the need for cold stratification, the seeds after sowing were stored at 2°C on a wet substrate for eight months, after which they were

**Table 1 – Germination of *Aristolochia contorta* seeds under different storage periods.**

Collection of seeds: October 2010, storage before sowing in laboratory; \* period from sowing until emergence of first seedling; \*\* germination in two stages with a pause between stages: first date: at the first stage, before pause; second date: at the second stage, after pause.

Number of test	Storage period, months	Sowing time	Start of germination (months*)	Pause (months)	Germinability %	Final germinability %
1	2	Dec. 2010	Apr. 2011 (4)	–	65.0±5.7	65.0±5.7
2**	12	Nov. 2011	1 Apr. 2012 (5) 2 May 2013 (18)	Sep. 2012 – May 2013 (9)	1. 82.2±8.0 2. 15.5±9.1	97.8±1.1
3	16	Feb. 2012	Apr. 2012 (2.5)	–	61.1±7.3	61.1±7.3
4	19	May 2012	Jun. 2012 (1)	–	60.0±6.9	60.0±6.9
5**	22	Aug. 2012	1 Sep. 2012 (1) 2 Apr. 2013 (7)	Nov. 2012 – Apr. 2013 (5)	1. 13.3±5.2 2. 32.4±5.7	45.7±6.3
6	25	Nov. 2012	Apr. 2013 (5)	–	37.3±4.7	37.3±4.7
7	26	Dec. 2012	Mar. 2013 (3)	–	28.0±2.0	28.0±2.0

transferred to laboratory conditions at  $27\pm 2^\circ\text{C}$ ; the control seeds remained in the laboratory during the entire period. To define the lifespan of the seeds, those collected in 2010 were germinated in December 2010; the experiment was repeated a year later in November 2011, after which the seeds were germinated every three months (table 1). In total, the experiments on germination were carried out for three years. The seeds were moistened with tap water when required during the course of the experiment. To determine the effects of low winter temperature on germinability, we collected seeds from fruits that wintered under natural conditions and used those seeds for germination experiments for two years.

To study the effect of gibberellin ( $\text{GA}_3$ ) on breaking of dormancy of seeds, the seeds were immersed in a  $\text{GA}_3$  solution (Gibberellic acid 90% PS, Panreac Quimica, Espana) that had a concentration 250 mg/L for 24 h prior to sowing. In the control test, the seeds were immersed in distilled water, and they were then desiccated and germinated; the experiment period was 3 months.

To study embryo growth, we focused on 290 seeds; to describe embryo forms, we examined 128 embryos. We conducted the experiment under the same conditions as those used for the germination tests. Observations of embryo growth were made every monthly; beginning with the protrusion of the radicle (the beginning of germination), germinated seeds were collected daily. Embryo growth was measured in the dry seeds after dispersal (stage I) and at three germination stages: stage II – seed coat rupture with no radicle protrusion, stage III – radicle tip protrusion; and stage IV – cotyledons emerging from seed coat. For the embryo growth rate measurements (absolute and the ratio to seed size, without the wing), we focused on 25 seeds at each stage. Selected seeds and seedlings were preserved in 70% ethyl alcohol. All of the embryo and seed measurements were obtained using a stereomicroscope Stemi 2000C (Carl Zeiss) with a digital camera AxioCam Icc 3 and software AxioVision 4.8.2. The results are provided as mean values with a standard error.

For cryopreservation, fresh seeds were placed in tightly closed aluminium foil bags, immersed directly into liquid nitrogen ( $-196^\circ\text{C}$ ), and stored for twelve months. The seeds were then warmed at room temperature for 2 h and placed in Petri dishes containing sand simultaneously with the control

seeds. The unfrozen control seeds were kept in paper packets in the laboratory. In the experiment and control, the seeds were germinated using three replicates each of fifty seeds.

We calculated germinability as the ratio of the number of germinated seeds to the total number of tested seeds (in %). The results are presented as mean values with a standard error. The reliability of the difference between the control and experimental values was assessed using the Student's *t*-test at  $P = 0.05$  (under  $n = 3$ ,  $t \geq 2.78$ ).

## RESULTS

### Germination

The germination test, based on known recommendations (Nikolaeva et al. 1992), revealed that the seeds did not germinate during long periods at a constant temperature of  $23^\circ\text{C}$ . After being subjected to a higher temperature ( $27\pm 2^\circ\text{C}$ ), the seeds began to germinate after two weeks; germinability was  $49.3\pm 4.4\%$  after 45 days. The cold stratification test revealed that control seeds had a germinability of 36.7%, and the stratified seeds had a germinability of 32%. We conclude that cold stratification does not promote the overcoming dormancy. In view of these test results, a subsequent germination study was conducted at  $27\pm 2^\circ\text{C}$  without cold stratification.

