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RESEARCH PAPER

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

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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 *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

РЕЗЮМЕ

Шелихан Л.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 ряда дальневосточных регионов. Целью работы была оценка репродуктивной эффективности *P. craspedosorum* в культуре *in vitro* с использованием спор в качестве эксплантов. 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 Мурасиге-Скуга с половинным содержанием макросолей (1/2 MS) без добавления нитрата аммония, витаминов и регуляторов роста, с добавлением 2 % сахарозы, 0,8 % агара, 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 наблюдали раньше в условиях *in vitro*.

Ключевые слова: *Polystichum craspedosorum*, культура *in vitro*, споры, гаметофит, спорофит

Polystichum craspedosorum (Max.) Diels is a fern species belonging to the family Dryopteridaceae. The species is distributed in East Asia: China, Japan, Korea, and the Russian Far East. This fern grows on the rivers banks and creeks, shady rocks and large stones of limestone. The species has been listed in the Red Data Books of the Jewish Autonomous Region (2006) and Khabarovsk Territory (2008). The influence of human activities and the decline of habitats lead to the decrease in the number the fern in nature (Kreschenok & Khrapko 2018). In Amur Province, the species was first discovered in 2011 in the Bureya river valley of the Bureysky district (Starchenko & Darman 2012). A significant part of the *P. craspedosorum* population area is currently flooded by the reservoir of the Lower Bureyskaya hydropower plant in 2017. Earlier this year, *P. craspedosorum* was included in the Red List of Threatened Species of Amur Province. Therefore, it is of great importance to develop effective procedures for the fern species propagation and conservation.

Propagation of *P. craspedosorum* from spores has been investigated for different growing substrates under non-sterile conditions (Kreschenok 2011). *In vitro* cultivation is an effective technique for conservation and propagation of ferns under aseptic controlled conditions (Shelikhan & Nekrasov 2018). The objective of this study was estimation of multiplication efficiency of the fern *P. craspedosorum* grown *in vitro* using spores as explants.

MATERIAL AND METHODS

Plant material. The object of this study was the fern *Polystichum craspedosorum* (Maxim.) Diels (Dryopteridaceae). Fronds with spores were collected in the Bureysky district of the Amur Province on the right bank of the Bureya River (50º08'29"N 130º09'69.4"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 (-NH_4NO_3), 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 (-NH_4NO_3; -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 florescent 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 (-NH_4NO_3; -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 (-NH_4NO_3; -vit.; -PGRs), supplemented with 2 % (w/v) sucrose, 0.8 % (w/v) agar, 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 UCMOS 14000KPA. Measurements were done with the softwares AxioVision Rel. 4.8 and ToupTek ToupView 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 %) withing 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 (-NH_4NO_3; -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 transfered 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 of 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 spatulated 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,

