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

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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.

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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.