J Plant Growth Regul (1996) 15:153–156

Journal of
Plant
Growth
Regulation

Short Communication

Effect of Brassinosteroids on Growth and Proton Extrusion in the Alga *Chlorella vulgaris* Beijerinck (Chlorophyceae)

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Received June 11, 1996; accepted September 18, 1996

Abstract. Brassinolide, as a plant hormone, promotes growth of a number of plant species. Similar effects are induced by its epimer 24-epibrassinolide. In this paper we discuss the effects of brassinosteroids on the growth and proton extrusion in the green alga *Chlorella vulgaris* (Chlorophyceae). At concentrations between 10^{-15} and 10^{-8} M, brassinolide and 24-epibrassinolide induce a significant stimulation of growth and H^+ extrusion. The growth was associated with an increase in the capability of algal cells to acidify the medium, where brassinolide is biologically more active than 24-epibrassinolide.

Key Words: Alga—Brassinosteroids—Growth—Proton extrusion

Since the discovery of brassinolide (BL) in 1979, more than 40 analog of BL (called brassinosteroids, BRs) have been isolated and identified from many species of plants, including algae, gymnosperms, monocots, and dicots (Kim 1991). BRs may thus be regarded as a new group of plant hormones with a regulatory function in cell elongation and division (Adam 1994, Cutler et al. 1991, Mandava 1988, Sakurai and Fujioka 1993). They may also have a role in the control of RNA and protein synthesis (Kalinich et al. 1986, Mandava et al. 1987). BRs interact with plant hormones and other growth substances (Adam 1994, Mandava 1988, Sakurai and Fujioka 1993). Recent work shows that BRs stimulate growth by cell enlargement and electrogenic H^+ extrusion in stems and roots (Cerana et al. 1983, 1984, Mandava 1988, Romani et al. 1983). The promotion of cell enlargement by other factors, either endogenous (auxins, gibberellins, and cytokinins) or added (phytohormones, fusicoccin) is almost always associated with, and probably mediated by, an increase in the capability of the tissue to acidify the incubation medium (acid growth theory) (Hobbie et al. 1994, Rayle and Cleland 1992). This corresponds to an increase in the activity of an electrogenic proton extrusion mechanism operating at the plasmalemma (Cerana et al. 1983, Romani et al. 1983).

We know that BRs have already been identified from a green alga *Hydrodictyon reticulatum* (Chlorophyceae) (Yokota et al. 1987). However, biochemical effects of native phytohormones of the BR type were not known until now in the alga. In this paper we report the results of experiment on the effects of BRs (BL and epiBL) on growth and acid secretion in the alga *Chlorella vulgaris* (Chlorophyceae).

Materials and Methods

Organisms and Culture Conditions

C. vulgaris Beijerinck (Chlorophyceae) was grown under controlled conditions at 25 ± 0.5°C. Illumination was supplied for a 16-h photoperiod (8-h dark period) by a bank of fluorescent lights yielding a proton flux of 50 μmol m^{-2} s^{-1} at the surface of the tubes. Permanent synchronous growth was established according to the method of Pirson and Lorenzen (1966) under the conditions developed by Sayegh and Greppin (1973). The culture medium used was modified Knop's. The pH of the medium was adjusted to 6.8 with 1 N NaOH. The *Chlorella* cells were cultured in an Erlenmeyer flask (500 ml) containing 250 ml of medium and were shaken at 50 rpm in a rotary shaker.

Abbreviations: BL, brassinolide; BR(s), brassinosteroid(s); epiBL, 24-epibrassinolide; DW, dry weight; IAA, indole-3-acetic acid.

