DOI: 10.1007/s11099-017-0710-7 PHOTOSYNTHETICA 56 (2): 660-669, 2017

Effects of excess cadmium in soil on JIP-test parameters, hydrogen peroxide content and antioxidant activity in two maize inbreds and their hybrid

M. FRANIĆ+, V. GALIĆ, M. MAZUR, and D. ŠIMIĆ

Agricultural Institute Osijek, Department of Maize Breeding and Genetics, HR31103, Južno predgrađe 17 Osijek, Croatia

Abstract

Excessive cadmium (Cd) content in soil leads to a number of phytotoxic effects and challenges agricultural production. Aim of this study was to investigate different responses of two maize inbreds and their hybrid to an elevated Cd content in soil by measuring photosynthetic and biochemical activity and to identify a Cd tolerance mechanism. Antioxidant status-related parameters varied significantly between inbreds and treatments. Dry mass increased in both inbreds, but remained unchanged in hybrid. After the Cd treatment, parameters of chlorophyll *a* fluorescence varied between inbreds and hybrid performance was similar to inbred B84. We concluded that inbred B84 is Cd-sensitive compared to Os6-2, which did not appear to be negatively affected by Cd treatment at this growth stage studied. We suspect that due to a dilution effect in the hybrid, there was no or very weak Cd stress detected by biochemical parameters, although stress was detected by chlorophyll *a* fluorescence.

Additional key words: antioxidant enzymes; heavy metal toxicity; JIP test; photosynthesis; photosystem II.

Introduction

Cadmium is a trace element known for its adverse effects in cellular systems of plants and animals. Once taken in by the plant, it causes damage in a number of molecular mechanisms and cell compartments (Das *et al*. 1998). Visual symptoms of Cd phytotoxicity include chlorosis, reduction of growth, leaf rolling, browning of root tips, and death in some cases. Key sources of excess Cd concentrations in soils are antropogenic activities, such as traffic, industry, and application of phosphate fertilizers (Di Toppi and Gabbrielli 1999). Its uptake by roots is mostly a transpiration driven passive process; plants possess no Cd-exclusion mechanism (Gallego *et al.* 2012). Other mechanism for Cd to enter plant cell is through the transport systems involved in micronutrient uptake, specifically through transmembrane divalent metal carriers although there is no evidence that these transporters can transport Cd above Fe (Verbruggen *et al.* 2009). Cd causes oxidative stress in plants by altering activities of antioxidant defense mechanisms resulting in hydrogen peroxide accumulation (Sandalio *et al.* 2001, Schützendübel and Polle 2002, Cho and Seo 2005) which eventually leads to an increase of protein (Romero-Puertas *et al.* 2002) and lipid peroxidation (Chaoui *et al.* 1997). Mechanisms that cope with elevated contents of hydrogen peroxide either catalyze its decomposition into oxygen and water or use substrate to reduce peroxide thus producing

Received 17 November 2016, *accepted* 19 January 2017, *published as online-first* 13 March 2017.
+Corresponding author; e-mail: mario.franic@poljinos.hr
Abbreviations: ABS/RC – absorption per active reaction centre; APX – ascorbate peroxidase; Car – carotenoids; CAT – catalase; Chl – chlorophyll; CK – control; DI_o/RC – dissipation per active reaction centre; DM – dry mass; FM – fresh mass; ET – electron transport; ET_o/ABS – quantum yield for electron transport; ET_o/RC – electron transport per active reaction centre; ET_o/TR_o – efficiency/probability for electron transport; ET_o (TR_o – ET_o) – electron transport beyond Q_A^-; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; M_o – approximated initial slope (ms^{-1}) of the fluorescence transient normalised on the maximal variable fluorescence F_v; PI_{ABS} – performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; POD – peroxidase; RC/ABS – density of reaction centres on chlorophyll *a* basis; RC/CS_o – density of reaction centres per excited cross section; ROS – reactive oxygen species; S_m – normalised total complementary area above the transient curve; TBARS – thiobarbituric acid-reactive substances; t_{max} – time (in ms) to reach the maximal fluorescence intensity F_m; TR_o/ABS – maximum quantum yield for primary photochemistry; TR_o/DI_o – flux ratio trapping per dissipation; TR_o/RC – trapping per active reaction centre; V_J – relative variable fluorescence at J step.
Acknowledgements: This research was funded by the Croatian Science Foundation (project No. 5707: „Genetics and physiology of multiple stress tolerance in maize“).

