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IN VITRO CULTURE AND APOGAMY-ALTERNATIVE PATHWAY IN THE LIFE CYCLE OF THE MOSS *AMBLYSTEGIUM SERPENS* (AMBLYSTEGIACEAE)

TIJANA CVETIĆ[1,2], M. SABOVLJEVIĆ[1,3], ANETA SABOVLJEVIĆ[1,4], and D. GRUBIŠIĆ[1,5]

[1]*Institute of Botany and Jevremovac Botanical Garden, Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia and Montenegro
[2]*Center for Multidisciplinary Studies, University of Belgrade,* 11000 Belgrade, Serbia and Montenegro
[3]*Nees Institute of Plant Biodiversity, Rheinische Friedrich Wilhelms University of Bonn,* 53115 Bonn, Germany
[4]*Institute of Botany, University of Cologne,* 50931 Cologne, Germany
[5]*Siniša Stanković Institute for Biological Research,* 11000 Belgrade, Serbia and Montenegro

Abstract - *In vitro* culture of the moss *Amblystegium serpens* (Amblystegiaceae) was established on hormone-free Murashige and Skoog (MS) medium that contained a half amount of MS micro- and macro- mineral salts and vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, and 0.70% (w/v) agar. Spores were germinated and primary protonema developed on the above medium at 16 h day/8 h night, 25±2ºC, 60-70% air humidity, and irradiance of 47 μmol/m^{2}s. Three months after development of primary protonema, seven sporophytes appeared directly from primary protonema without generation alternation. The phenomenon of apogamous sporophyte formation is very rare, both in nature and under *in vitro* conditions. This is the first report of apogamy induced by *Amblystegium serpens.*

Key words: Apogamy, life cycle, mosses, Amblystegiaceae, Serbia

UDC 58(Amblystegium serpens)

INTRODUCTION

Although the first axenic culture was established with bryophytes, mosses to be precise (Servettaz, 1913), there are many problems in dealing with *in vitro* culture of bryophytes. Servettaz (1913) himself referred to the first moss culture by Becquerel (1906) who described the development of pure culture of protonema of *Atrichum undulatum* and *Brachythecium velutinum*. After such a start, the liverwort *Marchantia polymorpha* was extensively grown in axenic conditions for various studies (Lilienstern, 1927).

It is not easy to get sterile parts of plants and to dispose of organisms on bryophytes (protozoa, beetles, fungi, algae) and there are still greater problems in disposing of endobionts. Even more problems occur in dealing with a pleurocarpous moss that has a stem densely covered with sharp leaves.

The problem after initializing the axenic culture of bryophytes is to induce bud growth from primary or secondary protonema, which can be achieved by addition of exogenous growth regulators in a certain ratio. It has been reported that better growth of bryophytes in axenic culture was achieved on media containing a mineral solution weaker than that needed for higher plants (Bates, 2000). However, it seems to be extremely problematic to get sex expression and to achieve gametophyte-sporophyte junction under *in vitro* conditions. Too many physiological and ecological factors influence this phenomenon.

Heteromorphic generation alternation in bryophytes with change in number of the chromosome set is well documented. However, phenomena like apogamy (formation of a sporophyte directly from gametophytic cells without the intervention of gametes) and apospory (development of a gametophyte from vegetative cells of the sporophyte without the intervention of spores) are rare in plants, and there are very few data concerning these processes in bryophytes. Both apogamy and apospory occur in nature extremely rarely (Chopra and Kumra, 1988).

Apogamous sporophyte formation in bryophytes was first reported in detail by Springer (1935) on the leaf and stem tips of naturally growing diploid gametophytes of the moss *Phascum cuspidatum* Hedw. No other apogamy of bryophytes in nature has been reported to date.

In many textbooks, gametophytic generation of plants is described as haploid and sporophytic as diploid generation. However, it is well documented that change in chromosome number is not necessarily correlated with change in life history phase (Graham and Wilcox, 2000). The nuclei of sporophytes and gametophytes of the brown seaweed *Haplospora globosa* (Tilopteridales) possess the same number of chromosomes. However, the DNA level of saprophytic nuclei is twice that of gametophytic nuclei (Kuhlenkamp *et al.* 1993). In seedless plants, apogamy and apospory are also observed, but gene dosage effects are important. Maintenance of sporophytic growth depends on the presence of at least two sets of chromosomes, whereas gametophytic growth in culture does not continue when four or more sets of chromosome are present (Bell, 1991).