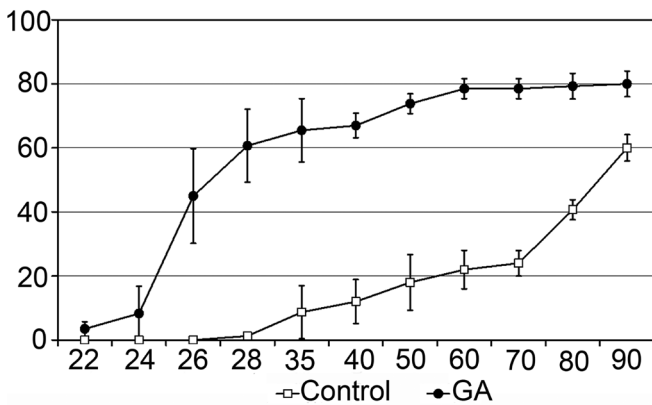
The 3-year germination tests of seeds collected in 2010 revealed that the most optimal temperatures for starting germination are within the range of  $26\text{--}29^\circ\text{C}$ . Seeds do not begin to germinate earlier than 1 month after sowing or later, depending on the moment of sowing (table 1) (i.e. they need warm stratification). We then observed extended germination for 3–5 months, or germination in two stages with a long pause between stages (test 3: 9 months, test 5: 5 months). We found that the most active germination occurred in April and was independent of the sowing time. Even if the seeds were sown in August, a few seeds germinated in autumn, but the majority of them germinated in April after the pause (test 5). High germinability was maintained for 1.5 years by storing seeds under laboratory conditions; after two years of storage, germinability decreased by approximately half (table 1).

In nature, not all seeds become separated from the fruits. A portion of seeds remain in the fruits during winter and are exposed to the effects of negative temperatures. When

**Table 2 – Germination of *Aristolochia contorta* seeds after action of negative winter temperatures.**

\* period from sowing until emergence of first seedling.

Number of test	Time of collection and sowing	Start of germination (months*)	Germinability %
1. (2011 seed harvest)	Feb. 2012	Apr. 2012 (2)	65.3±4.8
2. (2012 seed harvest)	Apr. 2013	May. 2013 (1)	72.0±2.0



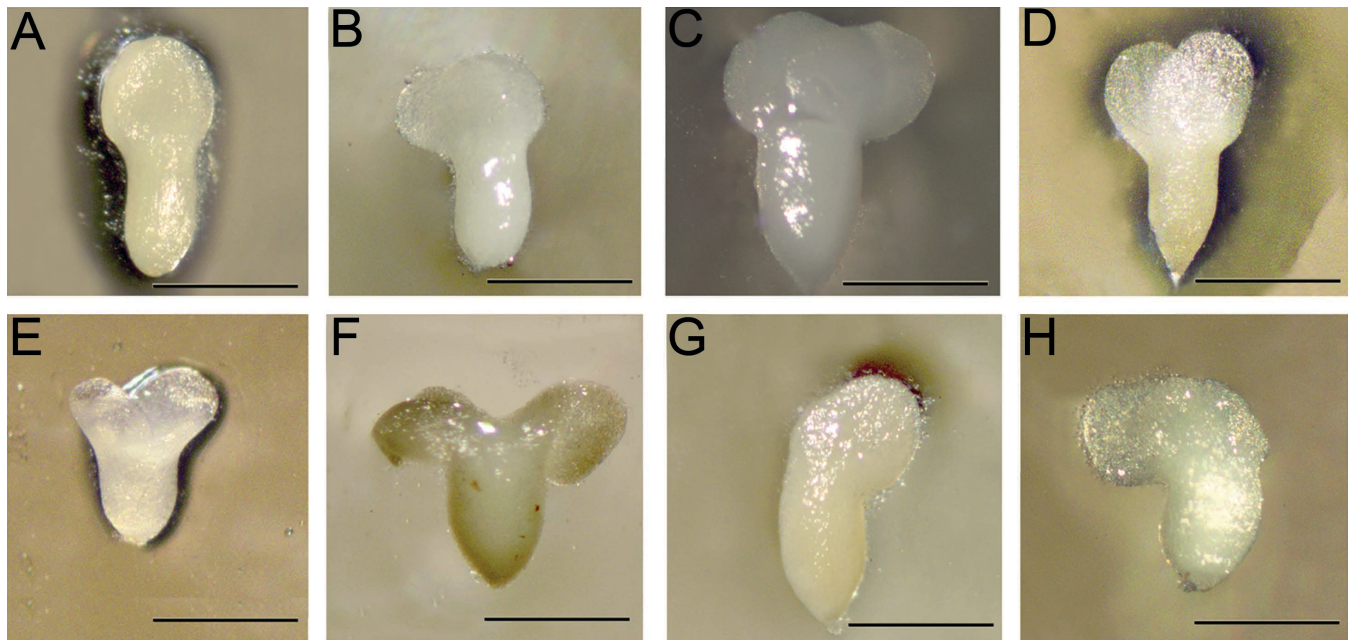
**Figure 1 – Effect of the gibberellin treatment on *Aristolochia contorta* seed germination.** Control: germination without treatment; GA<sub>3</sub>: seeds treatment by solution GA<sub>3</sub> (250 mg/l) during 24 h. On Y-axis: germinability, %; on X-axis: period of germination, days.