water and oxidized substrate. Enzymes that perform these actions are catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), while pigments from group of carotenoids (Car) act as nonenzymatic reactive oxygen species (ROS) quenchers (Anjum *et al.* 2015). Cd can also affect reduction of CO_2 fixation (Weigel 1985) and it is most probably due to modulation of key carboxylating enzymes of Calvin cycle, *i.e.* Rubisco and phospho*enol*-pyruvate carboxylase (PEPC) (Krantev *et al.* 2008).

Cd can even substitute the central Mg atom of chlorophyll (Chl) and this results in a breakdown of photosynthesis (Küpper *et al.* 1996). Every environmental change forces the photosynthetic apparatus to adjust its physiological state; parameters of fast polyphasic fluorescence induction transient appear to change accordingly (Strasser *et al.* 2004). Saturating light presents an energy influx to a dark-adapted photosynthetic apparatus, with one of its output being fluorescence along with dissipation in terms of heat. Measurement of fluorescence induction transients in dark-adapted samples spans for 1 s with data-point resolution of 10 μs and a key state identified as F_0 (initial fluorescence intensity) and F_m (maximal intensity) (Strasser *et al.* 2004). Between these two extrema, intermediate time-steps, such as K (at 300 μs), J (at 2 ms), and I (at 30 ms) (Fig. 1) related to electron flux between PSII components, can be observed and measuring these states resembles informative O-J-I-P curve (Strasser *et al.* 1995). Chl *a* fluorescence measurement is a suitable method for detection and evaluation of heavy metal stresses (Żurek *et al.* 2014) and it is sensitive to Cd stress (Larsson *et al.* 1998, Di Cagno *et al.* 1999, Burzyński and Żurek, 2007). Chl *a* measurements yield a large number of parameters that can be used to interpret the state of photosystems, such as experimental and normalized signals (F_o, F_m, F_v, F_v/F_m) as shown by Tuba *et al*. (2010) on bryophytes or transient fluorescence curves and phenomenological energy fluxes as shown on barley seedlings (Kalaji *et al*. 2007). There is a significant variation in affinity for heavy metals accumulation among as well as within plant species (Grant *et al.* 1998). Maize inbreds have been shown to differ in uptake of heavy metals (Florijn and van Beusichem 1993, Brkić *et al.* 2003) and temperate inbred lines, B84 and Os6-2, have been designated as different according to their respective ionomic profiles (Sorić *et al.* 2011, Šimić *et al.* 2012) and leaf Cd accumulation (Sorić *et al*. 2009).

The aim of our study was to evaluate effects of excessive Cd content in soil on young plants of maize inbred lines B84 and Os6-2 and their respective hybrid by means of Chl *a* fluorescence and activity of antioxidant enzymes.