The molecular basis of this phenomenon in bryophytes is not known. However, there are some scattered data for *Arabidopsis*. The LEC1 gene (*Leafy Cotyledon* 1) is known to be responsible for inducing embryo-like development from vegetative cells by encoding a transcription factor (Lotan *et al.* 1998).

Amblystegium serpens is a pleurocarp moss species that grows on rocks and tree barks. It is widespread in mild climates of the Holarctic.

Bryophytes are excellent material for experimental studies on morphogenesis, physiology, and molecular biology. However, to be able to perform experiments, controlled conditions over one species should be established.

MATERIAL AND METHODS

Amblystegium serpens was collected in the greenhouse of the Siniša Stanković Institute, Belgrade, in spring of 2004, and fresh unopened mature capsules were used as the starting plant material.

Axenic culture of the pleurocarp moss *Amblystegium serpens*, which previously has not been known to grow in axenic culture, was established using slightly modified versions of the methods of Sabovljević *et al.* (2002). Unopened capsules were sterilized using 25% commercial bleach (8% active chlorine). Capsules were opened with a sterile needle and transferred onto solid medium. The basal medium (BM) contained a half amount of MS (Murashige and Skoog, 1962) mineral salts and vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 0.70% (w/v) agar (Torlak purified, Belgrade) without any supplements of growth regulators. The medium pH was adjusted to 5.8 prior to autoclaving at 115ºC for 20 min. The cultures were grown at 25±2ºC under fluorescent light (47 μmol/m^{2}s irradiance) and a day/night regime of 16/8 h.

RESULTS AND DISSCUSION

Spore germination and development of primary protonema was successful under the conditions stated in material and methods, with no fungal or bacterial contamination. We were able to get primary protonema development. However, any attempt to induce bud formation failed. It seems that this species confirms the species-specific pattern in bryophyte development demonstrated previously (Bijelović *et al.* 2004).

Apogamy developed spontaneously while we were trying to induce bud formation on primary protonema (Fig. 1).

After one cushion of primary protonema was left on the medium long enough (ca. 3 months), seven sporophytes appeared directly from the primary protonema without generation alternation and change in the number of chromosomes.

Formation of apogamous sporophytes is a very rare phenomenon (both in nature and under in vitro conditions). *Phycomitrium coorgense* is possibly the only instance on record in which 50% of apogamous sporogonia were reported to produce viable spores (Tab. 1) (Lal, 1984). In apogamous sporophytes developing on diploid tissues, spore production has been reported in eight taxa (Tab. 1). These mosses were either diploid strains or were obtained aposporously. Haploids show a lack of fertility that is probably due to the absence of complementary alleles normally present in the second genome. However, duplication of the genome and its role in fertility remain to be ascertained.

Even though apogamy in the genus *Amblystegium* has been reported twice previously (Tab. 1), this is the first time that it was induced in *Amblystegium serpens* and the

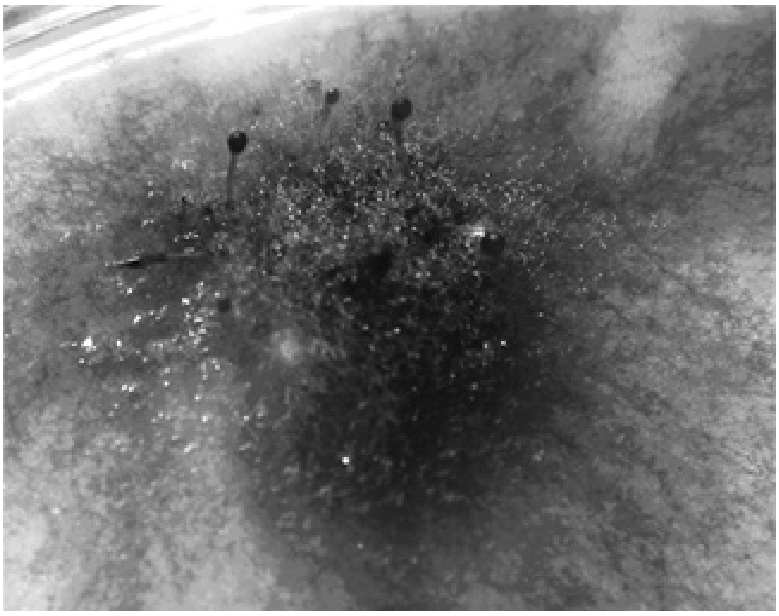

Fig. 1. Apogamous sporophyte in primary protonema of *Amblystegium serpens* haplophase in *in vitro* culture.

