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BRIEF COMMUNICATION

Effects of brassinosteroid infiltration prior to cold treatment on ion leakage and pigment contents in rape leaves

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Abstract

The effect of 24-epibrassinolide (BR_{27}) on cold resistance of rape seedlings was studied by ion leakage and photosynthetic pigment degradation measurements. Aqueous solutions of BR_{27} were injected into cotyledons or primary leaves of rape plants and these plants were incubated at 2 ºC or 20 ºC. Cold treatment (2 ºC) without BR_{27} injection elevated the membrane permeability in both primary leaves and cotyledons significantly. Surprisingly, injection of leaves with water or 0.467 % aqueous ethanol solution led to a massive increase in membrane permeability after cold stress at 2 ºC. The synergistic effect of leaf infiltration and cold on permeability was abolished by 0.05 and 1.00 µM of BR_{27} in primary leaves and by 1.00 µM of BR_{27} in cotyledons. On the other hand, BR_{27} solutions strongly elevated the membrane permeability at 20 ºC, while water and ethanol solutions brought about only negligible increases. Water or ethanol infiltrations strongly reduced the leaf contents of chlorophyll (Chl) *a*, Chl *b* and carotenoids at 2 ºC but less markedly at 20 ºC. However, in seedlings exposed to 2 ºC pigments content was significantly higher in BR_{27}-treated leaves as compared to water/ethanol control. There were no differences between pigment contents of leaves injected with BR_{27} solutions or only water/ethanol at 20 ºC. The above data strongly support the stress protecting effect of BR_{27}.

Additional key words: carotenoids, chlorophyll, cold stress, 24-epibrassinolide, ion leakage.

Brassinosteroids (BRs) were discovered by Grove *et al.* (1979). Currently BRs are considered as a new class of plant hormones (Bishop and Yokota 2001, Zullo and Adam 2002) and their functions in plants have been extensively investigated. BRs have several physiological activity. They stimulate plant growth and cell elongation (Grove *et al.* 1979, Hayat *et al.* 2001, Sasse 2003), the effectiveness of photosynthesis (Hayat *et al.* 2000, Fariduddin *et al.* 2004, Yu *et al.* 2004) and increase protein and sugar contents (Vardhini and Rao 1998). However, BRs can also inhibit the generative development in plants (Janeczko *et al.* 2003, Kęsy *et al.* 2003). Application of BRs can protect plants against stress conditions (Anuradha and Rao 2003, Bilkisu *et al.* 2003, Janeczko *et al.* 2005) and increase the quality and yield of different crops (Ramraj *et al.* 1997, Zullo and Adam 2002). The aim of our experiments was to study the effect of 24-epibrassinolide (BR_{27} according to Mandava 1988, Zullo and Kohout 2004) applied by infiltration on the cold resistance of rape seedlings. The stress severity was characterised by ion leakage tests and by following the degradation of photosynthetic pigments, since both factors are changing after cold treatment (Jatimliansky *et al.* 2004).

Spring rape (*Brassica napus* L. cv. Lycosmos) seeds were sown in soil and seedlings were kept in a greenhouse for 21 d under a 10 h-photoperiod and day/night temperatures of 20/17 °C. A 0.2 mM stock solution of BR_{27} (purchased from *Sigma*) was prepared in 96 % ethanol and this stock was diluted with distilled water. In all BR_{27} solutions the ethanol concentration were adjusted to 0.467 % according to the ethanol concentration of the 1.00 µM BR_{27} solution. The test solutions were fully injected into the apoplast of cotyledons or primary leaves by gentle pressure provided by a plastic syringe without needle. Three types of control were used – absolute, water and ethanol control. Plants of absolute control were not injected. In case of ethanol control the leaves were

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Abbreviations: BRs - brassinosteroids; BR_{27} - 24-epibrassinolide; PAR - photosynthetically active radiation.
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injected with an 0.467 % aqueous solution of ethanol. After injections the control and BR$_{27}$-injected plants were incubated at 20 °C (first group) or at 2 °C (second group) for different time periods in growth chambers under 100 µmol m^{-2} s^{-1} PAR at the plant level. Membrane damage was detected by leakage of ions in conductivity tests as described by Barna *et al.* (1993). Four days after injections cotyledons and primary leaves were cut and floated on 10 cm^{3} of deionized water in Petri dishes. Ion leakage was measured at various intervals in 3 parallels with a conductivity meter (*Radelkis OK-102/10*, Budapest, Hungary). For pigment degradation tests primary leaves of 21 d old rape plants were cut at 4 d after injection and incubated in growth chambers in the dark (until yellowing appeared) for 3 d at 20 °C or for 7 d at 2 °C. Leaves were lyophilised, weighed and homogenised in a mortar with 3 cm^{3} of an acetone-water 4:1 mixture. All samples were completed to a final volume of 5 cm^{3}. Homogenates were centrifuged for 20 min at 1100 *g*. Absorption of photosynthetic pigments in the supernatants were measured at 662 nm (chlorophyll *a*), 645 nm (chlorophyll *b*), 470 nm (carotenoids) according to Lichtenthaler and Wellburn (1983) with a *Biochrom Ultrospec II* spectrophotometer (*LKB*, Uppsala, Sweden) in 10 repetitions.

