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Suppression of *Wolffia arrhiza* growth by brassinazole, an inhibitor of brassinosteroid biosynthesis and its restoration by endogenous 24-epibrassinolide

Andrzej Bajguz [a,*], Tadao Asami [b]

[a] *Department of Plant Biochemistry, University of Bialystok, Institute of Biology, Swierkowa 20 B, 15-950 Bialystok, Poland*
[b] *RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

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Abstract

The effect of the brassinosteroid (BR) 24-epibrassinolide (epiBL; 10^{-13}–10^{-6} M) on growth and levels of chlorophylls, carotenoids, sugars and protein in *Wolffia arrhiza* after 7 days of cultivation is reported. Application of epiBL to *W. arrhiza* cultures stimulates the growth and increases the content of photosynthetic pigments, sugar and protein. The greatest effect of epiBL is observed at a concentration of 10^{-9} M. We tested the action of Brz2001, a specific BR biosynthesis inhibitor, in the range of 10^{-6}–10^{-4} M. Addition of Brz2001 to *W. arrhiza* cultures inhibits their growth after 7 days of cultivation. The inhibition of growth could be reversed by the addition of epiBL. Moreover, there was not complete recovery to the level of control, especially at 5×10^{-5}–10^{-4} M Brz2001. The effects of treatment with 10^{-9} M epiBL mixed with a mevalonate pathway inhibitor (mevinolin), or a 2-methylerythritol 4-phosphate pathway inhibitor (clomazone), were also investigated. Mevinolin did not inhibit growth of *W. arrhiza* after 7 days of cultivation. However, clomazone did. Addition of epiBL overcame this inhibition. These results suggest that the mevalonate pathway may not function well in *W. arrhiza* and that biosynthesis of BRs through the non-mevalonate pathway in *W. arrhiza* could be possible.

Keywords: *Wolffia arrhiza*; Lemnaceae; Brassinazole; Brassinosteroid; 24-Epibrassinolide; Growth; Inhibitors

1. Introduction

The Lemnaceae is a family of aquatic monocotyledons that show an apparent continuum of morphological simplification from *Spirodella* through *Lemna* to *Wolffia* and *Wolffiella*. The species in the genus *Wolffia* are, as the other genera in the duckweed-family, greatly reduced plants, that neither have leaves nor a stem and even lack roots. The whole plant is oval or nearly spherical and the dark green top-side is distinctly flattened. *Wolffia arrhiza* is a small plant that may set flowers and seeds, although rapid multiplication is archived by budding. At the end, the plant has a "pouch", in which the "daughter-plants" are formed. When the plant multiplies by budding, the new plant will often stay fixed to the "mother-plant". *W. arrhiza* plants float individually or as two in a cluster at the surface of the water. In response to unfavourable environmental conditions, the resting forms (turions) of *Wolffia* are formed.

Abbreviations: BR, brassinosteroid; Brz, brassinazole; Clo, clomazone; DMAPP, dimethylallyl diphosphate; epiBL, 24-epibrassinolide; HMG-CoA, hydroxymethylglutaryl CoA; IPP, isopentenyl pyrophosphate; MEP, 2-methylerythriol 4-phosphate; Mev, mevinolin; MVA, mevalonic acid.

* Corresponding author. Tel.: +48 85 7457334; fax: +48 85 7457302.
E-mail address: abajguz@uwb.edu.pl (A. Bajguz).

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Germinating turions emerge on the surface and give rise to vegetative floating fronds. Sometimes turions germinate at the bottom, giving rise to vegetative immersed fronds. Plants of this genus are the smallest flowering plants in the world (*W. angusta* or *W. globosa*; both less than 1 mm long). Owing to adaptation to specific environment conditions, *W. arrhiza* has undergone simplification process. Thanks to such properties as possibility of mixotrophic feeding, resistance to numerous toxic substances, fast multiplication in a vegetative way of life, *Wolffia* is used commonly in biotechnological treatment of sewage, especially of human and agricultural origin (Godziemba-Czyz, 1969, 1970; Frick, 1994; Fujita et al., 1999; Mical and Krotke, 1999; Lemon et al., 2001).

