PHOTOSYNTHETICA 39 (3): 363-368, 2001

Pre-treatment of bean seedlings with choline compounds increases the resistance of photosynthetic apparatus to UV-B radiation and elevated temperatures

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Abstract

Bean (*Phaseolus vulgaris* L. cv. Berbukskaya) seedlings were pre-treated with choline compounds, 19 mM 2-ethyltrimethylammonium chloride (Ch) or 1.6 mM 2-chloroethyltrimethylammonium chloride (CCh), during 24 h, then after 6 d the excised primary leaves were exposed to UV-B and high temperature stress. Chlorophyll (Chl) fluorescence, delayed light emission, accumulation of photosynthetic pigments, contents of thiobarbituric acid reactive substances, and activities of the active oxygen detoxifying enzymes (superoxide dismutase, ascorbate peroxidase, and glutathione reductase) were examined. Pre-treatment of plants with Ch or CCh enhanced the resistance of photosystem 2 (PS2) photochemistry to UV-B and heat injuries. The higher stress resistance can be explained by the increased activity of the detoxifying enzymes. The increased content of UV-B-absorbing pigments may also contribute to the enhanced resistance of choline-treated plants to UV-B radiation.

Additional key words: active oxygen species; 2-chloroethyltrimethylammonium chloride; 2-ethyltrimethylammonium chloride; heat stress; *Phaseolus vulgaris*; photosystem 2; ultraviolet radiation.

Introduction

Many stresses such as extreme temperatures and ozone reduce photosynthetic activity (Havaux *et al.* 1991, Runeckles and Krupa 1994). Among other climatic variables, limiting photosynthesis in environmentally stressed plants, ultraviolet (UV)-B radiation is important over the past decade. Studies of more than 300 plant species and cultivars revealed negative morphological and physiological changes in about 50 % of these plants (Tevini 1999). The ability of plants to prevent and/or to repair the stress-induced damage(s) is crucial for crop productivity and for plant survival. One of the methods to decrease stress injury may be using growth retardants that significantly enhance plant resistance to drought, frost, high salinity, and ozone (Mackay *et al.* 1987, Fletcher and Hofstra 1988, Rademacher 1990, Prussakova *et al.* 1993). The growth retardant types and their retardant effects are described in some reviews (cf. Rademacher *et al.* 1990). To our knowledge, however, the mode of action of growth retardants on stress resistance of leaf photosynthesis under different stress conditions is not yet clear.

The higher capacity to detoxify active oxygen species may contribute to the molecular basis of increased stress resistance in plants treated with growth retardants. For example, Mackay *et al.* (1987) found that pre-treatment of wheat seedlings with triazole growth retardant S-3307 stimulated the accumulation of lipid soluble antioxidants in microsomal membranes thus weakening the ozone-induced damage of membranes. Thompson *et al.* (1989) suggested that effects of antioxidative enzymes—SOD and catalase—may be involved in the heat-induced denaturation of photosystem (PS) 2 membrane protein complex.

Our objective was to study the influence of pretreatment of bean seedlings with growth retardants—CCh and Ch—on the susceptibility of leaf photosynthetic apparatus to UV-B radiation and elevated temperatures.

Received 15 February 2001, *accepted* 14 June 2001.
Abbreviations: AsP – ascorbate peroxidase; CCh – chlorocholine chloride; Ch – choline chloride; Chl – chlorophyll; DLE – delayed light emission; F_0 – initial level of chlorophyll fluorescence; F_V – variable chlorophyll fluorescence; GR – glutathione reductase; TBARs – thiobarbituric acid reactive substances; PS – photosystem; SOD – superoxide dismutase; UALM – unit leaf fresh (dry) mass per leaf area; UVP – pigments absorbing UV-B radiation.
Acknowledgements: The work was supported by the Russian Foundation of Fundamental Research (grant no. 01-04-48623).

