BRIEF COMMUNICATION

Protection of winter rape photosystem 2 by 24-*epi*brassinolide under cadmium stress

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Abstract

Seedlings of winter rape were cultured *in vitro* on media containing 24-*epi*brassinolide, EBR (100 nM) and cadmium (300 μM). After 14 d of growth, fast fluorescence kinetics of chlorophyll (Chl) *a* and contents of photosynthetic pigments and Cd in cotyledons were measured. Cd was strongly accumulated but its content in cotyledons was 14.7 % smaller in the presence of EBR. Neither Cd nor EBR influenced the contents of Chl *a* and *b* and carotenoids. Cd lowered the specific energy fluxes per excited cross section (CS) of cotyledon. The number of active reaction centres (RC) of photosystem 2 (RC/CS) decreased by about 21.0 % and the transport of photosynthetic electrons (ET_0/CS) by about 17.1 %. Simultaneously, under the influence of Cd, the activity of O_2 evolving centres (OEC) diminished by about 19.5 % and energy dissipation (DI_0/CS) increased by about 14.6 %. In the cotyledons of seedlings grown on media without Cd, EBR induced only a small increase in the activity of most photochemical reactions per CS. However, EBR strongly affected seedlings cultured with cadmium. Specific energy fluxes TR_0/CS and ET_0/CS of the cotyledons of plants Cd+EBR media were about 10.9 and 20.9 % higher, respectively, than values obtained for plants grown with Cd only. EBR also limited the increase of DI_0/CS induced by Cd and simultaneously protected the complex of OEC against a decrease of activity. Hence EBR reduces the toxic effect of Cd on photochemical processes by diminishing the damage of photochemical RCs and OECs as well as maintaining efficient photosynthetic electron transport.

Additional key words: carotenoids; chlorophyll fluorescence induction; photosynthesis; winter rape.

Brassinosteroids (BR) were found in rape pollen by Grove *et al.* (1979). These compounds, such as brassinolide or 24-*epi*brassinolide (EBR) are a new class of phytohormones (*e.g.* Khripach *et al.* 2000). BR exhibit a multitude of physiological activities but mainly influence plant growth and crop (Braun and Wild 1984, Ramraj *et al.* 1997, Vardhini and Rao 1998) and increase resistance to some stresses, among others, to heavy metals (Anuradha and Rao 2003, Bilkisu *et al.* 2003, Krishna 2003, Vardhini and Rao 2003). BR also stimulate photosynthetic pigment production in leaves (Krizek and Mandava 1983, Braun and Wild 1984, Fariduddin *et al.* 2003). Up to now, no more exact studies concerning the influence of these compounds on photosynthetic pathways in plants grown under stress have been carried out. In this work, the effect of EBR on photosystem 2 (PS2) of rape plants under Cd stress was examined.

Seeds of winter rape cv. Górczański were disinfected in ethanol for 5 min and rinsed with sterile water. Next, the seeds were inundated with *Domestos* preparation (*Unilever*, Bydgoszcz, Poland), diluted with distilled water (1 : 5, v/v) and after 20 min the seeds were again

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Abbreviations: ABS – absorption; BR – brassinosteroids; Car – carotenoids; Cd – cadmium ion (Cd^{2+}); Chl – chlorophyll; CS – cross section of the sample; DI_0 – energy dissipation; EBR – 24-*epi*brassinolide; ET – energy flux for electron transport; OEC – oxygen evolving complexes (centres); Q_A – primary bound plastoquinone; PS2 – photosystem 2; RC – reaction centre; Tr_0 – energy flux for trapping; φ_{Po} – maximum quantum yield of primary photochemistry; ψ_0 – efficiency with a trapped exciton can move an electron into the electron transport chain further than Q_A^-.