Inhibitors of the biosynthesis and metabolism of brassinosteroids (BRs) have complementary roles in the analysis of the functions of BRs in plants. Brassinazole (Brz) is a specific BR biosynthesis inhibitor, which blocks the conversion of campestanol to 6-deoxocathasterone, 6-deoxocathasterone to 6-deoxoteasterone, 6-oxocampestanol to cathasterone and cathasterone to teasterone (Asami and Yoshida, 1999). The application of Brz to plants resulted in growth inhibition or dwarfism, but exogenous brassinolide reversed this effect. This indicates the essential function of BRs to plant growth and development (Asami et al., 2000, 2001, 2003; Bajguz and Asami, 2004). This paper presents a study concerning the influence of 24-epibrassinolide (epiBL) upon the growth and chemical composition of *W. arrhiza* in the concentration range 10^{-13}–10^{-6} M. Data were determined for each of the parameters (the content of fresh weight, chlorophylls, carotenoids, sugar and protein) at the seventh day after the initiation of epiBL treatment. The greatest effect of epiBL is observed at a concentration of 10^{-9} M, this concentration has been selected to study the effect of application of Brz2001. We demonstrate that Brz, applied as a diastereomeric mixture of Brz2001 (10^{-6}–10^{-4} M) acts as an inhibitor of *W. arrhiza* cultures. The inhibition could be reversed by the addition of 10^{-9} M epiBL. We have also investigated the origin of BRs in *W. arrhiza* by testing the effects of a mevalonic acid (MVA) pathway inhibitor (mevinolin; Mev) and a 2-methylerythritol 4-phosphate (MEP) pathway inhibitor (clomazone; Clo) on its growth because isopentenyl pyrophosphate (IPP), a precursor of steroids, can be synthesized through the mevalonate pathway or the non-mevalonate pathway; Mev had no effect, but Clo caused growth inhibition that could be rescued by epiBL. This result suggests that in *W. arrhiza* only the MEP pathway may function or compensate the inhibition of MVA pathway and that BRs in *W. arrhiza* could be synthesized through the MEP pathway under the treatment of Mev.

2. Results

2.1. Appearance of *W. arrhiza* cultures

Microscopic observations indicate that there are no significant differences in morphology of epiBL-treated, Brz2001-treated or untreated *W. arrhiza* cultures (Fig. 1). Individual vegetative fronds of *W. arrhiza* are ellipsoidal, a little flattened at the top. Floating fronds are usually single or sometimes paired. The mean dimension of single vegetative floating fronds is $D = 1.72$ mm (length), $d = 1.35$ mm (width). EpiBL accelerates only the development of vegetative floating fronds. The impact of epiBL on immersed fronds was studied, but no effect was found. However, the whole plants treated with 5×10^{-5} M Brz2001 are nearly spherical. They are also less green than the control, or epiBL-treated, cultures. Furthermore, at concentrations above 5×10^{-5} M, Brz2001 is cytotoxic to *W. arrhiza* cultures, resulting in cellular fragmentation and lysis.

2.2. Growth of *W. arrhiza* cultures

Addition of 10^{-6} M epiBL to *W. arrhiza* cultures showed a weaker stimulatory effect on growth after 7 days of cultivation in comparison with the control (Fig. 2(a)). Higher concentrations than 10^{-6} M epiBL showed a lethal effect (data not shown). However, treatment with epiBL at concentrations 10^{-15}–10^{-14} M resulted in growth levels very similar to those of control cultures (data not shown). EpiBL mostly stimulates the growth of *W. arrhiza* at 10^{-12}–10^{-7} M. The most stimulation of growth of *W. arrhiza* was at 10^{-9} M, increasing fresh weight by 33%, relative to the control (100%). In turn, the stimulation by epiBL at a concentration of 10^{-12} M amounted to 108% of the control value.

The growth of *W. arrhiza* cultures was not affected by Mev treatment at 10^{-7} and 10^{-5} M (Table 1). The growth of Mev-treated *W. arrhiza* cultures was very similar to that of the control. These results suggest that in *W. arrhiza* cultures treated with Mev the MVA pathway may not play an important role for the growth. Application of a mixture of 10^{-9} M epiBL and Mev to a *W. arrhiza* cultures resulted in an increase in the fresh weight, as compared to untreated cultures. However, this mixture of compounds showed a stimulatory effect on the growth of *W. arrhiza*, which was similar to that observed in cultures treated with epiBL alone.

Application of Clo, at a range of concentrations from 10^{-6}–10^{-4} M, to *W. arrhiza* cultures caused growth inhibition (Fig. 3). In contrast, at concentrations below 10^{-6} M, Clo had no influence on the growth of *W. arrhiza*. Clo had the greatest inhibitory effect at a concentration of 10^{-4} M. The arrested growth of *W. arrhiza* treated with 10^{-6} M Clo was restored to the similar level of the control by the co-application of 10^{-9} M epiBL.