Tab. 1. Incidence of apogamy in haplophase and diplophase known to date. The sign in parentheses shows if the spores produced that way were viable (+) or not (-). The sign ! indicates our data on apogamy.

species	apogamy known		reference
	haplophase	diplophase	
Amblystegium juratzkanum	-	+ (+)	Lazarenko, 1963
Amblystegium riparium	-	+ (+)	Lazarenko, 1963
Ambystegium serpens	+ (-)!	-	Cvetić et al.
Brachythecium campestre	-	+ (+)	Lazarenko, 1963
Bryum sp.	+ (-)	- (-)	Bauer, 1967
Desmatodon randii	+ (-)	+ (+)	Lazarenko, 1963
Desmatodon ucrainicus	-	+ (+)	Lazarenko, 1963
Funaria hygrometrica	+ (-)	+ (-)	Bauer, 1959; Chopra and Kumra, 1988
Funaria hygrometrica x Physcomitrium pyriforme	-	+ (-)	Bauer, 1959
Grimmia pulvinata	-	+ (-)	Hughes, 1969
Phascum cuspidatum	-	+ (-)	Springer, 1935; von Wettstein, 1942
Physcomitrium coorgense	+ (+)	-	Lal, 1961
Physcomitrium pyriforme	+ (-)	+ (+)	Bauer, 1959
Pottia intermedia	+ (-)	+ (+)	Lazarenko, 1963
Pottia lanceolata	-	+ (-)	Lazarenko, 1963
Splachnum ovatum	-	+ (+)	Lazarenko, 1963
Splachnum pedunculatum	-	+ (-)	Lazarenko, 1963
Splacnum sp.	+ (-)	-	Bauer, 1967
Tetraphis pellucida	+ (-)	+ (-)	Hughes, 1969

first time it has been reported in its haplophase. Among factors so far known to induce apogamy are light, hydration, sugars, chloral hydrate, growth regulators, inorganic nutrients, and endogenous factors.

Dark can induce apogamous sporophytes, as can reduced hydration of the growth medium (von Wettstein, 1942; Chopra and Kumra, 1988). Sugars (sucrose and glucose) are speculated to induce apogamy to some extent. As shown by Sabovljević *et al.* (in press), sugars can induce gamete formation in some bryophytes. Since gametophyte differentiation starts with an apical cell with threecutting faces (in contrast to sporophyte differentiation, which starts with an apical cell with twocutting faces), it can be postulated that apogamy is expressed when the labile apical cell is influenced by certain internal and external conditions. This cell can even turn into a protonemal cell from highly differentiated tissues and develop a protonema cushion. It is present in the apical part of the protonemal filament and can differentiate or continue to produce other protonemal cells. An apical cell with threecutting faces passes through a stage with twocutting faces. If factors are conducive to apogamy, a two-cutting-face apical cell becomes stabilized and resorts turn to apogamy, otherwise three-cutting-face cells lead to formation of a gametophyte. However, both apogamous sporophytes and gametophytes have been observed to arise from the same protonema in *Physcomitrium pyriforme* (Menon and Lal, 1972). Growth regulators and inorganic nutrients are reported to have an apogamy - inducing role in apogamy formation, but there are no generalizations for bryophytes (Chopra and Kumra, 1988).