Materials and methods

Plant materials and growth conditions: Seeds of two maize (*Zea mays* L.) genotypes with different sensitivity to Cd in soil along with their hybrid were planted in trays (21 × 35 × 7 cm) filled with universal substrate and placed in a growth chamber [25°C, 16/8 day/night, 200 μmol (photon) m^{-2} s^{-1}]. Line B84 is a public line of Iowa Stiff Stalk Synthetic (BSSS) related to well-known reference line B73, while Os6-2 is a Lancaster inbred line related to line C103 and reference line Mo17, classified into two respective heterotic groups (Lee and Tracy 2009). Substrate properties were: nitrogen ($NH_3 + NO^{3-}$) at 70 mg L^{-1}, phosphorous (P_2O_5) at 80 mg L^{-1}, potassium (K_2O) of 90 mg L^{-1}, organic matter of 70% (dry mass, DM), and pH 5.7 ($CaCl_3$). Contents of heavy metals (Cd, Cr, Cs, Hg, Ni, Pb, Zn) and other toxic substances (polycyclic aromatic hydrocarbons, polychlorinated biphenyls) were below permitted amounts; Cd content was 0.2 mg kg^{-1}(soil DM). Total of 42 seeds were planted in one tray for each genotype for treatment and control (CK). Seeds (n = 42) were planted in a tray for each genotype, CK and Cd treatment were considered one replicate. Experiment was set in three replicates. Soil for CK was left uncontaminated and for the Cd treatment (Cd5) with 5 mg(Cd) kg^{-1}(soil), soil was weighed and spread in approximately 5-cm thick layer and sprayed with a solution of $CdCl_2$. $CdCl_2$ solution was made by dissolving 4.0805 g of $CdCl_2$ in 5 L of deionized water. For Cd5 treatment, 10 mL of prepared solution was sprayed using a spray bottle for every kg of soil. During the spraying of the solution, soil was mixed multiple times. Trays were watered with 200 mL of water every two days. Plantlets were grown for ten days after planting and on the last day, Chl *a* fluorescence measurements using the fluorimeter *Handy PEA* (*Hansatech*, King´s Lynn UK) were performed after which leaves were cut and stored in the freezer (–80°C) until further analysis.

Chl *a* fluorescence was measured on the middle section of the first fully developed leaf; 15 measurements per replicate (tray) were performed giving a total of 45 measurements for each genotype per treatment. After dark adaptation for 30 min, Chl fluorescence transient was induced by applying a pulse of saturating red light [peak at 650 nm, 3,200 μmol(photon) m^{-2} s^{-1}] on the leaf surface which was exposed by the leaf clip (4 mm in diameter). Saturating-light pulse induced fluorescence increase from minimal fluorescence (F_o), when all reaction centers are open, to maximal fluorescence (F_m), when all reaction centers are closed. During the 1-s measurement, 120 data points were collected. Chl *a* fluorescence data were processed with software provided with the fluorimeter.

Data obtained by Chl *a* fluorescence measurements was analyzed according to the JIP-test that outputs multiple parameters quantifying the photochemistry of PSII. The JIP-test was described by Strasser *et al.* (1995, 2004, 2010). JIP-test parameters are listed in Fig. 2, for definitions and formulas see Table 2S (*supplement avaliable online*).

Photosynthetic pigments and DM analysis: Collected samples of frozen leaves were powdered in a porcelain mortar in liquid nitrogen with addition of magnesium hydroxide carbonate. A sample (1 g) was put in an Eppendorf tube with 1 mL of cold acetone and vortexed. Tubes were placed on ice for 15 min, centrifuged for 10 min at 4°C and 20,000 × *g*. Precipitate was reextracted with the same procedure until the tissue lost its color. Concentration of photosynthetic pigments (Chl *a*, *b*, and Car) was determined spectrophotometrically (*Specord 200*, *Analytik*, Jena, Germany) according to Lichtenthaler (1987) with absorbance readings at 470, 661.6, and 664.8 nm in a glass cuvette. Acetone was used as blank.

Percentage of leaf dry mass (DM) was determined by weighing 1 g of ground fresh sample (ground in liquid nitrogen) in an Eppendorf tube and drying at 105°C for 48 h. Dry mass (DM) is expressed as % of fresh mass (FM). Three samples of pigments and DM were measured for each treatment, genotype, and replicate.

Assays of antioxidant enzymes activity, H_2O_2 concentration and TBARS content

Enzyme extractions: Tissue was macerated into fine powder in liquid nitrogen using mortar and pestle with the addition of polyvinylpyrrolidone (PVP). Approximately 0.2 g of powdered tissue was extracted for 15 min on ice with 1 mL of extraction buffer (for APX: 100 mM potassium phosphate buffer pH 7.0 + 5 mM Na-ascorbate + 1 mM EDTA, for CAT and guaiacol peroxidase: 100 mM potassium phosphate buffer pH 7.0). Afterwards it was centrifuged at 18,000 × *g* for 10 min at 4°C and supernatants were taken for APX, CAT, and guaiacol POD. Activities were measured spectrophotometrically using spectrophotometer (*Specord 200*, *Analytik*, Jena, Germany).