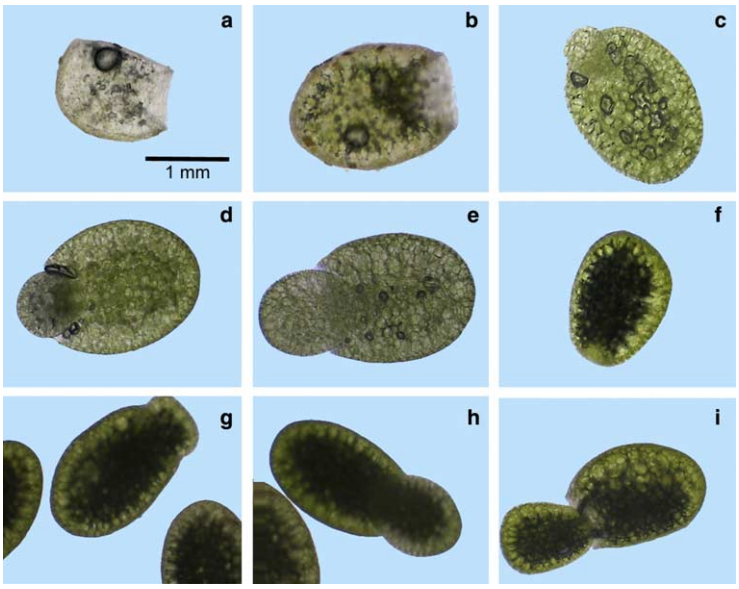

Fig. 1. Appearance and multiplication of *W. arrhiza* culture: (a) individual vegetative floating frond after treatment with 10^{-4} M Brz2001; (b) frond after treatment with 5×10^{-5} M Brz2001; (c) frond after treatment with 10^{-5} M Brz2001; (d, e) frond treated with a mixture of 10^{-9} M epiBL and 10^{-5} M Brz2001; (f)–(i) multiplication by budding (different stages of growth) in epiBL-treated or untreated cultures (there are no significant differences in morphology of these cultures).

The combination of 10^{-6} M Clo and epiBL did not appear to affect the epiBL-induced increase in the fresh weight. The combined treatment with Clo and epiBL suggested that epiBL overcame the inhibitory effect of Clo or that Clo inhibited the promotive effect of epiBL.

Application of 10^{-6}–10^{-4} M Brz2001 to *W. arrhiza* cultures inhibited their growth after 7 days of cultivation (Fig. 4(a)). However, treatments with Brz2001 at concentrations lower than 10^{-6} M resulted in growth levels very similar to that of control cultures (data not shown). Brz2001 most inhibited *W. arrhiza* growth at 10^{-5}–10^{-4} M, with a concentration of 10^{-4} M Brz2001 showing the greatest growth-inhibitory effect, resulting in growth levels very similar to that of cultures at the 0 day of cultivation.

A treatment of cultures with 10^{-6}-5×10^{-6} M Brz2001 and 10^{-9} M epiBL showed a weaker promotive effect on the growth than treatment with epiBL alone (Fig. 4(a)). Furthermore, application of a mixture of epiBL and 10^{-5} M Brz2001 to *W. arrhiza* resulted in growth levels very similar to that of the control culture. Treatment with a higher concentration of Brz2001 (5×10^{-5}–10^{-4} M) plus epiBL resulted in stronger suppression of growth. The combined treatment with Brz2001 and epiBL suggested that epiBL overcame the inhibitory effect of Brz2001.

2.3. Content of photosynthetic pigments

EpiBL had a stimulatory effect on the photosynthetic pigments in *W. arrhiza* cultures (Fig. 2(b)–(d)). BR at a concentration of 10^{-9} M the content of chlorophyll *a* and *b* (161%) and carotenoids (87%) increased. At higher (10^{-7} M) and lower (10^{-13} M) concentrations, epiBL had the least influence on the stimulation of the photosynthetic pigment synthesis, at ≈4–8%. No stimulation of chlorophylls and carotenoids synthesis was observed in the range of 10^{-15}–10^{-14} M (data not shown). Application of epiBL, at a concentration of 10^{-6} M, to *W. arrhiza* cultures caused less increase of chlorophylls and carotenoids content in comparison with the control.

Application of Brz2001, at a range of concentrations from 5×10^{-6}–10^{-4} M, to *W. arrhiza* caused reduction of chlorophyll (Fig. 4(b) and (c)) and carotenoid (Fig. 4(d)) content that was apparent after 7 days of cultivation. In contrast, at a concentration of 10^{-6} M, Brz2001 had a slight inhibitory effect on the pigment content as compared to the control culture. The greatest inhibitory effect on the pigment content has been observed at 10^{-4} M.

The reduction of chlorophylls and carotenoids content in *W. arrhiza* cultures treated with Brz2001 was prevented by the co-application of 10^{-9} M epiBL. EpiBL alone increased the content of chlorophyll *a* (76%) and *b* (72%) and carotenoids (112%). The combination of epiBL and Brz2001 showed a stimulating influence upon the content of photosynthetic pigments in the range of concentrations 10^{-6}-5×10^{-5} M Brz2001. On the other hand, application of a mixture of epiBL and 10^{-5} M Brz2001 to *W. arrhiza* cultures resulted in pigments levels very similar to that of untreated culture.

2.4. Content of sugar

EpiBL (10^{-13}–10^{-6} M) increased the sugar content in *W. arrhiza* cultures (Fig. 2(e)). The greatest stimulation of sugar content was at 10^{-9} M (39%). The lowest

