Original Article

ESTABLISHMENT OF THE MOSS *Polytrichum juniperinum* HEDW. UNDER AXENIC CONDITIONS

ESTABELECIMENTO E DESENVOLVIMENTO DO MUSGO Polytrichum juniperinum *HEDW. SOB CONDIÇÕES DE CULTIVO AXÊNICO*

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RESUMO: *Polytrichum juniperinum* Hedw. (Polytrichaceae) é uma espécie de musgo de ampla distribuição, ocorrendo em ambos os hemisférios. Culturas *in vitro* foram estabelecidas a partir de esporos de espécimes coletados na natureza. O desenvolvimento, tanto de protonema quanto de gametófitos, foi observado utilizando o meio básico MS em três tratamentos, livre de fitorreguladores, suplementados com uma fonte de auxina (AIA), suplementados com uma fonte de citocinina (BAP) e suplementado com ambos reguladores. Nos cultivos resultantes de meio livre de reguladores e de meios contendo auxina, foi observado o desenvolvimento total dos gametófitos, enquanto nos meios contendo citocinina não foram observados desenvolvimento e regeneração de gametófitos. Estes resultados sugerem a utilização do meio livre de reguladores para cultivo de *Polytrichum juniperinum* em cultivos axênicos.

PALAVRAS-CHAVE: Desenvolvimento *in vitro*. *Polytrichum juniperinum.* Meio MS. Cultura de tecidos de musgos.

INTRODUCTION

Micropropagation or *in vitro* vegetative propagation of plants constitutes a way of perpetuating healthy and aseptic explants, facilitating the application of regeneration techniques and genetic transformation. Aseptic culturing is necessary for certain experimental procedures (SABOVLJEVIĆ et al. 2006) and it is highly convenient for the maintainance of plant genotype collections free of different pathogens. The obtaintion of bryophytes isolated culturtes has been reported as a complex task by many investigators (GANG et al. 2003; BIJELOVIĆ; SABOVLJEVIĆ 2003; CVETIĆ et al. 2007, SILVA et al. 2009, SILVA et al. 2010), due to a possible interaction of these plants with other organisms in non-axenic conditions. Nevertheless, bryophytes have great advantages over vascular plants as models for plant biology investigations: (1) relatively simple structure compared to other higher plants, (2) haploid gametophyte as the dominant vegetative phase, and (3) lower chromosome numbers (GANG et al. 2003). The culture of bryophyte cells in suspension media, as well as the dominant gametophyte phase of mosses, have been reported as favorable model systems for genetic, biochemical, metabolic, and developmental studies (COVE et al. 2006; ONO et al. 1988).

The introduction of new species into axenic conditions and maintenance of stable cell is therefore essential as a start for in-depth investigation of the physiology and potential uses of bryophytes. *Polytrichum juniperinum* Hedw. (Polytrichaceae) is a common moss species with a worldwide distribution, adapted to open, dry and sandy environments, growing on a variety of peatlands, especially on drained habitats (VAN der VELDE; BIJLSMA 2003). The ancestral position of Bryophyta for land plants relatioship being that group as a target to understand the envolved processes to conquest the land enviroments by the plants. Early iniciatives aimed to verify the *in vitro* development for several moss species, such as *Physcomitrella patens* Brid. (COVE et al. 2006), *Ceratodon purpureus* (Hedw.) Brid. (SABOVLJEVIĆ et al. 2003), *Pogonatum urnigerum* (Hedw.) P. Beauv. (CVETIĆ et al. 2007) e *Atrichum* spp. (Ono et al. 1987; Gang et al. 2003; Sabovljević et al. 2006). The present study aimed to establish a *in vitro* culture for *P. juniperinum* and examine its development under axenic conditions.

MATERIAL AND METHODS

Fully developed *Polytrichum juniperinum* plants were indentified and collected by the first author in the autumm 2008 at two sites in Southern Brazil, Gramado (29° 23' S; 50° 52'W) and Canela (29° 21'S; 50° 50'W) in the highlands named Serra Gaúcha. Fresh, unopened sporophytes were surface sterilized as described by Cvetić et al. (2007) by dipping in 25% commercial bleach (8% active NaOCl) for 3 minutes, and thoroughly rinsed in

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sterile distilled water. The cap was then removed and the spores released on the nutrient medium.