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Table 1. Effects of BRs on the growth of *C. vulgaris* (expressed as mg/g DW ± S.E. (0.02–0.04))

Concentration (M)	12h		24h		36 h		48 h	
	BL	epiBL	BL	epiBL	BL	epiBL	BL	epiBL
0	2.62	2.62	4.97	4.97	7.45	7.45	9.94	9.94
10^{-15}	4.51	4.46	12.16	12.11	21.33	21.30	21.55	21.51
10^{-14}	4.56	4.51	12.22	12.17	21.38	21.34	21.59	21.54
10^{-13}	4.62	4.55	12.27	12.21	21.43	21.38	21.63	21.58
10^{-12}	4.67	4.60	12.31	12.27	21.48	21.42	21.67	21.62
10^{-11}	4.72	4.64	12.38	12.32	21.53	21.47	21.73	21.69
10^{-10}	4.77	4.68	12.42	12.37	21.58	21.51	21.77	21.72
10^{-9}	4.80	4.73	12.48	12.42	21.61	21.56	21.81	21.76
10^{-8}	4.85	4.78	12.52	12.47	21.65	21.60	21.85	21.82

Brassinosteroids

BL was obtained from Dr. Shozo Fujioka. BL and epiBL (Sigma) were prepared as 5 mM ethanolic stock solutions and stored at –20°C. The appropriate amount of BR stock solution, first as the strongest, was transferred directly into the culture medium, and then weaker solutions were prepared by serial dilution. Equal amounts of ethanol were added to the controls. The final ethanol concentration in the culture media did not exceed 0.001% (v/v), and this concentration did not affect on the growth.

Determination of Dry Weight

To determine the biomass dry weight (DW), duplicate known volumes of the algal culture were filtered through preweighed glass microfiber filters (Whatman, Maidstone, UK), washed with distilled water to remove nonbiologic materials such as mineral salt precipitates, dried overnight at 105°C, and then weighed (Lee and Low 1993).

H^+ Extrusion

H^+ extrusion was measured (Cole-Parmer pH Benchtop meter model 05669-20) as pH change (ΔpH) in the incubation medium during the treatment. ΔpH is the difference between the initial and the final pH. The S.E. for ΔpH values did not exceed ± 0.01.

The entire experiment was set up with six replicates. Where appropriate, data were analyzed using the analysis of variance and *t* test.

Results

The green alga *C. vulgaris* was used to investigate the effects of BL and epiBL on growth and proton extrusion. Growth of unicellular alga is manifested as an increase in the size of each cell followed by division into daughter cells. The specific growth rate was measured by cell weight, and the data are presented in Table 1. In the period from the 12th to the 36th h BRs induce intensive growth of the alga *C. vulgaris,* two to three times higher compared with the control. After 36 h a stagnation of growth is observed. In the control cultivation the weakening of the rate of growth of the alga does not occur until after 96 h, which is in agreement with its developmental cycle (data not shown). The results of the study indicate an intensive stimulation of the growth of the alga under the influence of BRs within the range of concentration 10^{-15} to 10^{-8} M. BL has slightly more biologic activity in relation to epiBL. Both BRs significantly shorten the development cycle of the alga. Negative impact of BRs upon the life of *C. vulgaris* contributes to the elimination of this alga as an element of the phytoplankton in the trophic ecosystem chain.

The intensity of H^+ secretion shows a correlation with the dynamics of growth of cells of *C. vulgaris* depending on the concentration of BRs and the time of their influence (Table 2). H^+ secretion (expressed as ΔpH) is stimulated intensively by BRs during the period from the 12th to 36th h of cultivation, reaching its maximum in the 36th h. After 48 h of BR activity a small inhibition of proton secretion is observed, with reference to the 36th h of the alga cultivation. Also, a slight difference was observed in the stimulation of proton secretion under the influence of BL compared with epiBL.

