In vitro culture of the rare fern
Polystichum craspedosorum (Maxim.) Diels.

Lyudmila A. Shelikhan

ABSTRACT
Cultivation of ferns in vitro is an effective method for their propagation and conservation. Polystichum craspedosorum (Maxim.) Diels is a rare fern for Amur Province, listed in the Red Data Books of several Far Eastern regions of Russia. The objective of the study was estimation of multiplication efficiency of P. craspedosorum in vitro using spores as explants. Surface sterilization of spores with 2% 1,3-dichloro-5,5-dimethylhydantoin in water for 2.5 min was effective. The spores were sown on the half-strength Murashige and Skoog’s medium (1/2 MS) ammonium nitrate-free, vitamin-free and plant growth regulators-free, supplemented with 2% sucrose, 0.8% agar, at pH 5.8 and incubated under a 16 h photoperiod. The germination rate of spores was 85%. The above medium was suitable for the growth of gametophytes and production of sporophytes. For acclimatization of gametophytes and young sporophytes, a mixture of peat and vermiculite (2:1, v/v) was used as substrate. The gametophytes ex vitro produced more sporophytes than the gametophytes grown in vitro. However, sporophyte initiation was observed earlier under in vitro conditions.

Keywords: Polystichum craspedosorum, culture in vitro, spores, gametophyte, sporophyte

REZУОМЕ
Шелихан А.А. Культура редкого папоротника Polystichum craspedosorum (Maxim.) Diels. в условиях in vitro. Культивирование папоротников in vitro является эффективным методом их размножения и сохранения. Папоротник Polystichum craspedosorum (Maxim.) Diels – редкий вид для Амурской области, занесенный в региональные Красные книги ряд дальневосточных регионов. Целью работы была оценка репродуктивной эффективности P. craspedosorum в культуре in vitro с использованием спор в качестве экскплантов. Поверхностная стерилизация спор 2% раствором 1,3-дихлоро-5,5-диметилгидантоина (сульфохлорантин-Д) в воде в течение 2,5 мин. была эффективна. Споры высевали на питательную среду Мурашисе-Скуга с половинным содержанием макроэлементов (1/2 MS) без добавления нитрата аммония, витаминов и регуляторов роста, с добавлением 2% сахарозы, 0,8% агар, при pH 5,8, и выращивали при фотопериоде 16 ч. Прорастание спор составило 85%. Та же питательная среда была пригодной для культивирования гаметофитов и получения спорофитов. Для акклиматизации гаметофитов и молодых спорофитов в качестве субстрата использовали смесь торфа и вермикулита (2:1, об./об.). Акклиматизированные гаметофиты давали большее число спорофитов, чем гаметофиты в условиях in vitro. Однако появление спорофитов наблюдалось раньше в условиях in vitro.

Ключевые слова: Polystichum craspedosorum, культура in vitro, споры, гаметофит, спорофит
MATERIAL AND METHODS

Plant material. The object of this study was the fern Polystichum craspedosorum (Maxim.) Diels (Dryopteridaceae). Fonds with spores were collected in the Bureysky district of the Amur Province on the right bank of the Bureya River (50°08′29″N 130°09′69.4″E) in September 2016. The fertile fronds were stored in a paper bag at room temperature until spores released. Spore viability was tested in Petri dishes with filter paper and distilled water.

Spore sterilization and culture initiation. Before sowing, the spores were washed with sterile distilled water, centrifuged in microtubes for 10 minutes. The spores were surface sterilized with 2% 1,3-dichloro-5,5-dimethylhydantoin (the commercial preparation Sulfochlorantin-D, Russia) in sterile distilled water (w/v) for 2.5 min. followed by washing with sterile distilled water three times. The spores were sown on the following sterile culture media in Petri dishes (30 ml per dish): a) half-strength Murashige and Skoog's medium (1/2 MS) (Murashige & Skoog 1962) ammonium nitrate-free (-NH₄NO₃), vitamin-free (-vit.) and plant growth regulators-free (-PGRs), supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar, adjusted to pH 5.8 (Makowski et al. 2016); b) 1/2 MS (-NH₄NO₃; -vit.; -PGRs), supplemented with 2% (w/v) sucrose, 0.35% (w/v) agar, adjusted to pH 5.8. The cultures were then incubated at a 16 h photoperiod (cool-white fluorescent light) and room temperature.