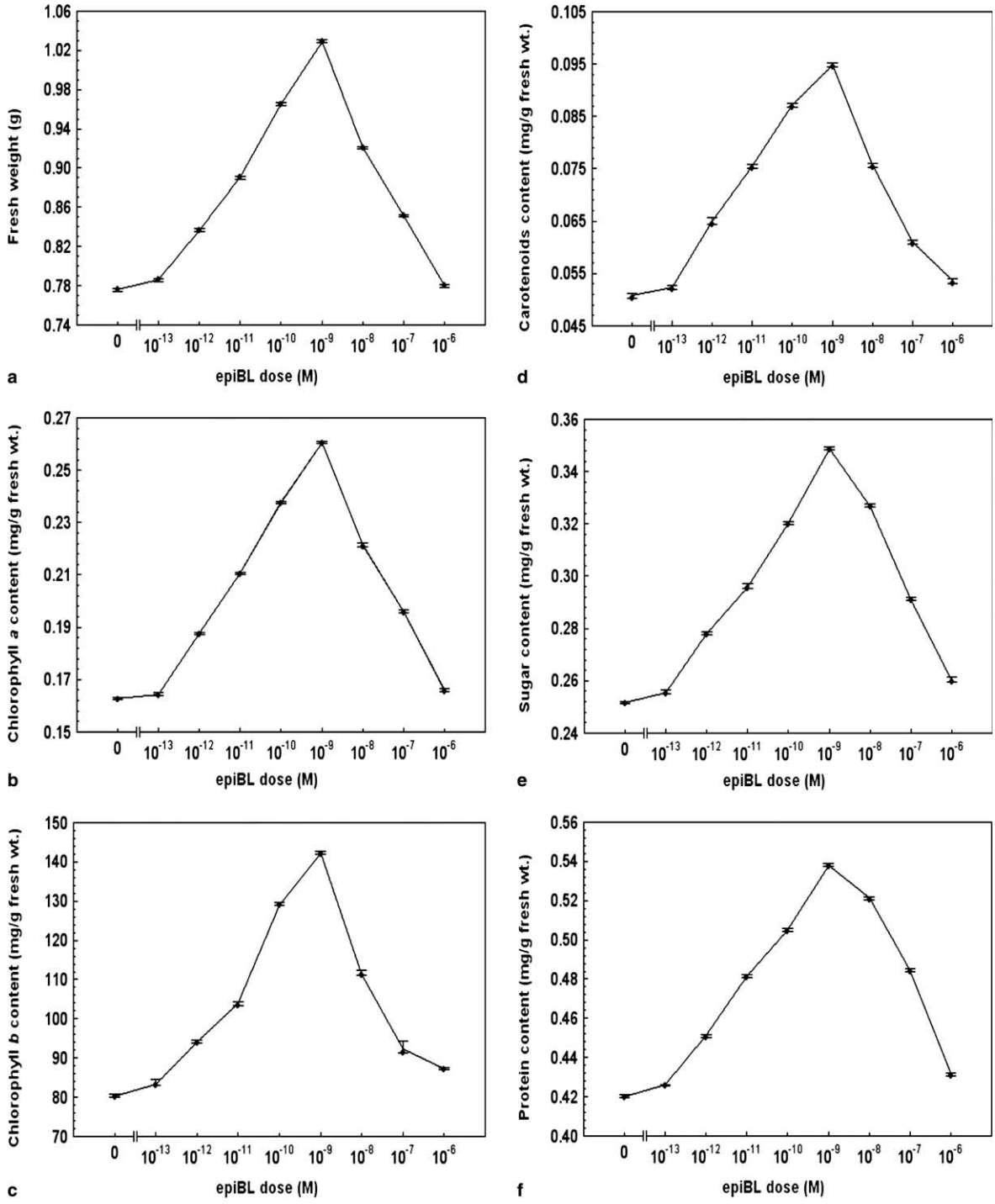

Fig. 2. Effect of epiBL on *W. arrhiza* after 7 days of cultivation. At the 0 day: growth expressed as a fresh weight was 0.5 g; the content of chlorophyll *a* was 0.097 mg/g fresh wt.; the content of chlorophyll *b* was 0.051 mg/g fresh wt.; the content of carotenoids was 0.035 mg/g fresh wt.; the content of sugar was 0.189 mg/g fresh wt.; the content of protein was 0.285 mg/g fresh wt. Data are presented as means of results from 10 replicate samples ± SE.

stimulation of sugar content in BR-treated *W. arrhiza* cultures was observed at 10^{-13} and 10^{-6} M. Furthermore, in the range of 10^{-15}–10^{-14} M, epiBL had no effect on sugar content after 7 days of cultivation (data not shown).

Application of 5×10^{-6}–10^{-4} M Brz2001 to *W. arrhiza* cultures decreased the content of sugar after 7 days of cultivation (Fig. 4(e)). The most significant reduction of sugar content in Brz2001-treated *W. arrhiza* cultures was observed at 10^{-5}–10^{-4} M, with a concentration of 10^{-4} M showing the greatest inhibitory effect. Furthermore, 10^{-6} M Brz2001 had no inhibitory effect on sugar content after 7 days of cultivation. The inhibitory effect

Table 1
Effect of Mev in the absence or presence of epiBL on the fresh weight of *W. arrhiza* after 7 days of cultivation

Fresh weight (g)					
Control (0)	10^{-7} M Mev	10^{-5} M Mev	10^{-7} M Mev + 10^{-9} M epiBL	10^{-5} M Mev + 10^{-9} M epiBL	10^{-9} M epiBL
0.770 ± 0.08	0.765 ± 0.05	0.768 ± 0.07	1.07 ± 0.04	1.03 ± 0.06	1.05 ± 0.05

Data are presented as means of results from 10 replicate samples ± SE.