Antioxidant enzyme activities: Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) by monitoring the decrease in absorbance at 290 nm due to ascorbate oxidation ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) during 2 min. Reaction mixture consisted of 50 mM potassium phosphate buffer (pH = 7.0) with 0.1 mM EDTA, 50 mM ascorbic acid, and sample extract. Reaction was started by adding 10 μL of 12 mM H_2O_2 to 990 μL of the reaction mixture. Enzyme activity was expressed as μM(ascorbate oxidized) min^{-1} g^{-1}(FM). Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by monitoring the decrease in absorbance due to decomposition of H_2O_2 at 240 nm ($\varepsilon = 0.036$ mM^{-1} cm^{-1}) during 1 min. Reaction was started with addition of sample extract to reaction mixture consisting of 50 mM potassium phosphate buffer (pH = 7) with 10 mM H_2O_2. Enzyme activity was expressed as μM(H_2O_2 decomposed) min^{-1} g^{-1}(FM).

Guaiacol peroxidase (POD; EC 1.11.1.7) activity was measured according to Siegel and Galston (1967). POD activity was determined by monitoring the increase in absorbance due to guaiacol oxidation ($\varepsilon = 26.1$ mM^{-1} cm^{-1}) at 470 nm over 2 min. Reaction mixture consisted of 5 mM guaiacol and 5 mM H_2O_2 in 0.2 mM phosphate buffer (pH 5.8). Reaction was started by adding protein extract to reaction mixture. Enzyme activity was calculated as μM(guaiacol oxidized) min^{-1} g^{-1}(FM).

H_2O_2 concentration was determined according to Velikova *et al.* (2000). Approximately 0.2 g of leaf tissue was powdered in liquid nitrogen and extracted with 2 mL of 0,1% trichloroacetic acid (TCA) on ice for 10 min. After the extraction, aliquot was centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant of 400 μL was transfered to a new Eppendorf tube and mixed with 600 μL of 10 mM potassium phosphate buffer and 1 mL of 1M potassium iodide. This reaction mixture was vortexed and left in the dark for 20 min. Absorbance was measured at 390 nm and 1 ml of 10 mM potassium phosphate buffer + 1 mL 1M potassium iodide was used as blank. H_2O_2 concentration was calculated using samples absorbance values from the standard curve and expressed as μmol g^{-1}(FM).

TBARS content was determined according to Verma and Dubey (2003). Samples were powdered in liquid nitrogen and extracted with 1 mL of 0.1% (w/v) TCA. After centrifugation at 6,000 × *g* for 5 min, 0.5 mL of the supernatant was added to 1 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA. The mixture was heated at 95°C for 30 min, then quickly cooled in an ice-bath and centrifuged at 18,000 × *g* for 15 min at 4°C. The TBARS content was determined by measuring absorbance at 532 and 600 nm. The concentration of lipid peroxidation products was expressed as total TBARS in terms of nmol g^{-1}(FM), using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis was performed using *R* (*R Core Team*, 2012). Analysis of variance (*ANOVA*) function from R´s car package was used for factorial *ANOVA* with three sources of variation: treatment, genotype, and replication. *Statistical Tool for Agricultural Research* (STAR 2014) was used to calculate *Tukey´*s honest significant difference (HSD) test at $P<0.05$ level ($HSD_{0.05}$). Measurements ($n = 15$) of Chl *a* fluorescence were performed per replicate. For antioxidant enzymes, TBARS, H_2O_2, pigments, and DM, 3 samples per replicate were measured. Since there were no significant differences between replicates, all Chl *a* fluorescence ($n = 45$) and biochemical analyses ($n = 9$) data were pooled to produce test statistics for graphs.