Subculture of gametophytes. Colonies of young gametophytes were subcultured in jars/flasks (100 ml) containing 30 ml of the medium: 1/2 MS (-NH₄NO₃; -vit.; -PGRs), supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar, adjusted to pH 5.8. The temperature and photoperiod were same as above.

Subculture of sporophytes. Sporophytes were separated from gametophytes with a scalpel. Sporophytes were then subcultured into jars/flasks (100 ml) containing 30 ml of the medium: 1/2 MS (-NH₄NO₃; -vit.; -PGRs), supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar, adjusted to pH 5.8. The temperature and photoperiod were same as above.

Acclimatization of gametophytes and sporophytes. One and a half months-old gametophyte colonies were transferred from in vitro to ex vitro conditions. Gametophytes were placed in plastic pots (1–5 colonies per pot) with a pre-autoclaved mixture of peat and vermiculite (2:1, v/v). The pots with substrate were placed in zip-lock bags and maintained at high humidity by spraying with water.

Young sporophytes with first leaves obtained in vitro were separated from gametophyte colonies and transferred (3–5 sporophytes per pot) to pots with substrate as above. The acclimatization was carried out at room temperature and a 16 h photoperiod.

Morphological observations. Microscopic observations were carried out with the light microscopes ZEISS Axio Lab.A1 and Nikon SMZ745T P-DSL32 (China), and images were captured with the attached digital cameras AxioCam ERC5s, ZEISS (China) and TOUPCAM UC-MOS 14000KPA. Measurements were done with the softwares AxioVision Rel. 4.8 and ToupleTou View 3.7. Photographs of colonies and plantlets were taken with the digital camera Sony Cyber-shot DSC-W630.

RESULTS AND DISCUSSION

The procedure of surface sterilization of spores with 2% 1,3-dichloro-5,5-dimethylhydantoin for 2.5 min was effective both for the elimination of microbial contamination and the retention of spore viability. To our best knowledge, this is the first report on the application of 1,3-dichloro-5,5-dimethylhydantoin for the sterilization of fern spores. The spores of P. craspedosorum began to germinate 16 days after the culture initiation and achieved maximum germination (85%) within 1.5 months. The germination of spores and the formation of protonema occurred according to the Vittaria-type (Nayar & Kaur 1971). Prothallium (Fig. 1A) development was of the Aspidium-type, which is characteristic for the Dryopteridaceae family (Nayar & Kaur 1971).

A culture medium may influence the spore germination and gametophyte growth (Shelikhan & Nekrasov 2018). Spores contain all the required nutrients for early development. Diluted Murashige and Skoog’s medium is suitable for in vitro propagation of ferns (Mikula et al. 2009, Makowski et al. 2016). The spore germination of P. craspedosorum was effective on the culture medium 1/2 MS (-NH₄NO₃; -vit.; -PGRs), supplemented with 2% sucrose, 0.8% agar (solid medium), and adjusted to pH 5.8. Some authors reported on effective germination of fern spores in the presence of liquid water on the medium surface (Fernandez et al. 1999) or in a liquid medium (Simoes-Costa et al. 2015). Reduction of agar content from 0.8 to 0.35% (liquid medium) achieved also spore germination of P. craspedosorum. However, gametophytes maintained in the liquid medium did not grow and became anoxic and died after 1–2 weeks. After germination in the liquid medium, gametophytes were to be transferred in 1/2 MS medium supplemented with 0.8% agar for survival.

Development of gametophytes. Gametophytes of P. craspedosorum were green. These gametophytes developed and formed rhizoids, antheridia or archegonia. Rhizoids in young prothallium are transparent; they contained a few chloroplasts (Fig. 1A). Rhizoids of mature gametophytes were brown and elongated, they lacked chloroplasts. The mature gametophytes were cordate (Fig. 1B) with numerous chlorophyllous, unicellular, glandular trichomes located both on the margins and on the surface of a thallus (Fig. 1C–E). Some marginal cells with trichomes were elongated, therefore the margins of a thallus looked irregularly (Fig. 1D). These trichomes contained few chloroplasts (Fig. 1E). The old gametophytes were ribbon-shaped, the margins were more corrugated. An initial step of the secondary gametophyte formation was represented by a proliferation of marginal cells into lateral filamentous branches (Fig. 1F), which then formed spatulate and cordate shape. The new gametophytes were not completely separated from the old gametophytes, so they formed colonies associated with the old thallus. The production of secondary young gametophytes was observed in the culture medium during 8 weeks of cultivation without subculturing. Starting from week 4 of cultivation (without subculturing), the production of new gametophytes was accelerated rapidly (Fig. 1G, H). After week 7 due to active proliferation, colonies of gametophytes were more dense,
In vitro culture of *Polystichum craspedosorum*