Materials and methods

Bean (*Phaseolus vulgaris* L. cv. Berbukskaya) seeds were germinated in distilled water at 24±1 °C in Petri dishes. After primary leaves appeared, water was replaced for 24 h by solutions of Ch (19.0 mM) or CCh (1.6 mM). Then the seedlings were carefully washed and planted in vermiculite in plastic pots. The pots were placed under controlled conditions of 10-h photoperiod, irradiance of 60 W m^{-2}, and the day/night temperatures of 23/20 °C. Seedlings were cultivated for 6 d and after that the primary leaves were harvested for analysis and further treatments. Total UV-B irradiance of 10 W m^{-2} was obtained by passing the emission from erythemic mercury lamp *DRP-250* (*Ksenon*, Russia) through an interference filter with maximum transmittance at 302 nm ($\Delta\lambda_{1/2}$ = 12 nm). Heat treatments were performed by placing intact leaves at 43 °C into a thermostated water bath (±0.1 °C). UV-B treatments were also performed using detached leaves placed into a thermostated water bath (±0.1 °C). Prior to UV-B or elevated temperature treatments, leaves were kept in dark for 30 min.

Chl fluorescence was detected in detached leaves *via* a single-beam mode using a laboratory-built set-up. Fluorescence was excited with blue radiation (λ_m = 480 nm). Irradiance was 30 W m^{-2} at the leaf surface. Chl fluorescence was passed through a 685 nm interference filter (10 nm band-pass) and monochromator ($\Delta\lambda$ = ±1 nm) and detected by a photomultiplier. The signal was fed to a storage oscilloscope (*Tektronix*, USA) or recorder *Endim 322-01M* (*VEB MS*, Germany). Parameters measured were F_0, F_v, and $F_v/(F_0+F_v)$. Delayed light emission (DLE) was measured using a phosphoroscopic laboratory-built device. Emission was excited by red radiation of 100 W m^{-2}. The duration of the excitation period was 0.9 ms and DLE was measured for 0.9 ms starting 2.5 ms after the end of the excitation period.

For enzyme and TBARs analysis a small portion (0.5 g) of freshly cut leaves was homogenised in a cooled mortar in 4.5 cm^3 of phosphate buffer (pH 7.4) containing 0.2 mM EDTA and 2 % polyvinylpyrrolidone. The homogenate was filtered through several layers of nylon. Part of homogenate was used for assay of TBARs, whereas the remainder was centrifuged for 20 min at 15 000×*g* before the assays of enzymatic activities. The supernatant (enzyme extract) was used for enzyme analysis. All operations were carried out at 4 °C. Activity of ascorbate peroxidase (AsP) was assayed as the decrease in absorbance at 290 nm due to ascorbate oxidation (Nakano and Asada 1981). Glutathione reductase (GR) activity was assayed by following the oxidation of NADPH (Foyer and Halliwell 1976) at 340 nm. Superoxide dismutase (SOD) activity was assayed spectrophotometrically (Giannopolitis and Ries 1977). Chls and carotenoids were assayed spectrophotometrically in ethanol extracts using the coefficients introduced by Lichtenthaler (1987). UV-absorbing pigments (likely, flavonoids) were extracted from leaf discs by keeping them for 24 h in acidified methanol (methanol : water : HCl, 78 : 20 : 2) at 4 °C according to Mirecki and Teramura (1984). The filtered extracts were then used for measuring the absorbance at 320 nm, which is indicative of relative concentration of UV-B absorbing pigments. TBARs were determined in leaf homogenate using a 2-thiobarbituric acid test developed by Dhindsa and Matove (1981).

Figures in tables are the means of 3-6 replicates. In each individual measurement, leaves from 5-10 plants were analysed to minimise probable ontogenetic differences between plants. The *t*-test was used.

