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Effects of different cytokinins on chlorophyll retention in the moss *Bryum argenteum* (*Bryaceae*)

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Abstract

Background and Purpose: *Cytokinins are a group of plant hormones that have an important role in plant growth and developmental processes. Chlorophyll content is an extremely important parameter in estimating the plant production level. Since bryophytes do not have such economical importance as vascular plants and their production in many ecosystems is small, they remain uninteresting for studying their chlorophyll level. The aim of this study was to compare the effect of different cytokinins on chlorophyll retention in moss* B. argeteum *gametophyte shoots grown in natural conditions with those grown in* in vitro *culture.*

Material and Methods: *The effect of different cytokinins: kinetin (KIN), 6-benzylaminopurine (BAP) and thidiazuron (TDZ) on chlorophyll retention of the moss* Bryum argenteum *Hedw. (Bryaceae) derived from* in vitro *culture or grown in nature was tested. Plants derived from* in vitro *culture were grown on Murashige and Skoog (MS) medium at 25±2°C. Gametophyte shoots were used in experiments where influence of different concentrations (0.001–10 μM) of three cytokinins was used to investigate their effect on chlorophyll-a, -b and total chlorophyll retention.*

Results and Conclusions: *Cytokinins had a positive but unequal influence on chlorophyll retention in both plant groups – plants derived from* in vitro *culture and plants grown in the nature. Kinetin proved to be the most effective cytokinin in chlorophyll retention. Exogenous application of kinetin increased chlorophyll content with concentration (0–10μM). BAP had similar trends in* in vitro *and native mosses, increasing chlorophyll content up to 1 μM and then significantly decreasing, although the chlorophyll content was greater in* in vitro *grown plants. TDZ showed significantly better effect in in vitro cultured moss shoots, but when applied in concentrations higher than 0.1 μM, total chlorophyll content decreased.*

INTRODUCTION

The level of chlorophyll content has been widely studied in vascular plants, while in bryophytes such studies are almost non-existent. Chlorophyll content is an extremely important parameter in estimating the plant production level. Since bryophytes do not have such economical importance and their production in many ecosystems is small, they remain uninteresting for studying their chlorophyll level.

However, bryophytes are very interesting considering some of their features which are not present in many vascular plants. Bryophytes can assimilate during very low light regime. Light saturation levels for many bryophytes have been found around 20% of full sunlight for a

wide range of bryophytes *(1)*. Rastorfer *(2)* gives similar data for *Bryum argenteum* Hedw. During periods of bright, dry, sunny weather, silver moss (*B. argenteum*) will generally be dry and metabolically inactive and whitish hyalinized parts of phylloid (bryophyte leaves) will protect the plants from high sunlight irradiance. *B. argenteum* is a homoiochlorophyllous plant, which means that it retains the chlorophyll and carotenoid content unchanged throughout the complete dessication-rehydratation cycle *(3)*.

The time of leaf senescence is longer in bryophytes compared to other plants. They can survive a longer period of drought or freezing without damaging their photosynthetic systems.

Cytokinins are a class of plant hormones that play a central role during the cell cycle and influence numerous developmental processes. Their effect on bryophytes is less studied *(4, 5)* compared to vascular plants, and there are many generalizations from other plant systems. They cause bud inductions on protonema which is documented in few mosses *(4, 6, 7)*. Also, there are some data that confirm exogenous cytokinin influence on plastid longevity in the protonema of moss system *Physcomiterlla patens (8)*. In mutant moss *P. patens,* exogenous cytokinin interacting with light provokes gene expression and plastid protein increase *(9),* although cytokinin sensitive mutant is defective in chloroplast division *(10)*.

Cytokinins have been implicated in the maintenance of chlorophyll, protein and RNA levels *(11),* all of which decline during senescence.

The senescence of bryophyte shoots involves changes in their photosynthetic apparatus.

The fact that cytokinins are so effective in delaying chlorophyll breakdown indicates that these growth regulators are somehow involved in maintaining the photosynthetic apparatus of plant organs. Cytokinin treatment can stimulate photosynthesis *(12, 13),* but also inhibit it in some tissues *(14)*.

Cytokinin treatment increases chloroplast DNA and protein synthesis, maintains pigment levels, alters membrane permeability, promotes chloroplast replication, grana formation and influences maturation *(15),* and consequently influences loss of chlorophyll, but also influences the formation of it.

To date there are no data on retention of chlorophyll in bryophytes, and relatively little work has focused on cytokinin metabolism in mosses *(16)*.

