GIBBERELLIN INFLUENCE ON THE MORPHOGENESIS OF THE MOSS BRYUM ARGENTEUM HEDW. IN IN VITRO CONDITIONS

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Abstract - The moss Bryum argenteum Hedw. was treated with gibberellins as well as some inhibitors of gibberellin biosynthesis in order to investigate their influence on B. argenteum morphogenesis. Generally, gibberellins have not been chemically identified in bryophytes, while other groups of classical phytohormones (auxins, cytokinins, abscisic acid and ethylene) have been chemically identified in these plants. The in vitro culture of the moss Bryum argenteum was established from sterilized spores. The apical shoots of untreated gametophytes grown in vitro were used to investigate the influence of different substances on secondary protonema and on the growth and multiplication of the gametophytes. B. argenteum reacts differently to the growth regulators applied. Both gibberellins applied in vitro (GA3 and GA7) have a positive effect on B. argenteum morphogenesis. Shoot multiplication was negatively affected by three tested growth retardants (ancymidol, BX-112 and chlorocholine chloride), while these substances did not have such strong effects on the moss protonema development.

Key words: Bryophytes, mosses, gibberellins, morphogenesis, Bryum argenteum.

INTRODUCTION

It is not commonly realized that the technique of culturing plant tissues and organs under axenic conditions was first established and profitably employed in bryophytes, especially mosses (Servettaz, 1913). However, the in vitro culturing of bryophytes has been recently highlighted in the protection of threatened species and the isolation of biologically active and new compounds (Gonzalez et al., 2006, Sabovljevic and Sabovljevic, 2008).

A few moss species have been used to investigate the influence of phytohormones on their development in vitro. Bopp and co-workers (Bopp, 1953, 1955, 2000; Bopp and Bohrs, 1965; Bopp and Jacob, 1986; Bopp et al., 1978, 1991) concentrated on the physiology of Funaria hygrometrica Hedw. and Reski and others (Reski 1998, 1999; Reski and Abel, 1985; Reski et al., 1991, 1994; Decker et al., 2006) have carried out substantial research using Physcomitrella patens. Several other species have also been used for such investigations, but reports are scattered and not detailed (Briere et al., 1977; Rahbar and Chopra, 1982; Alcalde et al., 1996; Sastad et al., 1998). The present knowledge of moss-phytohormone interaction is mainly based on auxins and cytokinins and there is no or very little data to be found for the application of other groups of growth regulators to bryophytes.

Gibberellins (GAs) are a large family of phytohormones involved in an array of various responses throughout the life cycle of plants. The general role of GAs can be summarized in germination stimulation, flowering time regulation and cell expansion. They were isolated from Giberella fungus but found afterwards in various bacteria (MacMillan, 2001) and many plant species including unicellular and multicellular algae (Radley, 1961; Kato et al., 1962; Mowat, 1965; Tarakhovskaya et al., 2007). However, the role of gibberellins in moss species is still unknown.
Almost no effects of GAs have been reported for mosses in contrast to ABA, cytokinins or auxins which are known to have an effect on the developmental stages of *Physcomitrella patens* (Decker et al., 2006; Yasumura et al., 2007). Chopra and Mehta (1992) and Chopra and Dhingra-Babbar (1984) mention the effect of GAs on moss growth. Chaban et al., (1998, 1999) reported on the interference with gravitropism when GA is applied to *Ceratodon purpureus* and *Pottia intermedia*.

In plant biology, growth retardants are a group of synthetic compounds known as inhibitors of gibberellin biosynthesis. Plant growth retardants are capable of reducing unwanted shoot growth and are applied in agronomy and horticulture (Rademacher, 2000). Depending on which step of gibberellin biosynthesis they block, there are four different groups of these compounds: “onium” compounds, compounds with a nitrogen-containing heterocycle, structural mimics of 2-oxoglutaric acid and 16,17-dihydro GAs. To date, there are no reports on the effect of growth retardants (GA biosynthesis inhibitors) on bryophyte development.

In this study, we have used three growth retardant compounds belonging to three different groups: Chlorocholine chloride (onium-type plant growth retardant), Ancymidol (belonging to group of N-containing heterocyclic compounds) and Prohexadione (a compound with structural mimics of 2-oxoglutaric acid).

**MATERIALS AND METHODS**

The moss species *Bryum argenteum* Hedw. was used for establishing in vitro cultures. Specimens were collected in November 2000 in Kalemegdan Fort Park (Belgrade). The voucher specimens are kept in the BEOU herbarium of Belgrade University.

This species was in the sporophyte phase, but with intact opercula. The cultures were initiated from almost mature spores, from unopened capsules that were taken for sterilization.