rinsed with sterile water. The rape was sowed on MS media including mineral components according to Murashige and Skoog (1962), 3 % sucrose, 0.6 % agar (pH 5.8). 3-d-old seedlings were transferred to sterile *Magenta* vessels on the following media: (*a*) MS (control), (*b*) MS+EBR (100 nM), (*c*) MS+$CdSO_4$ (300 μM), and (*d*) MS+EBR (100 nM)+$CdSO_4$ (300 μM) (*Sigma-Aldrich*, Poznań, Poland). 24-epibrassinolide (EBR) was dissolved in 96 % ethanol in order to obtain a stock solution with the concentration of 0.1 mg EBR per 1 cm^3. To 1 000 cm^3 of *in vitro* culture media 408.7 mm^3 of the stock solution EBR was added, whereas to all other media the same amount of 96 % ethanol was added. After 2 weeks of seedling growth at 20 °C with an 8 h photoperiod [150 μmol(quantum) $m^{-2}·s^{-1}$], the fast fluorescence kinetics of Chl *a* and contents of Cd and photosynthetic pigments in cotyledons were determined. The contents of chlorophylls and carotenoids (Car) were measured according to method described by Lichtenthaler and Wellburn (1983). The lyophilized plant material was homogenized in a mortar with 3 cm^3 of acetone : water mixture (4 : 1, v/v). Homogenates, filled to a final volume of 10 cm^3, were then centrifuged for 20 min at 1 100×*g*. The contents of pigments in the supernatant (10–11 repetitions) were estimated by measurements of absorption at 662, 645, and 470 nm using a *Biochrom Ultrospec II* spectrophotometer (*LKB*, Sweden).

The Cd contents were measured using atomic absorption spectrometry (*Varian 220FS*, Australia) and graphite furnace (*GTA 96*). The cotyledons were dried at 105 ºC for 24 h. Dry samples (about 0.2 g) were mineralized in hot mixture of nitric and perchloric acids (4 : 1, v/v). When the emitting fumes were white and the solution was clear, the cooled solution was filled up to the volume of 10 cm^3 with de-ionized water. Samples of acids were used as blank. Spinach leaves (cv. SRM 1570a) were used as standard reference material. Measurements were taken with 3 repetitions.

Chl *a* fluorescence was measured by a Plant Efficiency Analyzer (PEA; *Hansatech*, King's Lynn, Norfolk, England) with excitation irradiance of 3 mmol m^{-2} s^{-1} (peak 650 nm). Measurements on cotyledons were taken after 30 min of adaptation to darkness (clips with a 4 mm diameter hole), at a temperature of 20 °C. Fluorescence intensity was measured with a PIN-photodiode after being passed through a long-pass filter. Changes in fluorescence were registered during irradiation of 10 μs to 1 s. During the initial 2 ms, data was collected every 10 μs with 12 bit resolution. After this period, the frequency of measurements was reduced automatically. Measurements were taken with 25–30 repetitions. The registered data was analyzed with the use of a JIP test (Tsimili-Michael *et al.* 1988, Strasser and Strasser 1995, Lazár 1999, Lazár and Pospíšil 1999, Srivastava *et al.* 1999, Strasser *et al.* 1999, 2000). In the calculations, the following were used: fluorescence intensity in 50 μs (F_0) [it is assumed that, at that time, all reaction centres of PS2 (RCs) are open], maximal fluorescence (F_m) (when all RCs are closed), and fluorescence intensity after 300 μs (point K), 2 ms (point J), and 30 ms (point I). The following parameters were calculated:

(*a*) The specific energy fluxes (per RC), for absorption (ABS/RC), trapping (TR_0/RC), electron transport (ET_0/RC), and dissipation (DI_0/RC). All energy fluxes were also related to active cross section (CS), with the assumption that $ABS/CS \approx F_M$, $ABS/RC = (M_0/V_J)/[1 – (F_0/F_M)]$, $ET_0/RC = (M_0/V_J)/(1 – V_J)$, $TR_0/RC = (M_0/V_J)$, $DI_0/RC = ABS/RC – TR_0/RC$. M_0 is the initial slope, which is a measure of Q_A reduction in first 250 μs: $M_0 = 4\ (F_{300\mu s} – F_0)/(F_M – F_0)$. The multiplication by 4 gives the value for 1 μs. V_J corresponds to relative variable fluorescence at step J: $V_J = (F_{2ms} – F_0)/(F_M – F_0)$.

(*b*) Flux ratios or yields: the maximum quantum yield of primary photochemistry (φ_{Po}), the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^- (ψ_o), $\varphi_{Po} = (TR_0/RC)/ABS/RC$, $\psi_0 = (1 – V_J) = (ET_0/RC)/(TR_0/RC) = ET_0/TR_0$.

(*c*) The amount of active RCs per excited CS (RC/CS), $RC/CS = (ABS/CS)\ (TR_0/ABS)\ (RC/TR_0) = F_M\ \varphi_{Po}\ V_J/M_0$.

(*d*) The fraction of O_2 evolving centre (OEC). Using this information, one can calculate the fraction of OEC in comparison with the control sample (seedlings on media without Cd and EBR). $OEC = [(1 – (V_K/V_J)_{treated}/[1 – (V_K/V_J)_{control}]×100$. V_K is the relative variable fluorescence at step K, $V_K = (F_{300\mu s} – F_0)/(F_M – F_0)$.