Discussion

No experiments concerning the influence of BRs upon the alga have been conducted so far. It is only known that 24-epicastasterone and 24-ethylbrassinone occur in the green alga *H. reticulatum* (Chlorophyceae) (Yokota et al. 1987). The results presented in this paper show that BRs such as BL and epiBL significantly stimulate growth and proton secretion in cells of *C. vulgaris.* In this experimental model we have confirmed that BRs are active at extremely low concentration, 10^{-15} to 10^{-8} M, which is 100 times lower than for other plant growth regulators, and the structure-activity relationship between BL and epiBL. In many publications the growth-stimulating effects of BRs on higher plants have been described (Adam, 1994, Cutler et al. 1991, Sakurai and Fujioka 1993). The observed strong growth-promoting effects in culture of algae suggest that BRs probably play a physiologic role. Thus, we are pursuing further studies in this

Table 2. Effects of BRs on the proton extrusion (expressed as ΔpH) of *C. vulgaris*

Concentration (M)	12 h		24 h		36 h		48 h	
	BL	epiBL	BL	epiBL	BL	epiBL	BL	epiBL
0	0.12	0.12	0.15	0.15	0.18	0.18	0.20	0.20
10^{-15}	0.25	0.23	0.31	0.28	0.34	0.33	0.32	0.30
10^{-14}	0.27	0.25	0.33	0.30	0.36	0.35	0.34	0.32
10^{-13}	0.30	0.28	0.35	0.32	0.39	0.38	0.38	0.36
10^{-12}	0.33	0.31	0.37	0.35	0.42	0.40	0.40	0.38
10^{-11}	0.35	0.33	0.39	0.37	0.44	0.42	0.42	0.40
10^{-10}	0.37	0.35	0.42	0.40	0.47	0.44	0.44	0.42
10^{-9}	0.40	0.37	0.45	0.42	0.49	0.46	0.46	0.44
10^{-8}	0.43	0.40	0.48	0.45	0.52	0.49	0.48	0.46

direction. BR-induced growth stimulation depends at least partly on the decrease of pH in the wall space and thus on acid-induced wall loosening. The effects of BRs on proton secretion are associated with an early hyperpolarization of the transmembrane electrical potential. BR-induced proton excretion is also stimulated by the presence of K^+ in the medium (Romani et al. 1983).

For about the last 25 years the study of auxin action in the cell elongation has been dominated by the so-called acid growth theory. According to this theory auxin induces acidification of the free space in the cell wall, presumably by the activation of plasma membrane-bound proton pumps. The increase in the plasticity of the cell wall thus causes a rapid increase in elongation rate of the tissues. The acid growth theory suggests that proton pumps might be targets for auxin receptors (Hobbie et al. 1994, Rayle and Cleland 1992). In algae auxins stimulated the growth, and their metabolic activity is significantly higher than control culture (Czerpak et al. 1994). BR acts as an auxin when applied alone and as a gibberellin when applied together with auxin. By itself, it induces elongation, and it also enhances auxin-induced elongation, acting synergistically with the auxin in higher plants (Cohen and Meudt 1983, Katsumi 1985, 1991, Mayumi and Shibaoka 1995, Yopp et al. 1981). The relationships between BRs and the other plant hormones have been explored extensively (Cutler et al. 1991, Marquardt and Adam 1991, Sakurai and Fujioka 1993). It has been shown that both growth substances promote segment elongation and increase fresh weight and electrogenic proton extrusion in Azuki bean epicotyls (Cerana et al. 1983, 1984). Moreover, both plant hormones exhibit a strong synergistic action on segment elongation and ethylene production (Arteca et al. 1988). On the other hand, in contrast to the effect of IAA, BRs promotes elongation and H^+ extrusion (Romani et al. 1983). The actions of IAA and BRs are distinctive at the level of gene expression (Clouse et al. 1992), but more evidence is required to determine whether or not the mechanism of BRs action involves IAA.

Acknowledgments. We thank Dr. Shozo Fujioka (Institute of Physical and Chemical Research, RIKEN, Wako-shi, Saitama, Japan) for authentic samples of brassinolide and Agnieszka J. Bajguz for helpful technical assistance.

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