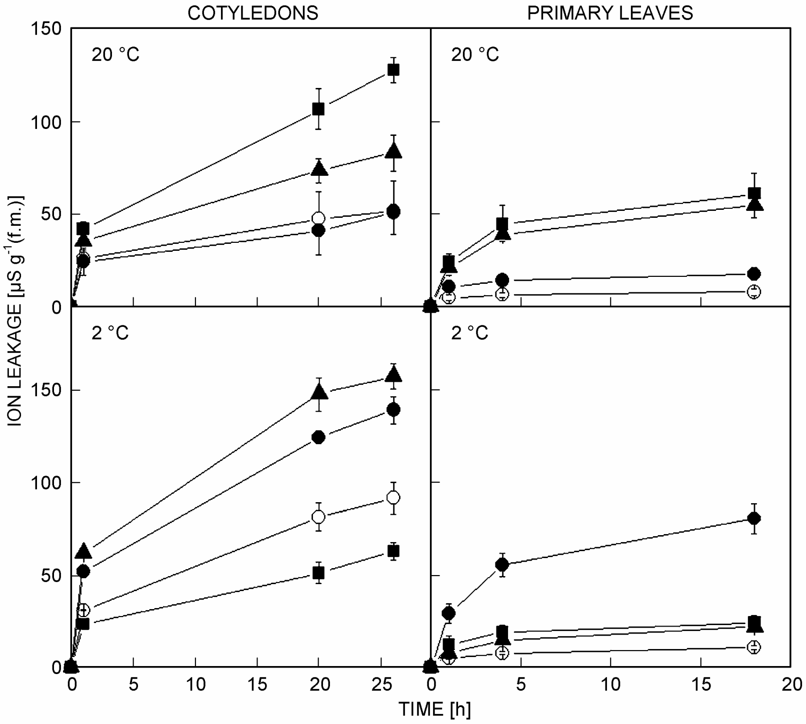

Fig. 1. Ion leakage from cotyledons and primary leaves of rape seedlings exposed to 24-epibrassinolide (BR$_{27}$) solutions for 4 d at 2 and 20 °C. The aqueous BR$_{27}$ solutions contained 0.467 % ethanol. Means of three replicate experiments ± SD are shown. Symbols: *open circles* - water control, *closed circles* - 0.467 % ethanol, *closed triangles* - 0.05 µM BR$_{27}$, *closed squares* - 1.00 µM BR$_{27}$.

Table 1. Contents of photosynthetic pigments [mg g^{-1}(d.m.)] in primary leaves of rape seedlings treated with 24-epibrassinolide (BR$_{27}$) and incubated at 2 °C for 7 d or at 20 °C for 3 d. Ethanol control: leaves injected with 0.467 % ethanol solution; BR $_{27}$ treatments: all BR $_{27}$ solutions contained 0.467 % ethanol. Values marked with the same letters (within each column) are not significantly different according Duncan test, *n* = 10, α = 0.05. In intact leaves (before dark treatment) the Chl *a*, Chl *b* and Car contents were 14.2 ± 0.9, 5.8 ± 0.3 and 4.7 ± 0.2 mg g^{-1}(d.m.), respectively.

BR$_{27}$ [µM]	Chl *a* 20 °C	5 °C	Chl *b* 20 °C	5 °C	Car 20 °C	5 °C
0 (intact leaves)	4.8^{a}	9.1^{a}	1.9^{a}	3.5^{a}	2.0^{a}	4.4^{a}
0 (ethanol control)	3.3^{b}	4.5^{c}	1.4^{b}	1.8^{c}	1.6^{b}	2.9^{b}
0.001	3.6^{b}	6.8^{b}	1.4^{b}	2.6^{b}	1.6^{b}	3.8^{ab}
0.010	3.1^{b}	7.4^{b}	1.2^{b}	2.7^{b}	1.5^{b}	3.9^{a}
0.100	3.2^{b}	7.7^{b}	1.3^{b}	2.8^{b}	1.6^{b}	3.9^{a}
1.000	3.6^{b}	7.5^{b}	1.5^{b}	2.8^{b}	1.6^{b}	4.3^{a}