Bryophytes are considered as higher plants but considerably less knowledge on their biology is available. Therefore, too many generalizations on bryophyte biology have been derived from tests done on vascular plants. In this study we have tried to examine the influence of selected phytohormones (namely kinetin, 6-benzylaminopurine and thidiazuron) in concentrations that are often used in similar studies with vascular plants, on chlorophyll retention of selected bryophyte model system *Bryum argenteum*. Bryophytes are able to survive in low light conditions and therefore we expected the chlorophyll retention time to be considerably higher and the phenomenon of senescence to appear, at all, later compared to vascular plants. Also, the influence of phytohormones on chlorophyll retention in bryophytes was not tested previously, and this was the reason why these experiments were performed.

The aim of this study was to compare the effect of different cytokinins, substituted adenines and novel phenylurea cytokinins on chlorophyll retention in *B. argeteum* gametophytes grown in native conditions with those grown in *in vitro* culture.

MATERIALS AND METHODS

In vitro growth conditions

Two groups of plant material were used in these experiments. The first group represented *B. argenteum* plants that were collected from native habitats. The second plant group consisted of shoots established in *in vitro* conditions, on Murashige and Skoog (MS) basic medium *(17)* that contained MS mineral salts and vitamins, 100 mg L^{-1} myo-inositol, 8 g L^{-1} agar, and was supplemented with 0.1 M fructose instead of sucrose. Fructose was selected as a carbon source in MS basic medium because it was previously shown that fructose has the best effect on *B. argenteum* development *in vitro (18)*. In order to determine cytokinin effects on chlorophyll retention, both plant groups were treated with different cytokinins before chlorophyll level was determined. The details on establishing and growing moss *B. argenteum* culture *in vitro* can be seen in Sabovljević *et al. (18, 19)*.

Bryophyte shoots grown *in vitro* were subcultered on the same MS basic medium, and after four weeks of subculture period plants were used for experiments with cytokinins.

Moss material collected in nature was brought to the laboratory and re-moistened if necessary, just before the start of the experiment. Similarly, plants grown in *in vitro* culture were harvested just before starting the experiment.

Experimental design and chlorophyll determination

Since it is widely known that cytokinins have an effect on chlorophyll retention from higher plants, we tested the effect of three different cytokinins belonging to either substituted adenines or to novel phenylurea cytokinins, on moss chlorophyll retention in two bryophyte groups: plants grown in nature and plants obtained from *in vitro* culture. While most of the natural and synthetic cytokinins are all substituted adenines, there are also phenylurea cytokinins, such as thidiazuron. In order to observe the influence of cytokinins on chlorophyll retention, different concentrations of benzylaminopurine (BAP), kinetin (KIN) or thidiazuron (TDZ) were used: 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM.

Moss shoots (3 g of fully hydrated 10 mm apical shoots for each treatment) were kept in the dark in a Petri-dish with certain concentrations of different cytokinins for 48 hours at 25°C. Pure water treatment was used as a control. At least three repetitions were done for each treatment.

Pigment analyses followed Arnon *(20)*. Bryophyte samples were extracted in 80% acetone and absorbance of acetone extract was measured at 645, 652, 663 and 720 nm with a spectrophotometer (UV visible Agilent 8453 Spectrophotometer) using 80% acetone as a blank. Chlorophyll concentration was calculated in (nmol g^{-1}).

Bryophyte shoot samples were homogenized in 3 mL of 80% acetone. The homogenates were incubated for 1 hour at room temperature before they were centrifuged for 10 min (Eppendorf Mini Spin F-45-12-11 Centrifuge). The supernatant was used for determination of chlorophyll -a, -b and total chlorophyll content which was estimated by Arnon *(20)*.

Statistical analysis

All data were analyzed using the statistic-graphic programme SigmaPlot (SPSS Inc., USA), version 8.0, using a multiple range test with significant level at $P < 0.05$. Mean values and standard errors were calculated for at least 3 replicates for each measurement. Three independent experiments were performed for each cytokinin essay.

RESULTS

Before starting the experiments the chlorophyll content of mosses from the two groups was different (Table 1).

Generally, chlorophyll content in bryophytes is much lower compared to vascular plants *(1)*. Also, the difference in pigment content among different moss populations in nature has already been reported, mainly due to the growing conditions *(21)*.

All tested cytokinins were effective in conducted experiments, but there were differences when comparing chlorophyll retention in plants grown in nature and these derived from *in vitro* culture. Chlorophyll- *a*, – *b* and total chlorophyll concentration was generally higher in plants obtained from *in vitro* culture, both in control conditions and when treated with cytokinins (Table 2).