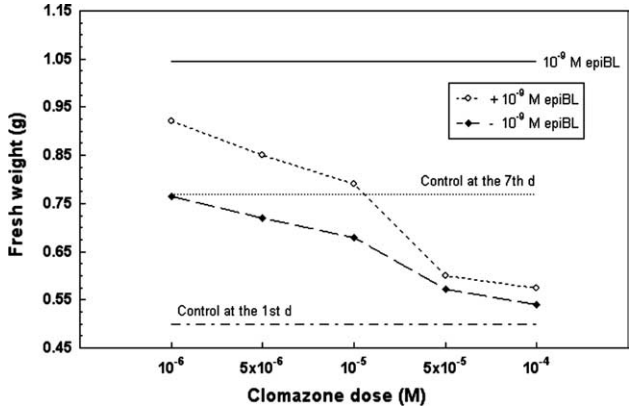

Fig. 3. Suppression of *W. arrhiza* growth by Clo and its restoration by the application of epiBL after 7 days of cultivation. Data are means of results from 10 replicate samples. Error bars of SE were smaller than the symbols for most points.

of Brz2001 on the content of sugar was suppressed by the co-application of epiBL. The combination of 10^{-6} M Brz2001 and 10^{-9} M epiBL appeared to have the highest stimulatory effect on the sugar content, as compared to the control. A decrease in Brz2001 concentration caused an increase in sugar content in *W. arrhiza* cultures, especially with interaction of epiBL.

2.5. Content of protein

EpiBL in the range of concentrations 10^{-13}–10^{-6} M enhances the content of protein in *W. arrhiza* cultures (Fig. 2(f)). BR displayed the greatest stimulatory activity at a concentration of 10^{-9} M (38%). EpiBL in the range of concentration 10^{-8}–10^{-6} M increased the protein content of *W. arrhiza* (30–3%).

Brz2001 displayed the greatest inhibitory activity at a concentration of 10^{-4} M (Fig. 4(f)). The lowest activity in decreasing protein content was demonstrated at a concentration of 10^{-6} M Brz2001. The inhibitory effect of Brz2001 (10^{-6}–10^{-5} M) on the protein content was suppressed by the co-application of epiBL. Cultures treated with epiBL and 10^{-5} M Brz2001 are characterized by slight increase of the protein content.

3. Discussion

Plant growth and developmental processes as well as environmental responses require the action and cross-talk of phytohormones including auxins, abscisic acid, BRs, cytokinins, ethylene and gibberellins. BRs are a unique class of plant polyhydroxysteroids that are structurally similar to the well-studied animal and insect steroids. In plants, many steroids have been identified, e.g., androgens, BRs, corticoids, ecdysteroids and estrogens. However, only BRs have unique growth promoting actions when applied exogenously. BRs occur in all parts of higher plants, including roots at extremely low concentrations (nanogram levels per kg). Till now, 65 free BRs and five BR conjugates have been characterized (Bajguz and Tretyn, 2003). BRs affect a variety of physiological processes at nanomolar to micromolar concentrations, including cell division, cell elongation, vascular differentiation, root growth inhibition, biotic and abiotic stress tolerance, reproductive development and modulation of gene expression (Krishna, 2003; Sasse, 2003). The molecular mechanisms of many hormone signal transduction pathways have been studied in detail. The diversity of BR-dependent responses suggests that the expression of many genes is regulated by BRs (Goda et al., 2002; Horvath et al., 2003; Müssig and Altmann, 2003; Nemhauser and Chory, 2004; Peng and Li, 2003). To date, animal steroids (androgens, estrogens and corticoids) have been isolated in *W. arrhiza* (Mical et al., 2000). However, *W. arrhiza* has not yet been examined for BR content.

No experiments concerning the influence of plant hormones upon *Wolffia* plants have been conducted so far. Microscopic observation of BR-treated, Brz-treated or untreated *W. arrhiza* cultures shows that the size of mother and adult daughter fronds is normally the same. The whole plant is oval. *W. arrhiza* may form two kinds of vegetative fronds: floating and immersed, which drop to the bottom. However, immersed vegetative fronds have not been observed in this study. EpiBL accelerates only the development of vegetative floating fronds, while Brz2001 inhibits. Impact of epiBL on immersed fronds was studied, but no effect was found. It has been presented that Brz2001 induces morphological changes in dicotyledones plants by interfering with the biosynthesis of BRs, and that Brz2001 is a more specific inhibitor of BR biosynthesis than Brz. The target site(s) of Brz2001 is still unclear; however, it may be the same as brassinazole (Asami et al., 2000, 2001). Treatment of wild-type *Arabidopsis thaliana* with Brz results in morphological changes, which are similar to the phenotypes of BR-deficient mutants of *A. thaliana*, such as light-grown plants exhibiting strong dwarfism with curly, dark-green