Results

PSII parameters derived by the JIP-test: Selected parameters of Chl *a* fluorescence and parameters derived by the JIP-test are shown in Figs. 1 and 2. In order to compare genotypes, values of parameters were normalized to their

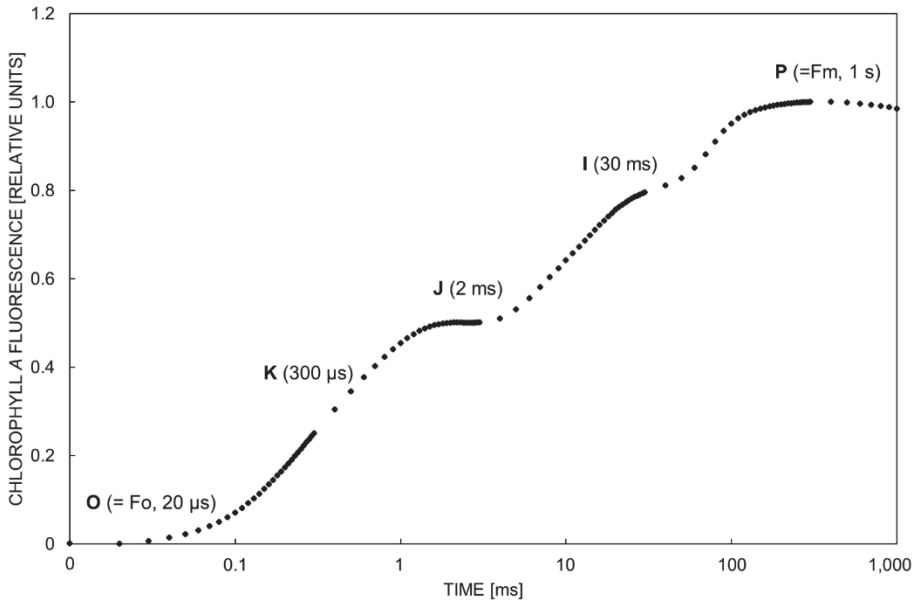

Fig. 1.Typical OJIP transient of chlorophyll fluorescence obtained by illumination of a dark-adapted leaf sample by saturating red light. Transient is plotted on a logarithmic time scale (20 ms to 1 s). Symbols O, K, J, I, P represent fluorescence intensities at 50 µs, 300 µs, 2 ms, and 300 ms, respectively. The figure was created using data measured with the fluorimeter *Handy PEA* (*Hansatech*, King´s Lynn UK) on maize leaf.