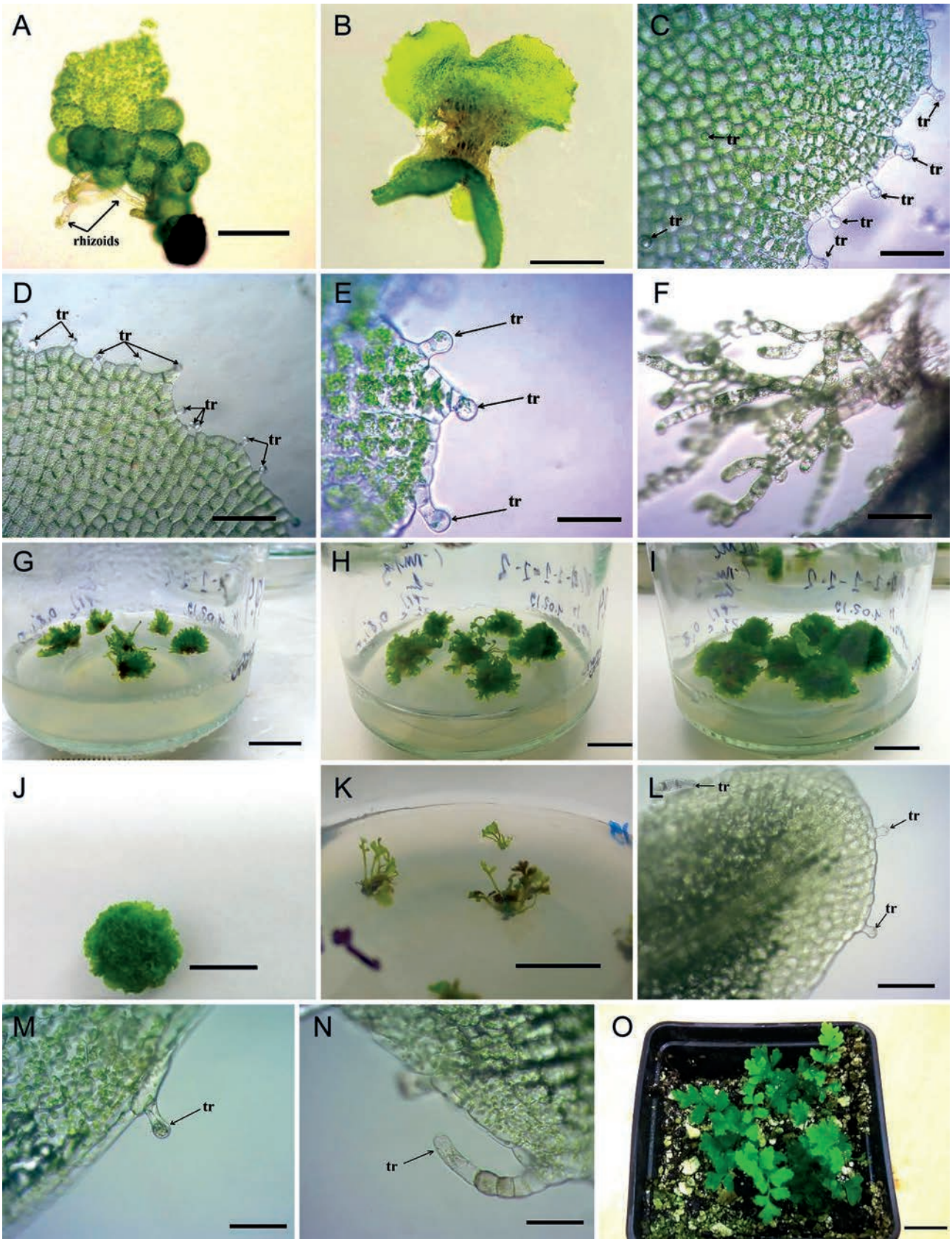

Figure 1 Development of *Polystichum craspedosorum* grown *in vitro*: A – prothallium; B – cordate gametophyte; C, D, E – unicellular glandular trichomes on the surface of a thalli; F – beginning of a secondary gametophyte formation (filamentous branches); G – 1 week of cultivation after separation of 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. 1I), 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_4NO_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 subculturing 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. 1L, 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, Kreschenok 2011) which is superior to the time interval found for gametophytes transferred from *in vitro* to *ex vitro* conditions (180–230 days). *In vitro* culture of the fern permits sporophyte production as earlier as 76–125 days starting from spore sowing in the medium thus providing a higher regeneration rate of new plantlets. Moreover, establishment of the *in vitro* culture opens opportunities for method development of long-term conservation of the rare fern.

Table 1. Sporophyte formation by gametophytes grown *in vitro* and *ex vitro*.

Gametophyte culture (substrate)	Emergence of sporophytes (fronds), weeks	Mean number of sporophytes produced per gametophytic colony
In vitro: 1/2 MS (-NH_4NO_3; -vit.; -PGRs), 2 % sucrose, 0.8 % agar, pH 5.8	5–12	5
Ex vitro: peat : vermiculite (2:1)	20–27	11

CONCLUSION

A *in vitro* production of *P. craspedosorum* plants from spores, described in this study, involves 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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