Results

Effects of cholines and UV radiation on the contents of pigments, TBARs, and enzyme activities: Plant pre-treatment with cholines increased remarkably the Chl (CCh and Ch) and carotenoid (CCh) contents calculated on a leaf area basis (Table 1). Effects of cholines on the pigments, calculated on a leaf fresh mass basis, were less significant. Hence the increased Chl and carotenoid contents calculated on a leaf area basis mainly result from leaf thickening. Indeed, we found that choline application reduced leaf area but unit leaf fresh (dry) mass per leaf area (UALM) was 1.6-1.8 times higher in Ch- and CCh-treated plants than in the control ones. Higher UALM indicates increased leaf thickness. In contrast to choline pre-treatment, exposure to UV-B somewhat decreased the Chl content calculated per unit of area or leaf matter. No clear effect of UV-B on carotenoid content and pigments absorbing UV-B (UVP) was apparent from our results. However, CCh itself stimulated the accumulation of UVP. The joint application of cholines and UV-B showed that choline pre-treatment did not protect leaves from partial damage of Chls by UV-B.

Accumulation of the products of peroxidation (TBARs) was stimulated by UV-B radiation in both control and CCh-treated leaves (Table 1). CCh itself also enhanced the formation of TBARs. At the same time both CCh and UV-B increased activities of all antioxidative enzymes (SOD, AsP, and GR). Also the content of total leaf proteins increased in CCh-treated seedlings (29 ± 4) compared to the control (17 ± 2) g kg^{-1}(f.m.).

Table 1. Changes in contents of chlorophylls (Chl), carotenoids (Car), thiobarbituric acid reactive substances (TBARs), UV-B-absorbing pigments (UVP), and in activities of antioxidative enzymes (SOD, AsP, and GR) in leaves of bean seedlings. After the first primary leaves had appeared, the seedlings were treated by 19 mM Ch or 1.6 mM CCh for 24 h and grown after this for 6 d. Then the primary leaves were exposed to UV-B (8 h, 10 W m^{-2}, λ_m = 302 nm) and kept in darkness for 48 h before harvesting. Numbers in parentheses represent SE (n = 3). Relative content of UVP was characterised by relative units of absorbance (A units) at 320 nm of leaf extracts prepared from equal leaf matter. * – insignificant differences between control and CCh, Ch, and UV-B (p>0.05); ** – insignificant differences between Ch (CCh) and Ch+UV-B (CCh+UV-B) (p>0.05).

Parameter	Control	UV-B	CCh	CCh + UV-B	Ch	Ch + UV-B
Chl (a+b) [mg m^{-2}]	353 (11)	305 (12)	556 (20)	477 (17)	477 (14)	397 (15)
Chl (a+b) [g kg^{-1}(f.m.)]	1.10 (0.04)	0.89 (0.05)	1.27 (0.04)	1.05 (0.035)	0.98 (0.04)*	0.83 (0.03)
Chl a/b	2.5	2.5*	2.4*	2.4**	2.3*	2.3**
Car [mg kg^{-1}(f.m.)]	156 (11)	124 (10)*	184 (19)*	171 (12)**	143 (8)*	111 (8)
Car [mg m^{-2}]	50 (5)	43 (3)*	78 (8)	72 (6)**	70 (5)*	57 (5)**
UVP [A unit g^{-1}(f.m.)]	0.86 (0.06)	1.06 (0.08)*	1.26 (0.1)	1.27 (0.1)**	1.1 (0.08)*	1.14 (0.07)**
TBARs [µmol kg^{-1}(f.m.)]	314 (15)	423 (25)	446 (24)	562 (27)		
SOD [unit g^{-1}(f.m.)]	114 (8)	270 (16)	185 (13)	363 (29)		
AsP	3.8 (0.3)	4.8 (0.3)*	5.8 (0.5)	6.7 (0.5)		
GR [µmol kg^{-1}(f.m.) s^{-1}]	72 (6)	93 (7)*	120 (8)	147 (10)		

Effects of UV-B radiation on DLE and Chl fluorescence: Two phases, fast and slow, were observed in the kinetics of DLE increase upon irradiation of control leaves (Fig. 1, curves *1* and *2*). The effect of excision