sowing seeds that were previously exposed to negative winter temperatures, the seeds began to germinate at the same time as those collected in autumn and those collected during April–May; these seeds also needed warm stratification (table 2).

The GA<sub>3</sub> treatment of seeds prior to sowing stimulated germination and the overcoming of dormancy (fig. 1). The first seedlings appeared after twenty days, the germination rate was high, and the final germination reached 80%, compared with 60% in the control. The differences observed for germinability were significant (t = 6.55).

**Embryo form**

In the studied embryos (136 in total), we observed different positions of the cotyledons with respect to the embryo axis, which causes variability of embryo forms. The forms could be classified into four types (figs 2 & 3). The first form is linear: the embryo is straight, and the axes of both cotyledons coincide with the embryo axis (figs 2A & 3A); this form was found in 43.7% of the seeds dissected. The second form is linear with a deviated cotyledon: the axis of one of the two cotyledons coincides with the embryo axis, and the second cotyledon deviates to the side in the plane of the seed (figs 2B, C & 3B, C); this form was found in 10.2% of the seeds. The deviation angle varied from 20–150° but was often small (20°, 30°, 45°). Such a form was noted earlier in our study (Nakonechnaya et al. 2013). The third form is fan-shaped: both cotyledons symmetrically deviate into opposite sides from the seed axis (figs 2D–F & 3D–F); this form was observed in 39.1% of the seeds. The angle between the cotyledon axes varied from 20 to 120°. Fan-shaped forms with angles of cotyledon divergence equal to 20–30° and 90–100° were most often noted. The fourth form is angular (knee type): both cotyledons deviate from the seed axis on the same side at the same angle of slope (figs 2G, H & 3G,



**Figure 2 – Embryo forms:** A, linear; B & C, linear with deviated cotyledon; D–F, fan-shaped form; G & H, angular. Scale bars: 0.5 mm.

**Table 3 – Change in the size of *Aristolochia contorta* embryo during germination.**

\* Embryo growth stages: I, embryo in the dry seeds; II, seed coat rupture, before radicle emergence; III, radicle protrusion; IV, cotyledon emergence; \*\* on stage IV the embryo length minus radicle/seed length ratio is given.

Size, mm	Embryo growth stage*			
	I	II	III	IV
Seed linear length (minus wing)	4.36±0.05	4.36±0.08	4.36±0.17	4.73±0.96
Embryo linear length	0.69±0.03	2.35±0.05	3.80±0.12	35.54±2.54
Embryo linear length / seed linear length ratio	0.16±0.01	0.54±0.01	0.66±0.03	0.93±0.01**
Cotyledon length	0.32±0.01	1.40±0.03	1.76±0.14	4.76±0.20
Axial structure length (hypocotyl+radicle)	0.38±0.02	1.03±0.03	1.74±0.05	31.33±2.44
Cotyledon length / radicle length ratio	0.84±0.03	1.38±0.04	2.10±0.15	0.16±0.01

H); this form was observed in 7% of the seeds. The angle of deviation from the embryo axis ranged from 15 to 90°, and the most frequent angles were 20° and 35°.

### Embryo development

In our data pertaining to freshly harvested seeds, the length of the embryo encompassed 16% of the total length of the mature seed (table 3), which was slightly less than that reported in previous studies (Nakonechnaya et al. 2013). During warm stratification, the embryo length increased by more than three-fold (table 3); by the development stage II (seed coat rupture stage, fig. 4E & F) the embryo length reached more than half the seed's length (54%). Embryo postdevelopment (i.e. the process in which the underdeveloped embryo grows and becomes fully developed inside the seed; Nikolaeva 2001) proceeded slowly prior to germination (1–18 months). The gradual growth of all embryo parts was observed, and the growth intensity differed. The cotyledons increased in sizes more rapidly than did the axial structure (i.e. the embryo grew mainly due to the elongation of the cotyledons). By the start of germination (stage II), the length of the

cotyledons exceeded the hypocotyl with radicle by 1.3-fold (seed coat rupture stage, fig. 4E & F).