According to the results obtained, in experimental conditions chlorophyll *a* retention was better in plants derived from axenic conditions compared to those collected in nature. The forty-eight hour treatment showed that with the increase in KIN concentration chlorophyll *a* concentration in plants from axenic conditions increased and just slightly decreased in the highest concentration (10 μM) (Table 2). The KIN influence on chlorophylls retention in *B. argenteum* grown in nature was less effective, and the differences among chlorophyll *a* concentrations were not so significant in this plant group (Table 2).

BAP had positive effect on chlorophyll *a* retention in mosses developed in *in vitro* conditions, but at the highest concentrations was not as effective as KIN. BAP effects on chlorophyll *a* retention of plants collected in nature was much better compared to KIN, especially at higher concentrations applied.

TDZ effect in two examined moss groups had no similar pattern. While in plants from *in vitro* culture TDZ was not as effective as the other two tested cytokinins, in plants from nature TDZ was the most effective compound with regard to chlorophyll *a* retention, especially when applied in high concentrations.

In the a case of plants grown *in vitro* all applied cytokinins had positive effect on chlorophyll *a* retention, whereas only cytokinins applied in higher concentrations (0.1 μM and higher) were effective for plants collected in nature.

The influence of different cytokinins on chlorophyll *b* retention was positive in both plant groups, but not significantly effective when compared with the cytokinin effect on chlorophyll *a* retention. Also, these two plant groups did not differ very much between themselves in chlorophyll *b* concentration. The efficiency of all tested cytokinins in plants from controlled conditions was very similar (Table 2). In contrast, in plants grown in nature, BAP and TDZ were more effective compared to KIN (Table 2).

Total chlorophyll (chlorophyll- *a* and -*b*) concentration in plants from *in vitro* culture varied from 8.28–16.02 nmol g^{-1} (Table 2), whereas in plants grown in nature it was 7.48–12.19 nmol g^{-1} (Table 2). In plants derived from *in vitro* culture, the most effective was kinetin, especially at the highest applied concentration (10 μM), while BAP and TDZ were less effective at very high concentrations (1 and 10 μM). The pattern of all three exogenously applied cytokinins in this plant group was similar: with the concentration increment, total chlorophylls retention also increased. Also, cytokinins had a positive effect on total chlorophyll retention in plants collected from nature, although the most effective was when applied at 0.1 μM concentration. Cytokinins applied at the highest

TABLE 1

The content of chlorophyll in two experimental moss groups prior the start of chlorophyll retention experiments.

Plant group	Chl *a* (nmol g^{-1})	Chl *b* (nmol g^{-1})	Chl *a*+*b* (nmol g^{-1})	Chl *a* / Chl *b*
In vitro culture	13.40±0.10	3.56±0.16	17.87±0.20	3.76
Nature	9.97±0.05	4.37±0.13	15.44±0.19	2.28

TABLE 2

Effects of KIN, BAP and TDZ on chlorophyll a, chlorophyll b and total chlorophyll (a+b) retention and on chlorophyll a / b ratios. The numbers with * represent results from the plants obtained *in vitro*, while numbers with + represent results from the plants obtained from nature. The values plotted in the table represent the percentage of the values prior to dark and cytokinins treatment that are presented in Table 1.

Conc. [μM]	Chl *a* (%)			Chl *b* (%)			Chl *a*+*b* (%)			Chl *a*/*b*		
	KIN	BAP	TDZ	KIN	BAP	TDZ	KIN	BAP	TDZ	KIN	BAP	TDZ
0	34.22*	35.04*	33.47*	72.33*	72.84*	74.11*	46.33*	48.57*	48.07*	1.75*	1.78*	1.67*
	37.71+	39.42+	37.91+	62.01+	62.70+	63.16+	48.46+	48.70+	48.96+	1.39+	1.43+	1.37+
0.001	45.38*	58.11*	51.04*	80.20*	77.41*	78.43*	63.12*	62.45*	62.62*	2.10*	2.78*	2.43*
	40.62+	46.04+	41.02+	62.47+	64.07+	64.76+	62.18+	59.65+	61.66+	1.48+	1.64+	1.45+
0.01	54.21*	69.20*	44.08*	79.19*	81.73*	77.41*	67.10*	68.38*	63.74*	2.54*	3.14*	2.11*
	45.04+	48.95+	39.32+	53.32+	68.42+	89.70+	71.50+	73.64+	62.43+	1.93+	1.63+	1.00+
0.1	67.90*	82.34*	52.09*	93.15*	96.45*	94.16*	74.93*	78.51*	75.66*	2.70*	3.17*	2.05*
	52.56+	58.07+	53.26+	67.51+	80.55+	88.79+	78.95+	78.56+	78.17+	1.79+	1.64+	1.37+
1	83.57*	82.00*	65.98*	96.70*	95.68*	89.34*	76.10*	79.02*	66.37*	3.20*	3.18*	2.74*
	38.21+	56.27+	74.12+	57.67+	80.09+	98.63+	61.66+	75.78+	62.18+	1.51+	1.60+	1.71+
10	80.77*	66.46*	53.05*	101.60*	106.60*	77.41*	89.65*	76.78*	62.51*	2.96*	2.31*	2.54*
	35.20+	70.41+	75.33+	48.74+	79.41+	81.69+	61.66+	69.95+	58.03+	1.65+	2.02+	2.10+