No visible symptoms were observed on the seedlings incubated at 2 °C for 5 d, neither on control nor on treated plants. However, the various treatments exerted significant effects on cell membrane permeability as indicated by ion leakage. At 20 °C BR_{27} strongly increased membrane permeability in both primary leaves and cotyledons in a concentration-dependent manner. Water or ethanol treatments alone slightly increased the leakage in primary leaves but not in cotyledons at 20 °C (Fig. 1). Water and ethanol (0.467 %) injections have always the same effect in each experiment, therefore only data of ethanol controls are shown. Cold treatment (2 °C) itself slightly increased the membrane permeability in untreated primary leaves and more strongly in cotyledons. It is noteworthy that leakage was generally more pronounced in cotyledons, which is probably due to their older age. Surprisingly, however, water or ethanol injection alone strongly increased the membrane permeability at 2 °C in both primary leaves and cotyledons as compared to absolute control. This strong effect was abolished by 0.05 and 1.00 μM of BR_{27} in primary leaves and by 1.00 μM of BR_{27} in cotyledons (Fig. 1). This means that BR_{27} exerted opposite effects on membranes at different temperatures; at 20 °C it increased, while at 2 °C decreased the permeability. In accordance with the literature, rape leaves stored in the dark at 20 °C lost pigments more quickly than leaves at 2 °C. Non-injected control leaves stored at 2 °C for 7 d contained higher pigment content than leaves stored at 20 °C only for 3 d (Table 1). Water or ethanol injections alone markedly reduced the contents of chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car) in leaves held at 2 °C and to a lesser extent in leaves held at 20 °C. This pigment degradation clearly indicated the stress caused by water or ethanol infiltration at 2 °C, confirming the results of ion leakage tests. Pigment contents of BR_{27} and ethanol-treated leaves did not differ significantly after incubation at 20 °C. However, at 2 °C the contents of Chl *a,* Chl *b* and Car were significantly higher in BR_{27}-treated leaves than those infiltrated with ethanol or water (Table 1), indicating the stress protecting effect of BR_{27}, similarly to the data of ion leakage tests.

BRs are water insoluble, hence it is necessary to dissolve them in organic solvent. However, both the solvent and the application method can exert some influence on the physiological factors studied. The above results show that ethanol or water injections exert a strong stress effect on rape leaf tissues at 2 °C but they have much weaker effect at 20 °C. These findings clearly emphasis the necessity of proper controls in experiments using leaf infiltration methods. It is noteworthy that injection or vacuum infiltration of leaves is a widely used method for bacterial infection, or testing the effect of various chemicals. Injection of ethanol or water alone had the same effect on leakage or photosynthetic pigments suggesting that ethanol at this low concentration has no additional toxicity on rape leaf tissues. As regards the mechanism of this unexpected finding one can suppose that infiltration of leaves has a moderate stress effect, which predisposes the plant tissue to the second stress, the cold treatment and the two stresses together have a stronger synergistic effect. The stressor feature of leaf infiltration is confirmed by the increased transcription of a respiratory burst oxidase homolog gene (*Nbrboh*) after water infiltration of *Nicotiana bethamiana* leaves (Yoshioka *et al.* 2003). BR_{27} treatments increased the membrane permeability of plant cells at room temperature. The increase in membrane permeability at 20 °C is probably due to the hormonal effect of BR_{27} on cell elongation. On the contrary, the elevated ion leakage induced by infiltration and cold stress at 2 °C can be alleviated or abolished by BR_{27} treatments, which shows the stress protecting effect of BR_{27}. There are indications in the literature on the stress protecting effect of BR, although very few data can be found as regards to cold treatment. Hotta *et al.* (1998) reported the increased cold tolerance of rice seedlings by brassinolide treatment. Markedly enhanced cell viability was observed in BR_{27}-treated bromegrass cell culture after high temperature stress (Wilen *et al.* 1995). As mentioned by Krishna (2003), treatments with BRs promoted growth recovery of maize seedlings following chilling treatment (0 - 3 °C). Cucumber seedlings germinated from seeds soaked in BRs grew better in cold (5 °C) as compared to controls. Furthermore, BRs had a growth-promoting effects in rice growing under low-temperatures (Krishna 2003). In our experiment the prevention of pigment loss was observed in leaves injected by BR_{27} and kept in cold. Similar phenomena is described by Anuradha and Rao (2003) in case of the salt stress and application of BRs in rice. Chl content was also maintained in BR-treated cucumber seedlings during the cold treatment, increasing even further during recovery from cold (Krishna 2003). The mechanism of such effects are not understood but in case of high temperature stress it has been reported that treatment of *B. napus* seedlings with BR_{27} led to an increased thermotolerance and a higher accumulation of the four major classes of heat shock proteins due to higher protein synthesis (Dhaubhadel *et al.* 1999, 2002). It has been also described that BRs increase the activities of antioxidative enzymes (Mazorra and Núñez 2000, Mazorra *et al.* 2002). Our data clearly indicate the protecting effect of BR_{27} against membrane leakage and degradation of photosynthetic pigments caused by cold and leaf infiltration stresses. In addition, our data strongly underlines the importance of proper controls in experiments using leaf infiltration methods.

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