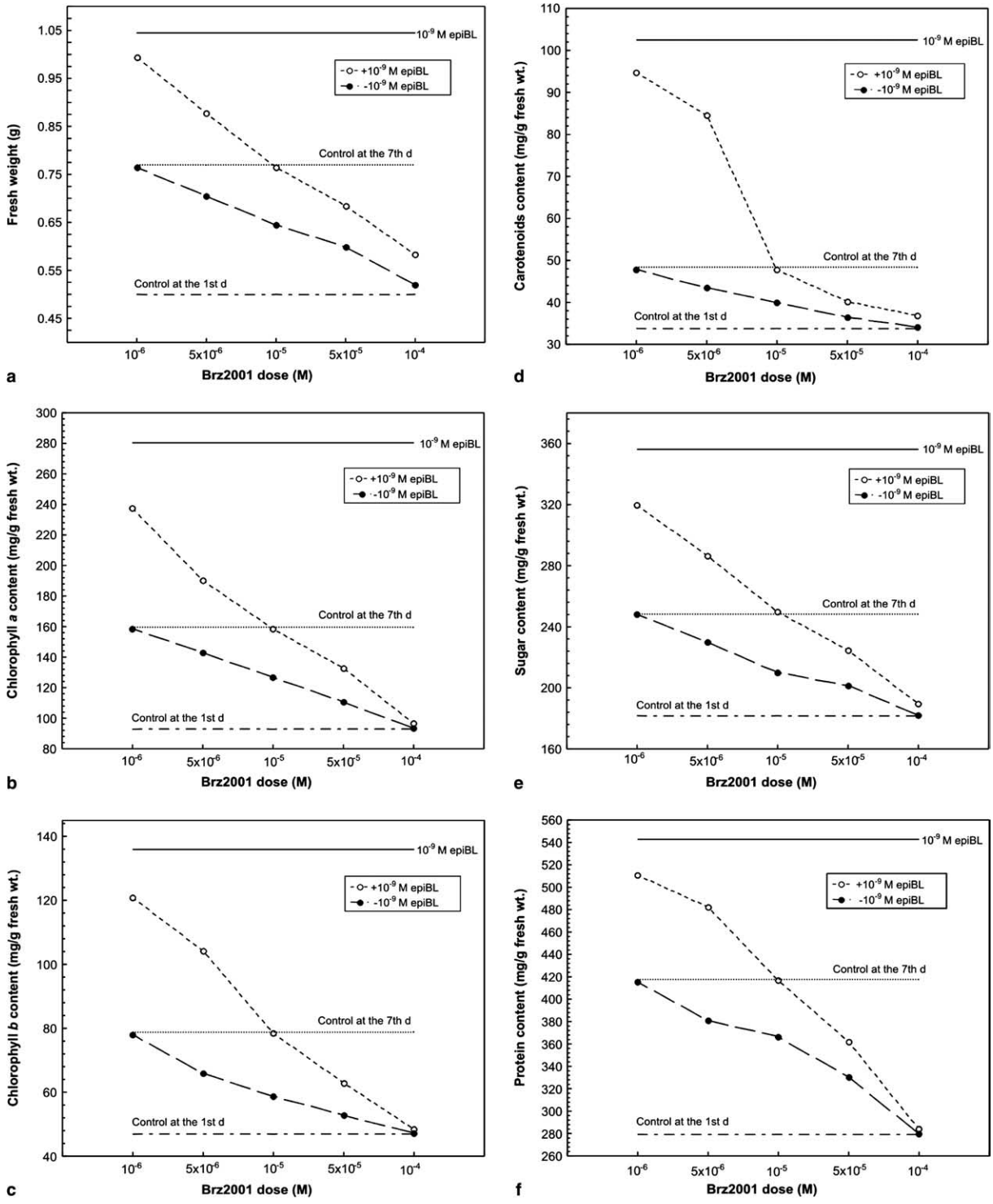

Fig. 4. Effect of Brz2001 in the absence or presence of epiBL on *W. arrhiza* cultures after 7 days of cultivation. Data are means of results from 10 replicate samples. Error bars of SE were smaller than the symbols for most points.

leaves, and dark-grown plants with a de-etiolated phenotype of short hypocotyls and open cotyledons, characteristic of light-grown plants (Asami et al., 2001; Nagata et al., 2000). Brz has also been found to induce dwarfism and curly in soybean (*Glycine max*) (Mazorra et al., 2004), dark-green leaves in light-grown cress (*Lepidium sativum*), cucumber (*Cucumis sativus*) and tobacco (*Nicotiana tabacum*) (Nagata et al., 2001; Sekimata et al., 2001). In the dark, Brz induces photomorphogenic changes in young seedlings of cress and cucumber (Nagata et al., 2001; Sekimata et al., 2001). The inhibition could be reversed, to some extent,

by the addition of BR. In contrast, Brz induces no morphological changes in rice (*Oryza sativa*) (Sekimata et al., 2001) and an alga *Chlorella vulgaris* (Bajguz and Asami, 2004). Brz2001 acts as a growth inhibitor of *C. vulgaris* cultures. Co-application of BR with Brz2001 results in normal growth in light-grown *C. vulgaris* cells (Bajguz and Asami, 2004).