After collection, the sporophytes were separated carefully from the gametophytes, placed in glasses, covered with cheese cloth and rinsed with tap water for 30 min. Then 10 sporophytes of *B. argenteum* were sterilized with 13% and 15% solutions of sodium hypochlorite, respectively. Finally the sporophytes were rinsed three times in sterile deionized water. For detailed procedures for *Bryum* and other mosses see Sabovljević et al., 2002, 2003, 2006; Bijelović and Sabovljević, 2003; Cvetić et al., 2007.

As a basal medium we used MS medium containing Murashige and Skoog (1962) mineral salts and vitamins, 100 mg dm$^{-3}$ myo-inositol, 0.70% (w v$^{-1}$) agar (Torlak purified, Belgrade) and 0.1 M fructose instead of sucrose. In experiments to observe the influence of the growth regulators we enriched the basal medium with phytohormones, gibberellins (GA$_3$ and GA$_7$), as well as with growth retardants, i.e. substances that inhibit gibberellin biosynthesis in different steps (Ancymidol, Prohexadione (BX-112) and Chlorocholine chloride (CCC)). The gibberellin concentrations were: 0.03 μM, 0.1 μM, 0.3μM, 1 μM, 3 μM and 10 μM, and the growth retardant concentrations were: 0.01 μM, 0.03 μM, 0.1 μM, 0.3μM, 1 μM, 3 μM and 10 μM. The effects of externally applied plant growth regulators were determined.

The pH of MS medium was adjusted to 5.8 before sterilization in an autoclave at 114°C and 108 kPa for 25 min.

The cultures were grown at 25±2°C under cool-white fluorescent light (47 μmol m$^{-2}$s$^{-1}$ irradiance) and a day/night regime of 16/8 h.

To study the influence of phytohormones and growth retardants on morphogenesis, 10 mm-long apical portions of shoots were used. For each concentration of the different phytohormones and growth retardants approximately 40 transplants of moss were cultivated in four petri dishes. Forty explants were used per treatment and each experiment was repeated three times. The influence of the exogenously applied phytohormones and growth retardants was quantified using an index of multi-
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Fig. 1. Effects of gibberellins (GA₃, GA₇) on the protonema diameter of B. argenteum in culture in vitro. Values are mean ± SD, with N = 120.

Fig. 2. Effects of gibberellins (GA₃, GA₇) on the multiplication index of B. argenteum in culture in vitro. Values are mean ± SD, with N = 120.

plication and the diameter of the secondary protonema, which were recorded after six weeks of culture. The index of multiplication represents the number of newly grown shoots originating from one shoot transplant. All data were analyzed using SIGMAPlot ver. 8.0 (SPSS, USA) as well as by analysis of variance (ANOVA), using a multiple range test with a significance level of P < 0.05, F = 9.331.

RESULTS AND DISCUSSION

The influence of various GA₃ and GA₇ concentrations on protonema diameter indicates that B. argenteum reacted similarly to both compounds (Fig. 1). With increasing gibberellin concentrations in the media, the protonema diameter of B. argenteum increased slightly.

The effects of gibberellins on protonema growth (diameter) are similar for both compounds examined, i.e. GA₃ and GA₇. The optimum concentrations were 3 μM and 10 μM, but in this case GA₃ had a slightly better effect (Fig. 1).

However, the effect of the two gibberellins on shoot multiplication shows different patterns in this species. When GA₃ is applied, the index of multiplication in B. argenteum first increases at the optimum concentration (3 μM), then slightly decreases to 10 μM. (Fig. 2). When GA₇ was applied in the MS medium, the index of multiplication values were two times less than with GA₃. However, under control conditions, in the medium without phytohormones the multiplication of B. argenteum is low and both applied gibberellins had a positive effect on the multiplication index.

In this study, we present for the first time the influence of plant growth retardants on bryophyte morphogenesis. All three tested compounds used in this study, ancyclidol, prohexadione (BX-112) and chlorocholine chloride, had similar effects in B. argenteum development in vitro. The growth of protonema was affected by the application of gibberellin biosynthesis inhibitors (Fig. 3). The protonema diameter was slightly smaller compared to control conditions when very high concentrations (3 μM and 10 μM) of ancyclidol, BX-112 and chlorocholine chloride were applied in the medium.

The effect of the three applied gibberellin biosynthesis inhibitors on shoot multiplication was stronger than their effect on protonema development (Fig. 4). In this experiment all three tested compounds had inhibitory effects on B. argenteum shoot multiplication in the in vitro culture. With the raising of ancyclidol, BX-112 and chlorocholine
chloride concentrations in the growth medium the levels of the multiplication index had lower values and were the smallest when 3 μM and 10 μM concentrations of these compounds were added in the medium (Figs. 5, 6, 7).