As basal medium for establishment of *in vitro* culture, the Murashige and Skoog (1962) basic medium containing 100 mg L^{-1} of inositol an 15 g L^{-1} of sucrose, solidified with 7 g L^{-1} of agar was used. In order to observe the influence of growth regulators on the *in vitro* development of this species the follow media composition were used: MS 1 (MS regulator-free); MS 2 (MS + 1.0mg L^{-1} AIA, 0.05 mg L^{-1} Kinetin); MS 3 (MS + 1.0 mg L^{-1} AIA, 0.1 mg L^{-1} Kinetin); MS 4 (MS + 1.0 mg L^{-1} AIA, 1.0 mg L^{-1} Kinetin); MS 5 (MS + 1.0 mg L^{-1} AIA, 1.5 mg L^{-1} Kinetin); MS 6 (MS+ 1.0 mg L^{-1} AIA); MS 7 (MS + 1.0 mg L^{-1} AIA, 1.0 mg L^{-1} BAP) and MS 8 (MS + 1.0 mg L^{-1} BAP). All media above were shed in 90x60 mm Petri dishes. Prior to the sterilization the pH was adjusted to 5.8.

Culture were grown at 25±1ºC under long-day conditions (16 h light/8 h dark) supplied by cool-white fluorescent tubes at a photon flow rate of 48 µmol m^2s^{-1}. When a protonemal mass formation was observed, it was subcultured monthly in the same medium until the rise of the first shoot. One unopened sporophyte per dish was used, in a total of four replicates for each treatment (completely randomized design). Calli development and regenerating gamethophyte amounts where evaluated in each medium proposed, and a test of comparison of means was performed using the Tukey test (5% of probability) with the aid of Statistix 9.0 *for Windows* software.

RESULTS AND DISCUSSION

The inoculated spores of *P. juniperinum* took 15 days to germinate and 20-40 days for completely protonemata formation (Figure 1A). After tree subcultures, the first gametophyte shoots rose in the MS regulator-free medium and in the MS6, MS7 and MS8 medium (Figure 1B). No differences were found for spore germination and protonemata development, when all media used were compared. Gametophyte regeneration was not observed in media containing Kinetin as a cytokinin source. In the medium containing AIA as auxin and BAP as an alternative cytokinin source, the regeneration was successful. The same was observed with regulator-free MS, with no significantly differences observed within other tested media (Table 1). On the other hand, calli formation were only found in media with 0.1 and 1.0 mg L^{-1} Kinetin.