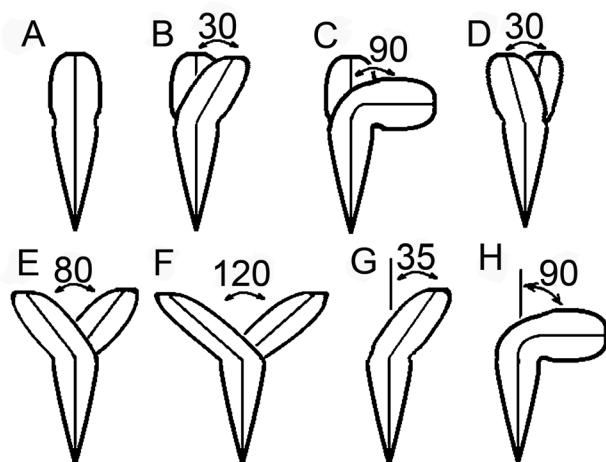
During germination (stages II–III), the cotyledons elongated by 126%, and the hypocotyl with radicle increased in size by 169% (table 3). The period from coat rupture, which indicates the start of germination (stage II), to radicle emergence (stage III) ranges from two to fourteen days. During this period, embryo changes consist mainly of the elongation of the axial structure (hypocotyl and radicle). Since germination (stage III), the embryo length had increased by 160% and encompassed 66% of the seed length (table 3, fig. 4G & H).

The development of the axial structures, hypocotyl and radicle, and cotyledons, which were still in the seed coat, proceeds very rapidly prior to stage IV. The period between stages III and IV varies from two to ten days. During this period, the seedling actively uses the nutrient substances of the seed through the cotyledons: the endosperm loses its dense structure and decreases, and the released space is occupied by the cotyledons, which significantly increase in size; their bases start emerging from the seed coat (fig. 4I & J).

Two cases of polyembryony were observed in *A. contorta* (fig. 5A & B). The seed contained two viable embryos – one of them smaller in size. The cotyledons of each of the two embryos differed in size, but these differences were greater than the differences in the cotyledon size of the normal embryos.

### Cryopreservation

The moisture content of *A. contorta* seeds was 5.63±0.08%. The germinability after deep freezing and of the control seeds, observed during two stages of germination, is presented in fig. 6. After twelve months of cryopreservation and control storage under laboratory conditions, the seeds were sown in November in the laboratory at 27±2°C; these seeds began to germinate simultaneously in April (after five months). By autumn, the seeds had stopped germinating and restarted only during the following year; the pause between the germination stages for the control seeds was nine months and for the frozen seeds was 10.5 months. The total period of seed germination was 21 months for the control seeds and 22 months for the experimental seeds. The final germinability



**Figure 3 – Diagram of the embryo forms: A, linear; B & C, linear with deviated cotyledon; D–F, fan-shaped form; G & H, angular.**

was rather high in the control (97.8±1.1%); after freezing, germinability reached 93.3±3.9%. No significant differences in germinability were observed ( $t = 1.52$ ).

DISCUSSION

Germination and seed dormancy

The study of germination biology consists of four primary aspects: identification of the type of seed dormancy, determination of the optimal conditions necessary for germination, identification of the characteristics of both germination

dynamics and germinability, and definition of the period of seed viability conservation. Germination delays associated with the properties of the embryo refer to the endogenous type of dormancy.

The embryo of *A. contorta* is well differentiated, and the embryo length is one-fifth of the seed length. In accordance with the classification of Baskin & Baskin (2004), the embryo of *A. contorta* can be described as underdeveloped: the embryo is differentiated (i.e. it has a discernible axis and two cotyledons), but does not encompass one-half of the seed length. Underdeveloped embryos, which are typical of many ancient angiosperms, are the cause of the morphological dormancy (MD) (Nikolaeva 2001). To break MD, it is necessary for the embryo to undergo post-development, which occurs in imbibed seeds at 15–30°C under the influence of warm stratification (Nikolaeva 2001, Baskin & Baskin 2004). If full development of the embryo occurs within 1–2 weeks and the seeds germinate within thirty days, they have only MD; if a further delay in germination due to the physiological state of the embryo is observed, this dormancy is defined as morphophysiological (MPD) (Nikolaeva 2001, Baskin & Baskin 2004). When only warm stratification is necessary for embryo development, MPD is simple, but if cold stratification or a combination of both cold and warm conditions occurs, MPD is considered to be complex. If MPD is non-deep,