**Figure 1** Development of *Polystichum craspedosorum* grown in vitro. A – prothallium; B – cordate gametophyte; C, D, E – unicellular glandular trichomes on the surface of a thallus; F – beginning of a secondary gametophyte formation (filamentous branches); G – 1 week of cultivation after separation of two-month-old gametophyte colonies with sporophytes; H – 4 week of cultivation; I – 7 week of cultivation (without subculturing); J – a colony of gametophytes; K – young sporophytes; L – trichomes on the margin of the first frond of a sporophyte; M – a unicellular glandular trichome on the margin of the first frond of the sporophyte; N – a multicellular filiform trichome on the margin of the first leaf of the sporophyte; O – young sporophytes growing in the pot. Scale bars: A, C, L= 100 μm; B= 1000 μm; D, F = 200 μm; E, M, N = 50 μm; G, H, I, J, K, O = 1 cm. Trichomes (tr.) are indicated with arrows.
globular (Fig. 1J), up to 1–2 cm (Fig. 1J). Colonies of young gametophytes were transferred into a fresh medium and grew further developing new gametophytes and forming a compact mass of old and new gametophytes (Fig. 1I–J). Thus, the culture medium 1/2 MS (-NH\textsubscript{4}NO\textsubscript{3}; -vit.; -PGRs), supplemented with 2% sucrose, 0.8% agar (solid medium) and adjusted to pH 5.8 was suitable for the growth and development of the *P. craspedosorum* gametophytes *in vitro*.

**Development of sporophytes.** The development of first fronds of sporophytes was observed 5–12 weeks after the subculturating of one and a half months-old gametophytes. One gametophyte produced only one sporophyte. Sporophytes together with portions of gametophytes were transferred into culture vessels, where they grew further and developed. Young sporophytes (Fig. 1K) were different from mature adult sporophytes, which indicates their juvenile nature. Two types of trichomes were found on the first fronds of young sporophytes: 1) unicellular glandular trichomes like those found on the gametophytes (Fig. 1I, M); 2) multicellular trichomes of filiform form consisted of 5–6 cells (Fig. 1L, N).

**Sporophyte development in vitro versus ex vitro.**

One and a half months-old gametophyte colonies were transferred to pots with the mixture of peat and vermiculite (2:1, v/v) for acclimatization under *ex vitro* conditions. The formation of first sporophytes on the substrate was observed after 20–27 weeks, which is later than *in vitro* in the culture medium and indicates a possible lag period required for gametophytes to adapt to the new conditions. First fronds of young sporophytes obtained *ex vitro* and *in vitro* were morphologically similar. The mean number of sporophytes formed *ex vitro* was significantly higher than *in vitro* (Table 1). In fact, there was always water droplets in the bags comprised potted gametophytes due to periodic spraying. The importance of free water in sporophyte induction has been noted for different substrates (Wu et al. 2010). Liquid water is needed to enhance fertilization rate, thus the availability of water in the culture vessels may be a limiting factor for sporophyte mass-production.

The survival rate for young sporophytes (Fig. 1O) separated from gametophyte colonies and transferred to a pot with substrate was high (80%) after 4 weeks.

Sporophyte formation of *P. craspedosorum* grown from spores under non-sterile conditions took 150 days in the best tested variant (a mixture of peat and sand in zip-bags, 1/2 MS (substrate) supplemented with 2% sucrose, 0.8% agar (solid medium) adjusted to pH 5.8 and sterilized with 1,3-dichloro-5,5-dimethylhydantoin and autoclaving. Spore surface sterilization with 1,3-dichloro-5,5-dimethylhydantoin and cultivation in the culture medium 1/2 MS with no ammonium nitrate, vitamins, and plant growth regulators added, supplemented with 2% sucrose and 0.8% agar, and adjusted to pH 5.8. The medium was suitable for spore germination, propagation of gametophytes and sporophyte formation. The procedure provides a high rate of spore germination (85%), early induction of sporophytes (5–12 weeks), and a high rate of sporophyte acclimatization (80%) in the mixture of peat and vermiculite (2:1, v/v). Alternatively, gametophytes obtained *in vitro* can be potted in the mixture of peat and vermiculite before the induction of sporophytes, which results in the growth of more sporophytes but their delayed formation (20–27 weeks).

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