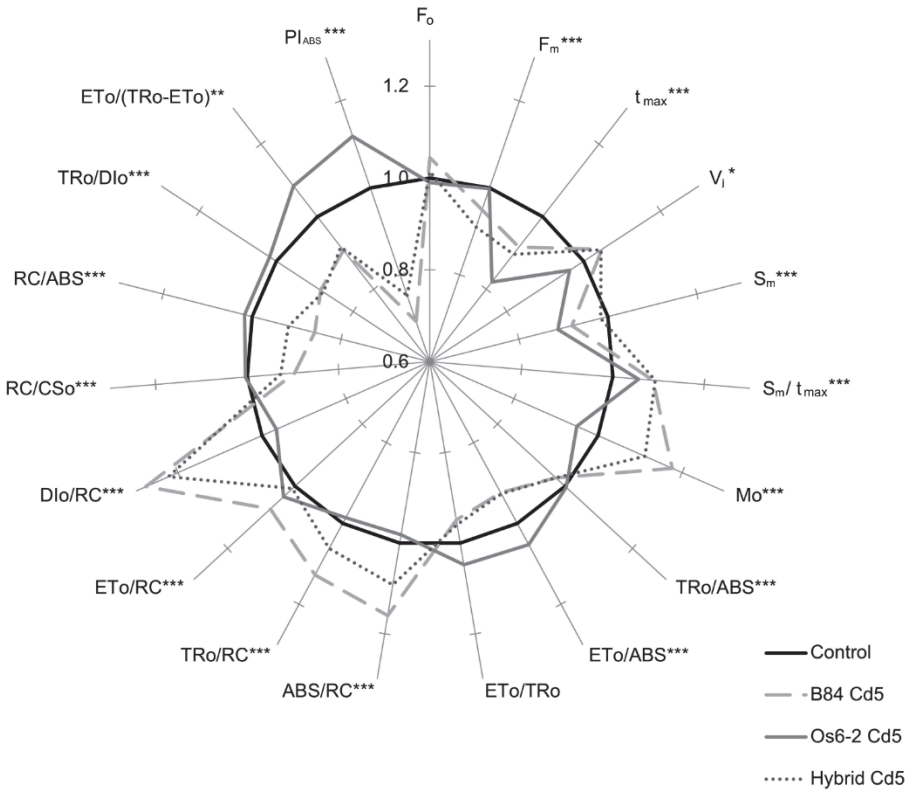

Fig. 2. Effects of elevated cadmium concentration in soil [5 mg(Cd) kg^{-1}(soil)] on selected functional and structural JIP-test parameters plotted (radar plot center = 0.6, maximum = 1.3) relative to their respective controls (set as reference black circle = 1.0). Values represent averages (n = 45). *, **, *** represent significant differences between control and Cd5 treatment at 0.05, 0.01, and 0.001, respectively. For definitions, formulas and abbreviations *see* also Table 2S.

control. Difference in patterns of stressed and control plants pointed out the impact of elevated Cd content in soil on each genotype. *ANOVA* for selected biophysical parameters showed significant effects and main sources of variation (treatment, genotype), as well as their interactions. Differences between CK and Cd5 treatment were confirmed (Table 1S, *supplement available online*). Effect of Cd treatment was not significant only for ET_o/TR_o, while for all other parameters, effect of treatment was significant at $P<0.01$ and in some parameters at $P<0.001$. The effect of the genotype was significant for all selected parameters. Interactions of main effects were significant for all selected parameters (Fig. 2). Values of directly measured parameters (F_m, t_{max}) showed a decrease caused by excess Cd content. B84 and hybrid values of F_m parameter decreased, while Os6-2 did not change compared with CK (Table 1S). Values of t_{max} in Cd5 treatment decreased in all three genotypes, Os6-2 value being the lowest one. Relative variable fluorescence at J step (V_J) increased in B84 and hybrid after the Cd5 treatment, while it was reduced in Os6-2. Normalized total complementary area above the transient curve (S_m) declined in Cd5 treatment for B84 and Os6-2, while it did not change in hybrid. Initial rate of primary photochemistry (M_o – initial slope) increased in Cd5 treatment for B84 and hybrid, but it was reduced in Os6-2 line. The Cd5 treatment caused the highest impact on dissipation per reaction center (DI_o/RC); B84 line and hybrid increased levels of dissipation, while Os6-2 was not affected. Both hybrid and B84 line showed elevated values of absorption per reaction center (ABS/RC) and trapping per reaction center (TR_o/RC) after Cd5 treatment, while Os6-2 line was not affected. Increase of electron transport per reaction center (ET_o/RC) occurred in B84 and Os6-2 line, while the hybrid was unaffected by the Cd5 treatment. Density of reaction centers on Chl *a* basis (RC/ABS) and density of reaction centers per excited cross-section (RC/CS_o) decreased in B84 line and hybrid after the Cd5 treatment, while Os6-2 line was unaffected. Maximum quantum yield of primary photochemistry (TR_o/ABS) decreased after the Cd5 treatment in B84 and hybrid and was more or less unaffected in Os6-2 line. Maximum yield of electron transport (ET_o/ABS) and efficiency of a trapped exciton to move an electron into the electron transport chain further than Q_A^- (ET_o/TR_o) decreased both in B84 and hybrid after the Cd5 treatment and increased in Os6-2 line. Flux ratio trapping per dissipation (TR_o/DI_o) decreased in the Cd5 treatment for B84 and hybrid, while it slightly increased in Os6-2 line. Electron transport beyond Q_A^- [$ET_o(TR_o - ET_o)$] decreased in B84 and hybrid in Cd5 treatment and increased in Os6-2 line. Performance index on absorption basis (PI_{ABS}) decreased in B84 and hybrid and increased in Os6-2 line after the Cd5 treatment.