Growth of *W. arrhiza* is manifested as an increase in the fresh weight. Plant growth is dependent upon the synthesis of nucleic acids and protein. It was demonstrated that the activation of the growth of plant tissue and higher levels of DNA and RNA polymerase is manifested by the increase of the content of DNA, RNA and protein. Enhancement of DNA and RNA polymerase activities may be the result of regulation of gene expression by BRs. It may be concerned directly, or indirectly, with growth promotion induced by BRs (Mandava et al., 1987; Kalinich et al., 1986; Bajguz, 2000). This study was conducted on *W. arrhiza* cultures. Our results show the stimulating role of BR in protein accumulation synthesis, and suggest an increase in the rate of the process of translation. We think that *W. arrhiza* can be a good model plant to learn the details of molecular mechanisms of BRs because the responses of *W. arrhiza* to BRs are different from those of reported plants such as *Arabidopsis*, rice or pea (Asami et al., 2001, 2003).

Monosaccharides produced during photosynthesis are building substances for a plant, as well as a source of energy necessary for inciting all the biochemical reactions and processes. BR-induced the accumulation of chlorophyll and photosynthetic assimilates in algae (Bajguz and Czerpak, 1998) and higher plants (Krizek and Mandava, 1983; Braun and Wild, 1984; Hayat et al., 2000; Fariduddin et al., 2003; Yu et al., 2004). BRs are effective in increasing photosynthesis, particularly the capacity of CO_2 assimilation in the Calvin cycle which were mainly attributed to an increase in the initial activity of Rubisco (Yu et al., 2004). BRs increased photosynthesis by sugar-signal-induced feedback regulation. The key enzyme in the source-sink regulation is an extracellular invertase, which is regulated by BRs. It is probably that increased photosynthetic CO_2 assimilation provided more carbohydrate for metabolism and export to sink. Sink strength could be stimulated due to direct effects of enhanced substrate availability, and also through the stimulation of the expression of genes encoding enzymes involved in the carbohydrate metabolism (Roitsch, 1999). We confirmed that the fresh weight and levels of chlorophylls *a* and *b*, carotenoids and sugar are stimulated by epiBL in *W. arrhiza*. Optimal stimulation is observed consistently at 10^{-9} M. At higher (10^{-8}–10^{-6} M) and lower (10^{-13}–10^{-10} M) concentrations than 10^{-9} M, the stimulation is gradually reduced. Furthermore, at concentrations $\geqslant 10^{-5}$ M, epiBL is cytotoxic to *W. arrhiza* cultures, resulting in cellular fragmentation and lysis (data not shown). In contrast, at concentrations below 10^{-13} M epiBL had no influence on *W. arrhiza*. Thus, there are two types of response to BRs in a dose-dependent fashion. One is the stimulation of growth, where stimulation is directly related to BR concentration (10^{-12}–10^{-7} M). The second response to BRs is toxicity of the cultures ($\geqslant 10^{-5}$ M). In general, the stimulatory effect of epiBL was characterized by little activity on the growth and biochemical changes in *W. arrhiza* cultures. However, knowledge of the role played by BRs in *W. arrhiza* is still fragmentary but the above results suggest that BRs are importantly involved in regulation of many processes in this plant. The further studies are required to explain unclear the mechanism of BR action in *W. arrhiza*.

In higher plants, BRs are synthesized via the MVA pathway of isoprenoid biosynthesis (Fujioka and Yokota, 2003). Among plant sterols, campesterol and its analogues are assumed to be the biosynthetic precursors of BL, based on the identity of the side-chain skeleton and campesterol is thought to derive from IPP (Asami and Yoshida, 1999). In order to obtain more information on the biosynthesis of IPP in *W. arrhiza*, we applied Mev, a highly specific inhibitor of HMG-CoA reductase, which catalyzes the reaction from HMG-CoA to MVA, but Mev did not show any effect on the growth of *W. arrhiza*. Similar to this result, Mev does not inhibit the growth of *C. vulgaris* cells. It suggests that *C. vulgaris* does not require the MVA pathway. In contrast, application of Clo to cultured*C. vulgaris* cells inhibits their growth (Bajguz and Asami, 2004). The reasons for this ineffectiveness of Mev are probably following: (i) HMG-CoA reductase of *W. arrhiza* may be resistant to Mev; (ii) detoxication of Mev may be active in *W. arrhiza*. In addition to these reasons, we can raise the possibility that *W. arrhiza* may primarily use the MEP pathway rather than MVA pathway for isoprenoid biosynthesis, or *W. arrhiza* may not have the MVA pathway. Therefore, a shorter experimental period may help for clarify the function of MVA pathway in *W. arrhiza*. The discovery of the MEP pathway revealed the fact that some organisms only use one of the two pathways for the biosynthesis of IPP (Lichtenthaler et al., 1997; Lichtenthaler, 2000). For example, animals use only the mevalonate pathway. Plants have been believed to use both the non-mevalonate and the mevalonate pathways, although they are localized in different compartments. It is generally accepted that in most plants the MEP pathway synthesizes IPP and dimethylallyl diphosphate (DMAPP) in the plastids, whereas the MVA pathway synthesizes cytosolic IPP and DMAPP (Lichtenthaler, 2000). By contrast, in green algae (*Chlorella*, *Chlamydomonas* and *Scenedesmus*) cytosolic isoprenoids (sterols) are only formed via the MEP pathway (Lichtenthaler, 2000; Schwender et al., 2001). In all organisms mitochondrial isoprenoids are synthesized from mevalonate-derived IPP that is imported