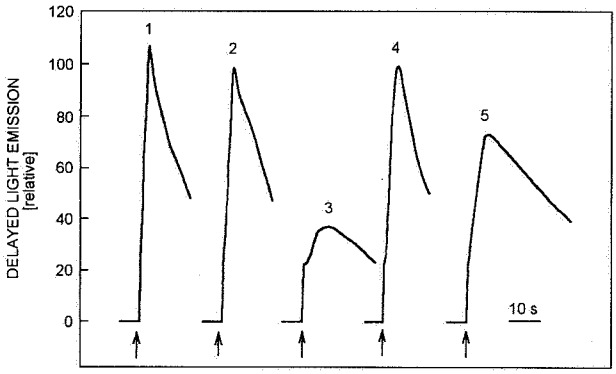

Fig. 1. Kinetics of light-induced changes in delayed light emission in detached leaves of bean seedlings. The seedlings were treated with Ch for 24 h after appearance of primary leaves. Six days later, primary leaves of both treated and non-treated with Ch seedlings were excised and a part of leaves was exposed to UV-B (10 W m^{-2}; λ_m = 302 nm) for 8 h. Curve *1* shows control leaves immediately after excision, before UV-B. After UV-B the leaves of non-treated (curves *2* and *3*) or treated (curves *4* and *5*) with Ch seedlings, both exposed (curves *3* and *5*) and non-exposed to UV-B (curves *2* and *4*) were placed into water bath and kept for 48 h in darkness at 20 ± 1 °C prior to measurements. Typical traces from 6 independent measurements. *Arrows* indicate the onset of excitation radiation.

(values not shown) and placing the detached leaves into water bath for 2 d had no significant effect on the magnitude of DLE (compare curves *1* and *2*). Plant pre-treatment with Ch did not affect both maximum magnitude of emission and relative magnitudes of two kinetic phases of DLE (Fig. 1, curve *4*). Exposure of control leaves to UV-B greatly diminished the magnitude of slow phase of DLE (Fig. 1, curve *3*). In contrast, the magnitude of fast phase was slightly affected by UV-B radiation. In Ch-treated leaves, the UV-B-induced suppression of slow phase was much smaller than in control ones (Fig. 1,

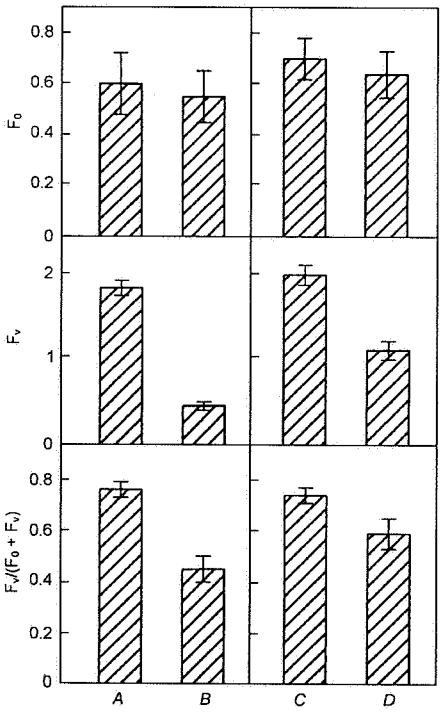

Fig. 2. Effects of UV-B on Chl fluorescence characteristics of leaves of bean seedlings non-treated (*A* and *B*) or treated with Ch (*C* and *D*), which were not exposed (*A* and *C*) or exposed (*B* and *D*) to UV-B radiation. Leaf treatments were done as in Fig. 1. Means ± SD, n = 6. Fluorescence parameters are given in relative units.

compare curves *3* and *5*). Similarly to Chl fluorescence, light-induced increase of DLE during slow phase to a maximum was slowed down after UV-B treatment.

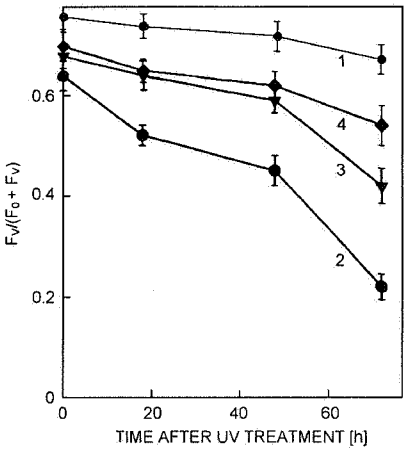

Fig. 3. Dependence of $F_V/(F_0 + F_V)$ chlorophyll fluorescence ratio on a time of dark exposure after treatments of detached bean leaves with UV-B. Curve *1* indicates detached control leaves non-exposed to UV-B from seedlings non-treated with Ch (CCh). Curves *2*, *3*, and *4* indicate leaves of seedlings non-treated (*2*) or treated with Ch (*3*) or CCh (*4*). Ch and CCh treatments and UV-B conditions were done as in Fig. 1. Leaves were exposed to UV-B for 8 h. Means ± SD ($n = 3$).