From the five main groups of phytohormones only auxins and cytokinins are documented as natural signal substances in mosses (Cove and Ashton, 1984). Not only do both hormone groups exist in mosses, but they also have basic functions in the regulation of normal development. Although Decker et al., (2006) reviewed phytohormones in Physcomitrella patens development, they do not mention any known role of gibberellins in the developmental processes of Physcomitrella patens. GAs in bryophytes have never been chemically identified (Anterola and Shanie, 2008). However, Ergun et al., (2002) reported that gibberellin-like substances are also detected in mosses, but the presence of GAs in an organism does not necessarily mean that it is responsive to these compounds. Since for a long time GAs have not been identified in mosses, Yasumura et al., (2007) state that the hormonal signaling pathway developed later in land plant evolution, but not completely de novo. Hence, it could be suggested that GA biosynthetic precursors, like ent-kaurene should be present in mosses (Vandenbussche et al., 2007). Anterola and Shanie (2008) reported that according to a survey of the Physcomitrella patens genome, at least this moss species may have a shorter version of the gibberellin biosynthetic pathway relative to that of higher plants.

To understand any hormonal effect in more detail it is important to know more about as aspects of synthesis, metabolism, and transport. For this purpose, mutants have been introduced into moss research. There are large numbers of auxin and cytokinin mutants that have a low/high degree of auxin/cytokinin production. According to Anterola and Shanie (2008), with the identification of putative gibberellin biosynthetic genes in P. patens, it is now possible to knock them out and make a functional characterization of these genes in order to find out whether or not GAs are necessary for moss growth and development. Although the P. patens (a model bryophyte plant) genome is published (Rensing et al., 2008), the experiments presented in this study which indicate the classical physiological effects of gibberellins in bryophyte development (bryophyte species other than P.
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**Fig. 5.** *B. argenteum* grown on MS medium supplemented with gibberellin biosynthesis inhibitors (1 μM ancimol) a) petri dish, b) moss plant detail.

**Fig. 6.** *B. argenteum* grown on MS medium supplemented with gibberellin biosynthesis inhibitors (3 μM ancimol) a) petri dish, b) moss plant detail.

*patens* are also very important and useful, especially as there are still not enough data on the role of gibberellins in the developmental process of this group of land plants.

While it is still not known whether or not bryophytes produce GAs, there are reports that some of them contain GA-related diterpenoids as secondary metabolites. Von Schwartzenberg et al., (2004) report that in the moss *Physcomitrella patens* the tetracyclic diterpene is produced as a secondary volatile compound in huge amounts and Hayashi et al., (2006) reported on the bifunctional ent-kauren synthase in the same species.

In seed plants, GAs are critical hormones since they are necessary for many morphological and physiological processes. According to the *P. patens*
sequenced genome, this moss species has a protein that is closely related to gibberellin receptors, although some orthologues of the key elements in the GA biosynthetic pathway are missing from *P. patens* (Vandenbussche et al., 2007). Also, Vandenbussche et al., (2007) state that the GA signaling pathway is conserved in many groups of land plants, but not in mosses. Pathway components seem to be missing in the moss *P. patens*, apart from the receptors. Hence, it could be that bryophytes may have an evolutionary onset to respond to GAs.

According to our results gibberellins have positive effects on moss morphogenesis, at least for the tested species, *B. argenteum*. Tested GA concentrations (up to 10μM) are usually used in experiments where phytohormone effects are examined.

In the experiments where gibberellin biosynthesis inhibitors were applied, it was shown that the tested retardants had inhibitory effects on shoot multiplication *in vitro*. However, these substances have almost no negative effect on protonema morphogenesis while in vascular plants these substances have extremely negative effects on the morphogenetic process. This type of *B. argenteum* reaction to applied growth retardants could be explained by the fact that the GA signaling pathway, although well conserved in many groups of land plants, is not conserved in mosses. It would also seem that mosses lack many crucial components of the GA pathway. Hence, it could be that applied GA biosynthesis inhibitors do not block gibberellin biosynthesis in the same way they do in vascular plants, or that maybe mosses overcome the retardant inhibition and “find” some optional pathways.

The results given above highlight many new problems not known to date, e.g. does protonemal growth increase due to cellular expansion or division; what can be expected when GAs and inhibitors are applied synergistically; or do GA inhibitors block the biosynthesis or the action of GAs. Further studies in bryophytes should provide answers to these questions.

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