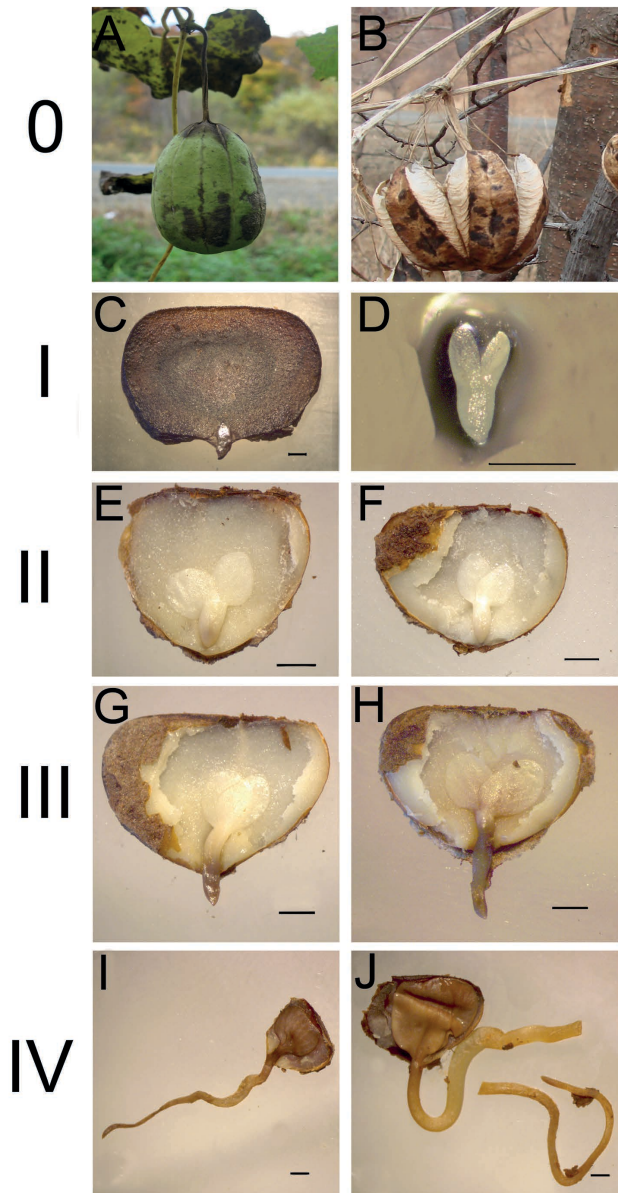


Figure 4 – Embryo development stages in *Aristolochia contorta*. Stage 0: A, fruit; B – dehiscent dry fruit. Stage I: C, freshly harvested seed; D, embryo in freshly harvested seed. Stage II: E & F, embryo in seed coat rupture stage. Stage III: G & H, embryo in radicle tip protrusion stage. Stage IV: I & J, seed in cotyledons emerging from seed coat stage. Scale bars: C, D = 0.5 mm; E–J = 1 mm.

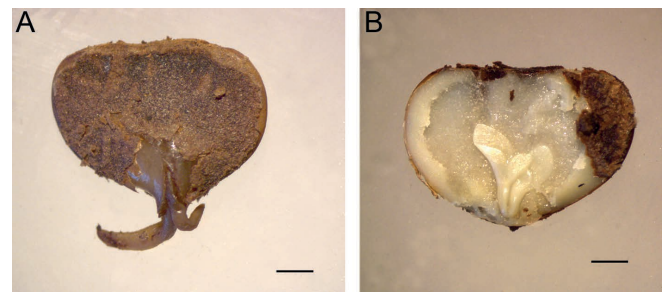


Figure 5 – A & B, two cases of polyembryony in *Aristolochia contorta*. Scale bars: 1 mm.

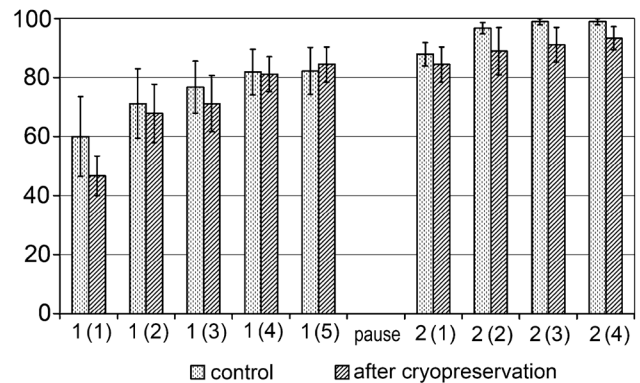


Figure 6 – Effect of the cryopreservation on *Aristolochia contorta* seed germination. Control: germination without preliminary treatment; after cryopreservation: pre-sowing freezing of the seeds in the liquid nitrogen (-196°C) during twelve months. On Y-axis: germinability (%); on X-axis: period of germination; 1: before pause, 2: after pause; in parentheses: number of months.