In preliminary experiments, the influence of different concentrations of Cd (10, 100, and 300 μM) and EBR (1, 10, 100, and 1 000 nM) on rape seedling growth was visually estimated. Cd in lower concentrations (10 and 100 μM) did not visibly influence growth, but at 300 μM inhibited plant growth by about 27 %. 1 000 nM EBR also inhibited rape growth. For the next experiments, 100 nM EBR and 300 μM Cd were used. Seedlings growing on media without Cd and EBR were taken as the control. After two weeks of growth, the seedlings had developed cotyledons and first leaf. The second leaf was in germ form. Cd inhibited the growth of seedlings and was strongly accumulated. The average content of Cd in cotyledons was 582 mg kg^{-1}(DM) but depended on the presence of EBR in the medium (Table 1). EBR lowered the Cd content in tissues by about 14.7 %. Cd and EBR did not influence the contents of Chl and Car in cotyledons. When the cotyledons of rape seedlings were exposed to saturating "actinic light" the Chl *a* fluorescence curves increased from F_0 to a peak (P or F_M). When the curves were plotted on logarithmic scale, two intermediate steps F_J and F_I were seen between F_0 and F_M. Fluorescence signal curves for all treatments were running similar, but Cd induced decrease of fluorescence in seedlings growing on EBR-free medium. In plants growing on media containing EBR and Cd, EBR caused significant increase of value F_M and simultaneously decrease of parameters F_0,

M_0, and V_J (Table 1).

Table 1. The interaction of cadmium (Cd) and 24-*epi*brassinolide (EBR) treatments on content of Cd [mg kg^{-1}(DM)] and photosynthetic pigments [g kg^{-1}(DM)], chlorophyll (Chl) fluorescence parameters, and JIP test parameters in cotyledons of rape after 14 d growth *in vitro* on media containing these chemicals. In parentheses are changes of values of parameters [%] under influence of 24-*epi*brassinolide. V_I – the relative variable fluorescence at K step. Values marked with the same letters (within parameters) are not significantly different (α<0.05) according to the multiple Duncan test.

	EBR [nM]	Cd [µM] 0		300	
Cd	0	-		628.1 [a]	
	100	-		535.9 [b]	
Chl *a*	0	16.6 [a]		15.8 [a]	
	100	16.6 [a]	(0)	16.3 [a]	(+3.2)
Chl *b*	0	5.6 [a]		5.1 [a]	
	100	5.6 [a]	(0)	4.9 [a]	(-3.9)
carotenoids	0	4.4 [a]		4.6 [a]	
	100	4.2 [a]	(-4.5)	4.9 [a]	(+6.5)
F_0	0	1033 [b]		1184 [a]	
	100	1183 [a]	(+14.5)	1012 [b]	(-17.2)
M_0	0	1.038 [b]		1.259[a]	
	100	1.105 [b]	(+6.4)	0.981 [b]	(-28.3)
V_J	0	0.423 [b]		0.458 [a]	
	100	0.426 [b]	(+0.7)	0.410 [b]	(-11.7)
V_I	0	0.793 [a]		0.824 [a]	
	100	0.794 [a]	(+0.1)	0.792 [a]	(-4.0)
ABS/CS	0	3541 [b]		3391 [c]	
	100	3778 [a]	(+6.7)	3461 [c]	(+2.1)
TR_o/CS	0	2508 [a]		2208 [d]	
	100	2594 [b]	(+3.4)	2449 [c]	(+10.9)
DI_o/CS	0	1033 [b]		1184 [a]	
	100	1183 [a]	(+14.5)	1012 [b]	(-14.5)
ET_o/CS	0	1449 [a]		1201 [b]	
	100	1491 [a]	(+2.9)	1452 [a]	(+20.9)
RC/CS	0	1038 [a]		820 [b]	
	100	1013 [a]	(-2.4)	1053 [a]	(+28.4)
ABS/RC	0	3.5 [bc]		4.3 [a]	
	100	3.8 [b]	(+8.6)	3.4 [c]	(-20.9)
TR_o/RC	0	2.4 [b]		2.7 [a]	
	100	2.6 [ab]	(+8.3)	2.4 [b]	(-11.1)
DI_o/RC	0	1.0 [b]		1.6 [a]	
	100	1.2 [b]	(+20.0)	1.0 [b]	(-37.5)
ET_o/RC	0	1.4 [a]		1.5 [a]	
	100	1.5 [a]	(+7.1)	1.4 [a]	(-6.7)
φ_{Po}	0	0.709 [a]		0.645 [b]	
	100	0.687 [a]	(-3.1)	0.707 [a]	(+9.6)
ψ_o	0	0.577 [a]		0.542 [b]	
	100	0.574 [a]	(-0.5)	0.590 [a]	(+8.9)
OEC	0	100.0 [ab]		80.5 [c]	
	100	90.4 [b]	(-9.6)	104.2 [a]	(+29.4)