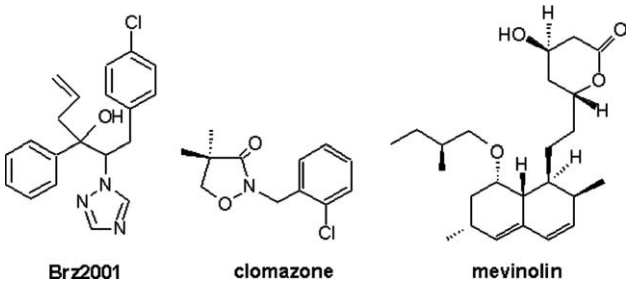

Fig. 5. Chemical structures of the inhibitors used in the experiments.

from the cytosol. Some exchange of IPP or a common downstream intermediate does also appear to take place between the plastids and the cytoplasm (Rodríguez-Concepción and Boronat, 2002; Laule et al., 2003). The dynamics and the regulation of the crossflow of common intermediates between cell compartments may vary dramatically in different species, cell types and/or developmental stages. This exchange may explain, in part, why the MEP pathway for IPP biosynthesis was completely overlooked (Lichtenthaler et al., 1997; Rodríguez-Concepción and Boronat, 2002; Laule et al., 2003; Bajguz and Asami, 2004). Our future efforts will be focused on the biosynthesis of IPP via the cytosolic classical acetate/mevalonate pathway and the communication between the two pathways in *W. arrhiza*. Furthermore, on the basis of the action of the BR biosynthesis inhibitor – brassinazole, the existence of BRs in *W. arrhiza* is probable. Nonetheless, the occurrence of BRs in *W. arrhiza* has not been yet detected.

4. Experimental

4.1. Chemicals

All reagents used in analytical methods were purchased from Sigma. The brassinazole (Brz) used in this study was a diastereomeric mixture of Brz2001 (Fig. 5), synthesized using a published method (Min et al., 1999). Mevinolin (Mev) was purchased from Sigma. Clomazone (Clo) was purchased from Fluka Chemie (Switzerland). Chemicals have been prepared as a DMSO stock solution and stored at −20 °C. Stock solutions were prepared gravimetrically. Weaker solutions were prepared by serial dilution. An equal amount of DMSO was added to the controls.

4.2. Plant material and growth conditions

Wolffia arrhiza (L.) Hork. ex Wimm. (*Lemnaceae*) was grown in small, sterile, plastic vessels (Phytatray™, Sigma) containing 150 ml of culture solution. The cultures were grown under controlled conditions at 28 ± 0.5 °C, 16-h photoperiod (photon flux of 100 μmol m^{-2} s^{-1}). The culture medium used was Hutner's medium in the following nutritive solution: 500 mg/l EDTA, 500 mg/l $MgSO_4 \cdot 7H_2O$, 400 mg/l KH_2PO_4, 354 mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 200 mg/l KOH, 200 mg/l NH_4VO_3, 65.9 mg/l $ZnSO_4 \cdot 7H_2O$, 25.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 24.9 mg/l $FeSO_4 \cdot 7H_2O$, 17.9 mg/l $MnCl_2 \cdot 4H_2O$, 14.2 mg/l H_3BO_3, 3.95 mg/l $CuSO_4 \cdot 5H_2O$, 0.2 mg/l $Co(NO_3)_2 \cdot 6H_2O$ (Hutner, 1953). 1/20 Dilution of the medium was used in this study. The total volume of Hutner's medium was 125 ml. About 0.5 g of *W. arrhiza* cultures were treated with epiBL and/or Brz2001, epiBL and/or Mev, epiBL and/or Clo. Analysis of growth and selected biochemical parameters was performed after 7 days of cultivation.

4.3. Analytical methods

For fresh weight determination, the test plants were filtered and washed three times with distilled water, kept on filter paper for a few minutes to remove excess liquid and weighed. For photosynthetic pigments determination, the cultures were first collected by filtration and then the pellets (0.1 g) were homogenized in methanol. The absorbance of the extract was measured at 470, 653 and 666 nm. The amounts of chlorophylls *a* and *b* and carotenoids present in the extract were calculated according to the equations of Wellburn (1994). For sugar determination, the cultures were first collected by filtration and then the pellets (0.1 g) were assessed using the Somogyi (1954) method. Measurement of protein content was done by extracting the pellets (0.1 g) overnight in 0.1 M NaOH at 4 °C. Concentration of cellular protein was determined by the method of Lowry et al. (1951) with a protein kit calibrated with bovine serum albumin as standard.

4.4. Replication and statistical analysis

Each treatment consisted of two replicates and each experiment was carried out at least five-times at different times. Minitab statistical package was used to carry out a one-way ANOVA. Significance was determined using *t* tests and LSD values based on the ANOVA data.

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