**Table 4 – Seed germination of *Aristolochia* species.**

\* period from sowing until emergence first seedling; \*\* use of slash means the fluctuating temperature treatment, day/night; n-dash (–): no data, WS: seeds were germinated without stratification; MD, MPD: morphological and morphophysiological dormancy. The names of subgenera and sections are given according to Schmidt (1935), González (1991), González & Stevenson (2002).

Subgenus, section, species	Start of germination, days*	Germinability, %	Temperature of germination, °C**	Note	Dormancy	Reference
Subgenus <i>Orthoaristolochia</i> O.C.Schmidt						
Section <i>Diplobolus</i> Duch.						
<i>A. contorta</i> Bunge	120	65.0±5.7	27±2	WS	Non-deep simple MPD	Our data
<i>A. baetica</i> L.	70–80	50.0±8.37	24/19	WS, summarized after 2 periods	MD and non-deep simple MPD	Berjano Pérez (2006)
<i>A. debilis</i> Siebold & Zucc.	11–19	64	25±2	WS	MD	Zhou et al. (2014)
<i>A. paucinervis</i> Pomel	–	75.2±6.3	24/19	Only after GA <sub>3</sub> treatment	Intermediate complex MPD	Berjano Pérez (2006)
Section <i>Gymnolobus</i> O.C.Schmidt						
<i>A. esperanzae</i> Kuntze	–	96	30	WS	MD	Maekawa et al. (2010)
<i>A. fimbriata</i> Cham.	–	95	27/18	WS	MD	Bliss et al. (2013)
<i>A. galeata</i> Mart.	12	62±14.4	27/20	WS	MD and non-deep simple MPD	Alves-da-Silva et al. (2011)
<i>A. gigantea</i> Mart.	9	50	28±2	WS, after pre-germination treatment	MD	Gois & Almeida (2016)
<i>A. triangularis</i> Cham.	16	78.9	30/20	WS	MD	Scalon et al. (2007)
Subgenus <i>Siphisia</i> O.C.Schmidt						
Section <i>Siphisia</i> (Raf.) Duch.						
<i>A. californica</i> Torr.	– 6–7	74 97–98	10 15	WS after warm+cold stratification	Intermediate and deep complex MPD	Adams et al. (2005a)
<i>A. macrophylla</i> Lam.	10 3–4	60–70 95–98	25/15; 30/15 25/15	WS after cold stratification	MD and non-deep simple MPD	Adams et al. (2005a)
<i>A. manshuriensis</i> Kom.	– 6–7	53 95–98	25/15; 15/6 25/15	WS after cold stratification	MD and non-deep simple MPD	Adams et al. (2005a)
<i>A. tomentosa</i> Sims	10 3–4	78 97–100	35/20 25/15	WS after cold stratification	MD and non-deep simple MPD	Adams et al. (2005a)

MPD can be easily broken by gibberellin treatment of the seeds (Nikolaeva 2001, Baskin & Baskin 2004).

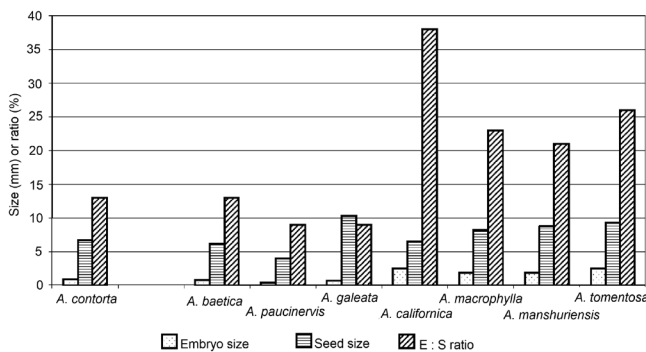
The presence of MD or MPD has been described for previously studied *Aristolochia* seeds (table 4). Some of these seeds exhibited MD only, and another fraction of the seeds demonstrated a continuum of physiological states according to the degree of dormancy depth (Adams et al. 2005a, Berjano Pérez 2006, Alves-da-Silva et al. 2011). *Aristolochia galeata* seeds of the subgenus *Orthoaristolochia* (of which *A. contorta* is also a member) exhibit high variability in their time to germination (Alves-da-Silva et al. 2011). According to Zhou et al. (2014), *A. debilis* exhibits increased germinability by more than 90% if treated with different concentrations of GA<sub>3</sub>.