Fluorescence parameters calculated from Chl fluorescence induction curves are shown in Fig. 2. The $F_V/(F_0 + F_V)$ ratio that reflects the activity of PS2 was about 0.75. This ratio was barely affected by a plant treatment with Ch or CCh, while it was dramatically reduced by exposure of control leaves to UV-B. The loss of PS2 activity developed gradually after plant exposure to UV-B and was similar in Ch and CCh-treated leaves (Fig. 3). Excision of leaves had no significant influence on fluorescence curves (values not shown). When the detached leaves were kept in darkness, a change in F_V magnitude was much less pronounced than in experiments with UV-B treatment (Fig. 3, curves *1* and *2*). Importantly, the reduction in activity was mainly caused by reduced F_V magnitude, whereas F_0 level was slightly affected by UV-B radiation. Much smaller UV-B-induced decline of F_V magnitude and the $F_V/(F_0 + F_V)$ ratio was observed in choline-treated plants compared to control ones. In addition to reducing F_V magnitude, UV-B treatment slowed the irradiation-induced rise of F_V. The half time of that rise increased after exposure to UV-B radiation from 0.45 s to 1.10 s and 0.80 s in control and Ch-treated leaves, respectively.

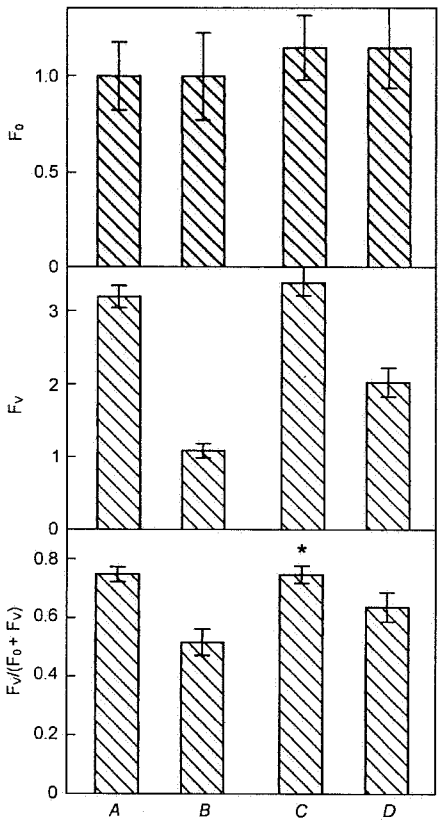

Fig. 4. Effects of heat on chlorophyll fluorescence characteristics of leaves of bean seedlings non-treated (*A* and *B*) or treated with 1.6 mM CCh (*C* and *D*), which were not exposed (*A* and *C*) or exposed for 6 min to 43 °C (*B* and *D*). The seedlings were treated by CCh for 24 h after appearance of primary leaves. Six days later, primary leaves were excised and kept for 30 min in darkness before heat treatment. Fluorescence parameters are given in relative units. Means ± SD ($n = 7$). The star means insignificant difference between control and experiment ($p > 0.05$).

Effects of elevated temperatures on Chl fluorescence: After a 6-min exposure to 43 °C, heat injury markedly reduced F_V magnitude and the $F_V/(F_0 + F_V)$ ratio (Fig. 4). At all time exposures to 43 °C, heat-induced inhibition of F_V was less in Ch- (CCh-) treated leaves compared with control ones. Similar tendencies (the higher heat-resistance of PS2 in choline-treated plants) were also observed at other temperatures: 41 and 46 °C (values not shown).