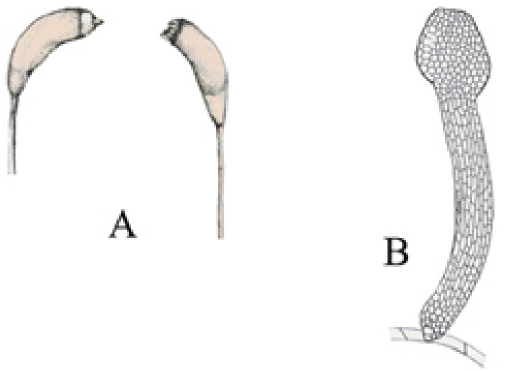

Fig. 2. The look of normal (A) sporophytes (6x) (left mature unopened capsule with operculum and led and right mature opened capsule with peristome) and apogamous (B) sporophyte (15x) of *Amblystegium serpens.*

The presence of the sporogon factor (an adenine type of cytokinin) or mixes of various metabolites in a certain ratio is assumed to be a possible cause of apogamy in mosses (Menon and Lal, 1972). Long growth of a moss on the same medium affords the possibility of exogenous accumulation of some metabolite that can later induce the phenomenon of apogamy. Also, age of the tissue seems to play a role in apogamy (Chopra and Rashid, 1967; Rashid and Chopra, 1969; Kumra and Chopra, 1980). Since, apogamy is known only in one diploid moss, it can be considered that chromosome numbers play an important role in apogamy induction (Lal, 1961; Lazarenko, 1963). However, according to Hughes (1969), moss species known to express apogamy are natural polyploids. In liverworts (in which polyploidy is rare), chromosome numbers are uniformly low and apogamy is conspicuously lacking (Lal, 1984). However, data reported by Ripetsky and Metasov (1973) do not support this conclusion.

In *Amblystegium serpens*, the apogamous sporophyte is morphologically different from the normal one (Fig. 2). It is very simple compared to the normal one developed through gametes. Also, the sporophyte-gametophyte junction is completely distinct, and the apogamous sporophyte always has a tender tiny body. The apogamous sporophyte is short-lived, while the normal sporophyte remains standing on the gametophyte long after finishing its role.

CONCLUSIONS

Axenic culture of the moss *Ambystegium serpens* has been established for the first time. It was initiated from sterile spores, and only primary protonema could be obtained. Further plant development failed. Apogamous sporophyte development was obtained directly from protonema in the haplophase for the first time in the moss *Amblystegium serpens*.

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IN VITRO КУЛТУРА И АПОГАМИЈА – АЛТЕРНАТИВНИ ПУТ У ЖИВОТНОМ ЦИКЛУСУ КОД МАХОВИНЕ *AMBLYSTEGIUM SERPENS* (AMBLYSTEGIACEAE)

ТИЈАНА ЦВЕТИЋ[1,2], М. САБОВЉЕВИЋ[1,3], АНЕТА САБОВЉЕВИЋ[1,4] и Д. ГРУБИШИЋ[1,5]

[1]*Институт за ботанику и Ботаничка башта "Јевремовац", Биолошки факултет, Универзитет у Београду*, 11000 Београд, Србија и Црна Гора
[2]*Центар за мултидисциплинарне Студије, Универзитет у Београду*, 11000 Београд, Србија и Црна Гора
[3]*Nees Institute of Plant Biodiversity, Rheinische Friedrich Wilhelms University of Bonn,* 53115 Бон, Немачка
[4]*Институт за ботанику, Универзитет у Келну*, 50931 Келн, Немачка
[5]*Институт за биолошка истраживања "Синиша Станковић"*, 11000 Београд, Србија и Црна Гора

In vitro култура маховине *Amblystegium serpens* (Amblystegiaceae) је успостављена на подлози Murashige и Skoog (MS) (без биљних хормона) којој су додати MS микро и макро минералне соли и витамини (у два пута мањој концентрацији од уобичајене), 100 mg/l мио-инозитола, 30 g/l сахарозе и 0.70% агара. На наведеној подлози, у условима дугог дана (16h дан/ 8h ноћ), на температури од 25±2ºC, при влажности ваздуха од 60-70% и на светлости интензитета 47 μmol/m^2s дошло је до клијања спора и до развића примарне протонеме. Три месеца након развића примарне протонеме, спорофити су се развили директно из примарне протонеме, а да се није догодила смена генерација. Феномен апогамног развића спорофита је веома редак, како у природи, тако и у условима *in vitro*. Ово су први пут забележени резултати да је апогамија индукована код врсте *Amblystegium serpens*.