Cadmium caused also reductions in the specific energy fluxes calculated per CS in comparison to control. Under the influence of Cd, the number of active RCs of PS2 (capable of Q_A^- reduction) decreased by about 21.0 % and photosynthetic electron transport by about 17.1 %. Simultaneously, Cd lowered OEC by about 19.5 % and increased this part of excitation energy which is dissipated by cotyledons as heat (Di_0/CS) by about 14.6 %. This was accompanied by a decrease in the efficiency of the quantum yield of primary photochemistry (φ_{Po}) by about 9.0 % and the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^- (ψ_0) by about 6.0 %. Due to significant damage in some PS2 RCs of cotyledons caused by Cd, and a small reduction of photon absorption by the antenna system (ABS) per CS, the absorption of photons by the antenna system (ABS/RC), trapping (TR_0/RC), and dissipation (DI_0/RC) in relation to single

active RC all increased. In the cotyledons of seedlings grown on media without Cd, EBR induced a small increase in the activity of most photochemical reactions in relation to CS, but did not significantly influence the specific energy fluxes per RC. A stronger effect of EBR occurred in seedlings cultured on media with Cd. This depended on reducing the effects of inhibition of photochemical processes caused by Cd. The cotyledons of seedlings grown with Cd+EBR had higher values of TR_0/CS and Et_0/CS, 10.9 and 20.9 %, respectively, and φ_{Po} increased by 9.6 %, whereas the efficiency with which a trapped exciton moves an electron into electron transport chain (ψ_0) increased by 8.9 % in relation to the value obtained for the cotyledons of seedlings growing on media with Cd but without EBR. EBR limited the increase of dissipation (DI_0/CS) induced by Cd by about 14.5 %, as well as protected OECs from a decrease in their activity. The protective effect of EBR on photochemical reactions was sometimes so effective that the values of selected JIP-test parameters for plants growing on media with Cd and EBR and control plants (media without Cd and EBR) did not significantly differ. This concerned DI_0/CS, ET_0/CS, the flux ratios or yields (φ_{Po}, ψ_0), OEC activity, and also the number of RCs on CS. Our results indicate that the EBR added to plant growth media can completely or partially limit the toxic effect of Cd on photochemical processes in rape cotyledons.

As described, EBR (100 nM) lowered the content of Cd in plant tissue by about 15 %. Similar results were obtained by other authors. In experiments on *Brassica campestris* L., EBR (10^{-5}, 10^{-7}, and 10^{-9} M) applied with Cu, Ni, and Co limited the uptake of these metals into plant tissue (Kaur and Bhardwaj 2003). According to Bajguz (2000), in the presence of EBR (10^{-8} M) the amounts of Cu, Cd, and Zn accumulated by the cells of *Chlorella vulgaris* Beijerinck decreased by about 50 %.

In our experiment, Cd did not significantly affect the contents of Chl and Car in cotyledons, although it is a typical reaction of many plant species to stress caused by Cd and other heavy metals (Clijsters and van Assche 1985, Padmaja *et al.* 1990, Baszyński 1994, Larsson *et al.* 1998, Ducsay and Kováčik 2001, Sandalio *et al.* 2001). One of the possible reasons is the different morphological structure and physiological function of cotyledons in comparison to leaves. Measurements of fluorescence confirm the toxic effect of Cd on the photosynthetic activity of plants (*e.g.* Weigel 1985, Sheoran *et al.* 1990, Krupa *et al.* 1993a, Moya *et al.* 1993, Skórzyńska and Baszyński 1993, Siedlecka and Krupa 1996, Krupa and Moniak 1998). Cadmium inhibits both the "light" and "dark" reactions of photosynthesis, but the Calvin cycle is more sensitive to its activity (Weigel 1985, Siedlecka and Krupa 1996, Krupa and Moniak 1998). The inhibition of photochemical processes by Cd may result from the limitation in the use of ATP and NADPH by the Calvin cycle and accompanying increase of pH gradient across the thylakoid membranes (Krupa *et al.* 1993a,b).