Discussion

Our study revealed a high activity of Ch and CCh as growth retardants. This agrees well with our recent findings on the action of Ch and CCh on morphology of wheat seedlings (Kobzar' *et al.* 1999).

We confirmed previous conclusion of several authors that PS2 is one of the most heat- and UV-sensitive components of photosynthetic apparatus (Bornman 1989, Renger *et al.* 1989, Strid *et al.* 1994, Yamane *et al.*

1998). Indeed, loss of Chls induced by UV-B was much less pronounced than the decrease in PS2 photochemistry (compare Table 1 and Figs. 1, 2, and 3). Since the inhibitory action of UV-B developed during several days after UV irradiation (Fig. 3), loss of PS2 photochemistry can be attributed not to primary, but rather to secondary effects of UV-B likely due to oxidative damage of photosynthetic apparatus, first PS2 (Tevini 1999).

Treatments with Ch or CCh barely affected photochemical efficiency of PS2 as evidenced by similar and high $F_V/(F_0 + F_V)$ ratios in leaves of control and Ch- or CCh-treated plants (see Figs. 2 and 3). At the same time, Ch or CCh significantly weakened the inhibitory effect of UV radiation on PS2 photochemistry. On the contrary, the losses of Chl in control and Ch-treated plants were similar. UV-B radiation induces a wide range of oxidative stress injury in plant cells (Larson 1988, Tevini 1999). Therefore we suggest that choline pre-treatment decreases the oxidative injuries of PS2 caused by UV-B radiation.

Changes in kinetic curves of DLE induction indicate that a damage of thylakoid membrane occurs in UV-B-treated leaves. Indeed, the slow component of DLE kinetic curve was greatly reduced in control leaves exposed to UV-B (compare curves *2* and *3* in Fig. 1). This phase appears from the development of a pH gradient across the thylakoid membrane (Wright and Crofts 1971). Thus, higher proton leakage from intra-thylakoid space seems to proceed after leaf treatment with UV radiation. Partial protection of thylakoid membranes against UV-B stress by choline compounds is thus evident from Fig. 1, because the UV-B-induced decline in the magnitude of the slow DLE phase was less pronounced in Ch-treated leaves than in control ones. Thus, there is experimental evidence that choline-treated plants show higher resistance of PS2 and thylakoid membranes to UV-B stress compared with untreated plants.

Antioxidant enzymes are crucial components in preventing oxidative stress in plants (Allen 1995). In our study, plant treatments with Ch or CCh significantly enhanced activities of SOD, AP, and GR (Table 1). This finding evidences that growth retardants specifically activated the three defence enzymes located mainly in chloroplasts and thus protected PS2, one of most sensitive constituents of leaf cells. This agrees with the higher content of TBARs found in the leaves of these plants (Table 1). Another choline-dependent mechanism that may be involved in protection of leaf cells against UV is an enhanced accumulation of UVP, indicated in leaves of CCh-treated seedlings (Table 1). The accumulation of UVP such as phenylpropanoids and flavonoids is a well-known mechanism protecting against UV-B radiation (Strid *et al.* 1994, Tevini 1999).

We found that plant treatments with Ch and CCh increase the tolerance of PS2 not only to UV-radiation, but to heat stress as well (see Fig. 4). This action cannot be attributed to oxidative stress, because the primary target of short-term heat action on photosynthesis is probably the donor side of PS2 (see Bukhov and Mohanty 1999 for recent review). The damage of PS2 is probably related to the release of Mn ions or 33 kDa polypeptide from the water-splitting complex (Enami *et al.* 1994, Yamane *et al.* 1998). Those processes occur very rapidly at high temperatures and do not require oxidative injury. On the other hand, significant contribution of SOD, which catalyses the dismutation of O_2^- radicals to H_2O_2, to protection of thylakoid membranes to heat action was proposed by Thompson *et al.* (1989). This possibility must not be ruled out in our experiments since choline pre-treatment increased SOD activity. Further studies seem to be necessary to reveal how cholines are able to effectively protect PS2 from heat stress.

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