By studying *A. contorta* seeds, we also determined a variety of dormancy states during germination. Our results revealed that the seeds start to germinate in 1–5 months after being sown under warm temperatures and that the seeds have an extended period of germination, sometimes with the long pause. Based on the obtained data, the dormancy of *A. contorta* seeds can be referred to as the non-deep, simple morphophysiological type (C<sub>1b</sub>B–C<sub>1b</sub>, according to the classification of Nikolaeva 2001), and dry storage with warm stratification conditions are sufficient to break this dormancy. The effectiveness of treating *A. contorta* seeds with GA<sub>3</sub> indicates a slight level of the depth of MPD of this species. The seeds are evidently in different states of dormancy due to the level of the MPD depth in a similar manner as seeds of

*Aristolochia* species studied previously (Adams et al. 2005a, Berjano Pérez 2006, Alves-da-Silva et al. 2011). High variability in the depth of dormancy and the extended germination period of *A. contorta* seeds can be considered to be adaptive strategy for both survival in unfavourable conditions and the renewal of germination under optimal conditions. A similar strategy of *A. galeata* seeds associated with dry spells in Brazil, according to the authors (Alves-da-Silva et al. 2011), is 'ecologically advantageous' and allows seedlings to survive due to their gradual emergence.

The activation of *A. contorta* seed germination in April, regardless of habitat, year, and time of collection, indicates seasonal dynamics of germination. The stored seeds of *Dendrocalamus strictus* Nees (Rawat & Thapliyal 2003) and *Schismus arabicus* Nees exhibited similar annual periodicity in germinability, possibly regulated by endogenous rhythm (Bai et al. 2015 and references herein). However, the seeds of *A. contorta* cannot germinate in April under natural conditions because of the low temperatures in the southern portion of Primorsky Krai. Suitable temperatures in the environment arrive only much later, which reduces the period of seedling establishment before the cold winter season. Our data from studying *A. contorta* ontogenesis under greenhouse conditions revealed that only 11% of seedlings entered into subsequent ontogenetic state (immature state) by the end of the vegetative period in October (Nakonechnaya et al. 2012). It is likely that most of the seedlings and juvenile plants die with the coming of winter in nature. Difficulties in the germination process related to mismatched climate conditions that differ from optimal conditions for *A. contorta* may constitute one of the reasons that the species is rare in nature.

The germination level of fresh *A. contorta* seeds is moderate (within 40–60%) (table 1, fig. 1). However, nearly all the seeds from 2010 stored for twelve months germinated (germinability was 97.8%, table 1, fig. 6). The germinability of the *Aristolochia* species previously studied ranged from 50 to 100% at germination temperatures of 25–35°C (table 4), and the germinability of the species from the subgenus *Orthoaristolochia*, section *Diplolobus* under the same conditions was similar and ranged from 50 to 75%.



**Figure 7** – Mean length for fresh embryos and seeds, and their ratio, of *Aristolochia* species: *A. contorta* (our data), *A. baetica*, *A. paucinervis* (Berjano Pérez 2006), *A. galeata* (Alves-da-Silva et al. 2011), *A. californica*, *A. macrophylla*, *A. manshuriensis*, *A. tomentosa* (Adams et al. 2005a).

### Embryo form

Only the linear form of the embryo was described for seeds of *Aristolochia* species studied previously (Martin 1946, Adams et al. 2005a, 2005b, Berjano Pérez 2006, Alves-da-Silva et al. 2011). The *A. contorta* embryo forms with deviated cotyledons were observed in 56.3% of cases. We hypothesise that the cotyledon deviation from the axis may represent an adaptation to successful growth and development of the embryo in the flattened triangular heart-shaped seed. The most commonly encountered fan-shaped form (69.4% among the forms with deviated cotyledons) is the optimal form with respect to the spatial constraints in the seeds of *A. contorta*. Therefore, the cotyledons are able to fully exploit the storage tissues, which are located in the lateral lobes of the seed. The *A. contorta* plants studied in our experiment were collected from the north limit of the distribution. The northern edge of the range of the species is the place where different kinds of adaptations to more severe conditions of survival have been established in rare far-eastern plants (Artyukova et al. 2012). The transformation of the linear embryo form into another form allows the embryo to maximally use internal seed resources during the long period of embryo post-development and may be associated with the process of the adaptation of its morphological structures. Perhaps the mechanism is not yet stable; therefore, we are accordingly observing a variety of forms that have deviating cotyledons. The study of embryo forms from other parts of the distribution range could clarify whether the variety of forms is an adaptation in the northern border of the range or a feature of the seeds of this species as a whole. Such intraspecific variation of embryo form is rather rare event. There are data on variety of embryo form within families of other angiosperms, for examples, for representatives of family Solanaceae (Sokolova 2010).