** in vitro*
\+ nature

concentration (especially KIN and TDZ) were not as effective as in the case of plants from *in vitro* culture.

Chlorophyll *a* / *b* ratios were in the range 1.67–3.20 for plants obtained in *in vitro* conditions (Table 2) and 1.00–2.10 for plants collected in nature (Table 2). It is reported that bryophytes typically have low chlorophyll *a* / *b* ratios *(16)*, which is in accordance with our results.

In plants grown in controlled light conditions the chlorophyll *a* / *b* ratios were higher than in plants from nature, which infers that the breakdown of chlorophyll *b* into chlorophyll *a* by chlorophyll *b* reductase is probably increased, as stated by Hörtensteiner *(22)*.

DISCUSSION

It is interesting that in some cases TDZ was not as effective as the other two tested cytokinins in *B. argenteum* chlorophyll retention. While the natural as well as synthetic cytokinins based on them are all substituted adenines, there are several phenylureas that are shown to have cytokinin activity *(23)*. Although it is known that induction of shoot buds from the filamentous protonema of moss represents cytokinin bioassay, to date there has only been sporadic use of this bioassay with the phenylurea cytokinins (no activity: 24, 25; low activity: 26). Christianson and Hornbuckle *(6)* reported that phenylurea cytokinins (thidiazuron, TDZ and chloro-pyridyl--phenylurea, CPPU) induce bud formation in moss *Funaria hygrometrica* grown *in vitro*. Same authors report that treatment with TDZ, like treatment with substituted adenine cytokins (BA), results in concentration-dependent stimulation of buds. However, they report some differences in the activity of TDZ and BA: TDZ is active over a slightly higher range of concentrations than BA, but at the same time BA is more effective at the same applied concentrations. This difference might reflect differences in affinity for hormone receptors, but it could also reflect differences in hormone uptake or metabolism *(6)*. This could provide the answer of why TDZ was not as effective as BAP in our experiments with cytokinin-induction of chlorophylls retention.

Considering the fact that many moss species are drying- or freezing – desiccation tolerant species *(3, 27, 28)*, it seems that cytokinins play an important role in chloroplast preparation for the inactive dry period and its quick activation when good conditions return.

Chlorophyll retention is only one indicator of leaf photosynthetic function, as photosynthesis may decline long before chlorophyll content declines *(29)*.

Many regulators in vascular plants, required to induce control chlorophyll breakdown, remain to be discovered *(22)*, and even less are known in bryophytes. The results presented in the paper towards elucidation of chlorophyll retention processes should provide stimulus for more investigations of chlorophyll eco-physiology and biochemistry in bryophytes, which share general biological patterns with vascular plants, but which also have many peculiarities. Hörtensteiner *(22)* states that chlorophyll binding protein is in close interconnection between chlorophyll and apoproteins.

In bryophytes, which have low matter turnover and energy flow, chlorophyll retention is a very important process, since rapid loss of chlorophyll demands much energy for new synthesis, and make the plant less competitive in harsh environments. Moss plants are known as resurrection plants, so the inactivation vs. activation of metabolic processes is quick and not clear, especially not for such important systems such as chloroplasts. On the other hand, breakdown of chlorophyll qualifies as detoxification mechanism, which is also vitally important for further plant development and survival.

The influence of phytohormones on chlorophyll retention in bryophytes was not previously tested, and this was the reason why these experiments were performed. All tested cytokinins (KIN, BAP and TDZ) have a positive effect on chlorophyll retention in both tested *B. argenteum* groups; plants grown in native conditions and plants derived from *in vitro* culture. Generally, chlorophyll – *a*, – *b* and total chlorophyll concentration, as well as chlorophyll *a* / *b* ratio were higher in *B. argenteum* plants grown *in vitro*, both in control conditions and when treated with cytokinins.

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