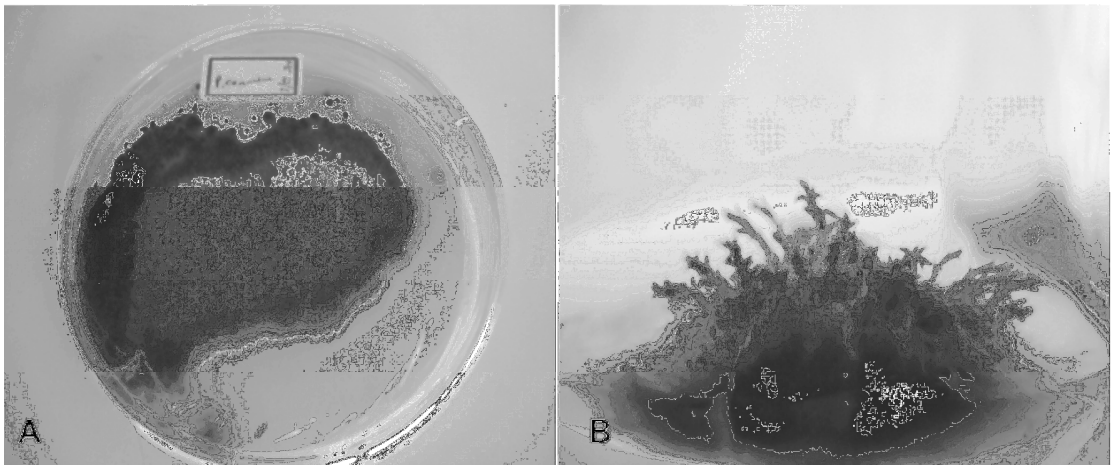

Figure 1. A. *Polytrichum juniperinum* Hedw. protonemata obtained after 40 days *in vitro* cultive. **B.** *Polytrichum juniperinum* Hedw. regenerate gametophytes by pure MS medium.

Table 1. *In vitro* micropropagation results for *Polytrichum juniperinum* Hedw. Positive (+) and negative (-) signs indicate a positive or negative response to a given medium, respectivel, (N/A) indicates a unsuccesfull gametophyte regeneration.

	MS1	*MS2*	*MS3*	*MS4*	*MS5*	*MS6*	*MS7*	*MS8*
Germination	+	+	+	+	+	+	+	+
Callus formation	-	-	+	+	-	-	-	-
Gametophyte formation	+	-	-	-	-	+	+	+
Nr of regenerating gametophyte	66 a	N/A	N/A	N/A	N/A	66 a	60 a	68 a

Means followed by the same letter do not differ by the Tukey test ($p<0,05$).

The results demonstrate that the fully in vitro development for *P.juniperinum* can be reach using regulator-free medium. Early studies reports the importance of hormonal requirements for shoot regeneration and multiplication (BOPP; ATZORN 1992). Cytokinins have been shown to induce bud formation in protonemata cultures of some moss species (SPEISS 1976; BIJELOVIĆ; SABOVLJEVIĆ 2003). In the present study these growth regulator not demostrate a diferencial shoot formation response. The cytokinins dosages used were not sufficient for buds development, suggests a diferencial requirement for bud induction in *P. juniperinum* where compare with other mosses species.

In studies of cytokinin action on different moss species (SPEISS 1976), calli were obtained with most of the species, except for the polytrichaceous species studied. However, Gang et al. (2003) demonstrate for *Atrichum undulatum* (Hedw.) P. Beauv. that calli was obtained when the growth medium contained Benzyladenine (BA). The callus formation was obtained to *Pogonatum urnigerum* (Hedw.) P. Beauv., when a medium with low sugar values was used (CVETIĆ et al. 2007). For *P. juniperinum* calli was observed only in the media when the Kinetin contents was increase (MS4 and MS5). In our study is the new report for calli occurrence in Polytrichaceae species in axenic conditions.

Cvetić et al. (2007) reported a fast calli senescence formation and relatively fast senescence of protonemata, probably due to the fact that the protonemata of moss species are not persistent in nature. The positive effect found on the AIA and Kinetin ratio tested in MS4 and MS5 media probably due to an increase in protonemata growth, delaying the senescence and keeping the calli viable, which was not observed for the other tested media in the present study.

The spore germination and protonemata development was observed on all media used. Similar results were found in other initiatives to establish an *in vitro* protocol for moss development and gametophyte regeneration (CVETIĆ et al. 2005, 2007; SABOVLJEVIĆ et al. 2003, 2006), when the use of growth regulators were not necessary. These results indicate that it is possible to use simple media for spore germination and gametophyte regeneration of *P. juniperinum* in axenic conditions.

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ABSTRACT: *Polytrichum juniperinum* Hedw. (Polytrichaceae) is a moss with a worldwide distribution. *In vitro* culture was established from *P. juniperinum* spores collected in nature. Both protonema and gametophore stages of gametophyte development were obtained. The Murashige-Skoog regulator-free nutrient medium or supplemented with AIA and BAP conferred a fully development and regeneration of gametophytes. Tissues grown on cytokinin did not produce any gametophytes. These results indicate the possibility to use a medium without growth regulators to obtain gametophytes for this species in axenic conditions.

KEYWORDS: *In vitro* development. *Polytrichum juniperinum*. MS medium; Mosses tissue culture.

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