### Embryo development

Seed and embryo size varied among *Aristolochia* species (fig. 7). Embryo:seed ratios were consistently approximately 10% and were similar among the four species from the subgenus *Orthoaristolochia*, but these ratios were smaller than those of the species from the subgenus *Siphisia*. For these species, embryo:seed ratios ranged from 20 to 40%. The study of embryo development during the stages of both seed coat rupture and emergence of the both radicle and cotyledons in *A. galeata* from the same subgenus (Alves-da-Silva et al. 2011) revealed that the values of embryo size were closest to those obtained for *A. contorta* (table 3); the embryos of both species of this subgenus increased by five and seven fold until the time of germination. The data concerning the embryo size in the dry seed and at the moment of germination of the three species of the subgenus *Siphisia* (*A. californica*, *A. macrophylla*, *A. tomentosa*) (Adams et al. 2005a) show that the embryos of these species increased by 2–2.5 fold.

The data obtained in our study concerning the duration of the stages of *A. contorta* embryo development are similar to the values of *A. galeata* obtained earlier (Alves-da-Silva et al. 2011): the period from the seed coat rupture until radicle protrusion ranged from two to fourteen days and from three to fourteen days, respectively, and the period from radicle

protrusion until cotyledon emergence ranged from two to ten days and from four to ten days, respectively. We noted a difference in the length of the period of the embryo post-development stage (stage I) between these species, which is caused by the depth of the *A. contorta* seed dormancy.

### Cryopreservation

Seed viability following deep freezing depends on the moisture of the seed, as only a certain range of moisture allows the seeds to be resistant to ultralow temperatures, thereby preserving the initial properties of the seed. The moisture of *A. contorta* seeds does not exceed 10% (i.e. the seeds are referred to as the orthodox type). Orthodox seeds are more resistant to freezing than are recalcitrant seeds (Tikhonova 1999). Exposure to ultralow temperatures did not cause *A. contorta* seeds to die; these seeds maintained the ability to germinate and demonstrated a high final germinability close to that of the control seeds. After deep freezing of the seeds of *Schefflera octophylla* (Lour.) Harms, a representative of Araliaceae, an ancient family of angiosperms whose seeds also contain an underdeveloped embryo and are in the MD state, germinability decreased by 50% compared with that of control seeds (Chen et al. 2007). This outcome was due to the high initial moisture content of the seeds and sensitivity of these seeds to drying to 6%. Studying the mutual effects of cryopreservation and stratification on seed germination of more than 200 plant species from the state of Ohio revealed that in the most cases exposure to liquid nitrogen enabled the conservation of seed viability; however, this exposure did not help break MD or MPD (Pence 1991). Therefore, for successful germination, it is necessary to use the methods of breaking dormancy after freezing. In our experiments, cryopreservation also helped to maintain the viability of *A. contorta* seeds, but it did not lead to the breaking of the MPD.

### CONCLUSION

Studying the biology of *A. contorta* seed germination revealed that the seeds are in a state of non-deep, simple MPD of different degrees of depth. One method of breaking dormancy involves dry storage of the seeds and warm stratification, during which embryo postdevelopment occurs and the physiological inhibiting mechanism of germination is overcome. The variability in the depth of seed dormancy, gradual maturation of the embryo, a long period of germination, relatively high germinability (near 60%), and preservation of seed viability for up to three years can be considered as adaptations, which allow *A. contorta* to survive in unfavourable conditions in the northern boundaries of the range.

The length of the embryo reaches  $3.80 \pm 0.12$  mm by the time of germination (i.e.  $0.66 \pm 0.03\%$  of the seed length). The contribution of the morphological structures to the process of embryo growth gradually changes: at the stage of embryo postdevelopment, embryo growth proceeds mainly due to the increase of the cotyledons; at the stage when the radicle emerges from the seed, the embryo growth is mainly due to radicle lengthening. We have revealed here for the first time a variety of embryo forms of *Aristolochia* species, as well as polyembryony in *A. contorta* seeds.

Cryopreservation did not have a detrimental effect on *A. contorta* seeds. After deep freezing of seeds in liquid nitrogen ( $-196^\circ\text{C}$ ) for twelve months, germinability did not differ from the control. The resistance of *A. contorta* seeds to ultralow temperatures provides an opportunity to preserve the seed material with maximum guarantee, which is especially important for these rare relict species.

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