In our experiments, cotyledons were adapted to the dark for 30 min before fluorescence measurements. During this period, in photosystems intensive relaxation processes, accompanied by energy-dependent quenching (q_E) and protective sub-components of photoinhibitory quenching ($q_{I,p}$) occurred (Ting and Owens 1994). q_E relaxes within seconds to minutes and $q_{I,p}$ relaxes within tens of minutes (Müller *et al.* 2001). Simultaneously, in the dark relaxation of damaged sub-components of $q_{I,p}$ did not occur. $q_{I,p}$ is irreversible in the dark, requiring light-dependent chloroplast protein synthesis to repair damaged PS2 RC complexes (Krause and Weis 1991, Leitsch *et al.* 1994). For this reason, calculated JIP test parameters reflect primarily photochemical changes accompanied by damages brought about by Cd in photochemical RCs. Cd damaged the centres of photochemical reactions, lowered the activity of OEC and transport of electron per CS of cotyledons (Table 1). The above mentioned data is compatible with the results of the JIP test performed on *Spirodela polyrhiza*, which grew in the presence of chrome (Appenroth *et al.* 2001). Cd induced a reduction in the absorption of photons by the antenna system of cotyledons of seedlings cultured without EBR by about 4 %. This was consistent with the lack of changes in pigment contents under Cd stress (Table 1). Simultaneously, the absorption of photons per RC (ABS/RC) or the antenna size in these plants increased. This indicated that the number of active RCs per absorption decreased (Krüger *et al.* 1997). Due to a reduction in the efficiency of energy flow through PS2 caused by Cd there had to be a strong increase of dissipation with reference to a single, active RC. EBR lowered the toxic effects of Cd on photochemical reactions. EBR action depended primarily on the prevention of RC damage and maintaining TR_0/CS and ET_0/CS. The described decrease of Cd accumulation in tissue by about 15 % would probably not cause the observed limitation of Cd toxicity symptoms for photochemical processes (Table 1). Lowering this disorder resembles the abilities of BR to lower stress effects in other physiological processes. According to Bilkisu *et al.* (2003) brassinolide during Al-related stress stimulated growth in *Phaseolus aureus*. However, growth stimulation did not occur when only brassinolide was present in the medium (Bilkisu *et al.* 2003). The molecular mechanism for the protection of photosynthetic reactions by EBR might exist in the ability of BR to increase activity of antioxidant enzymes (peroxidase, superoxide dismutase, catalase) (Mazorra *et al.* 2002). In this way, the extent of oxidative stress and the inhibition of the antioxidant systems induced by Cd (Sandalio *et al.* 2001) might be limited.

Heavy metals' contamination or thermal stress caused the increase in content of heat shock proteins, HSPs (Tseng *et al.* 1993, Neumann *et al.* 1994, 1995). Their role relies, among others, on protecting some proteins against denaturation and the renaturation of proteins which were partly denaturated. In seedlings of tomato and

Brassica napus, EBR increased accumulation of HSPs under thermal stress which directly affected the growth of tolerance to the stress agent (Dhaubhadel *et al.* 1999, 2002). Thus, it cannot be ruled out that EBR increases *via* HSPs the efficiency of reparation of the structural and functional PS2 proteins, which have been damaged by Cd. On the other hand, we confirmed that EBR stimulates photochemical reactions in the cotyledons of seedlings untreated by Cd. Our measurements do not give data to explain this phenomenon. According to other authors, BR can increase the intensity of CO_2-fixation (Braun and Wild 1984, Hayat *et al.* 2000). Higher efficiency of these processes favours better use of absorbed photon energy to photochemical pathways, preventing in this way the formation of potentially damaging reactive oxygen species (Baker 1994). The protective effect of EBR on plants under Cd stress may appear only in older rape tissue. Fluorescence measurements of the first rape leaves, which accumulated Cd on a level similar to cotyledons (measurements taken on the same day, data not shown) did not show significant disorder of photochemical activity. This phenomenon may result from the correlation between increasing Cd toxicity and the age of tissue (Krupa and Moniak 1998). Older tissues and tissues exposed to heavy metal action for longer time are the most sensitive (Krupa and Moniak 1998). Our results show the toxic activity of Cd on many photochemical pathways in rape cotyledons. EBR totally or partially eliminates the toxic effect of Cd, mainly by diminishing the damage in RCs and OEC as well as maintaining efficient photosynthetic electron transport. Probably, the effectiveness of the protective action of EBR increases in older tissue, which is more susceptible to damage by cadmium.

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