Photosynthetic pigments and DM: In control treatment, DM was the lowest in B84 line, while Os6-2 and hybrid had similar values. Cd treatment caused an increase of DM

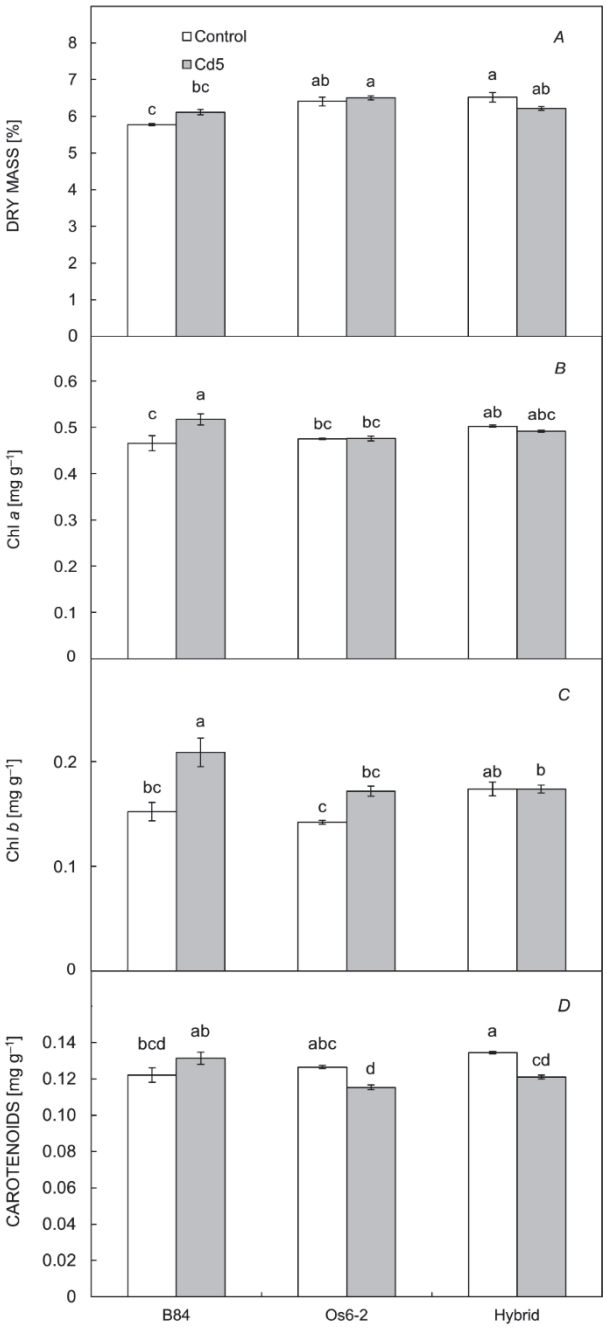

Fig. 3 Effects of elevated cadmium concentration in soil [5 mg(Cd) kg^{-1}(soil)] on dry mass (*A*), chlorophyll (Chl) *a* content (*B*), Chl *b* content (*C*), and carotenoid content (*D*). Means (± SE, $n = 9$) with *the same letters* are not significantly different (*Tukey´*s $HSD_{0.05}$).

in B84 and Os6-2 lines and in hybrid (Fig. 3*A*). *ANOVA* showed that there was no significant effect of the treatment, although genotype and genotype × treatment interaction showed significant effects (Table 1). Spectrophotometric data for Chls and Car revealed changes in pigment concentrations induced by Cd which were genotype dependent. In CK, hybrid had the highest concentration of Chl *a*, Chl *b*, and Car, while after the Cd5

Table 1. Analysis of variance for the effects of cadmium in soil and genotype on hydrogen peroxide (H_2O_2), dry mass (DM), antioxidant enzymes, thiobarbituric acid reactive substances (TBARS), and photosynthetic pigments. APX – ascorbate peroxidase; POD – guaiacol peroxidase; CAT – catalase; Chl – chlorophyll; Car – carotenoids. n.s. – insignificant; *, **, *** – significant differences between control and Cd5 treatment at 0.05, 0.01, and 0.001 levels, respectively.

Source of variation	df	H_2O_2	DM	APX	POD	CAT	TBARS	Chl *a*	Chl *b*	Car
Cd	1	*	n.s.	***	***	*	n.s.	*	***	**
Genotype	2	***	***	***	***	***	***	*	**	**
Cd×Gen	2	***	**	***	n.s.	***	***	**	***	***

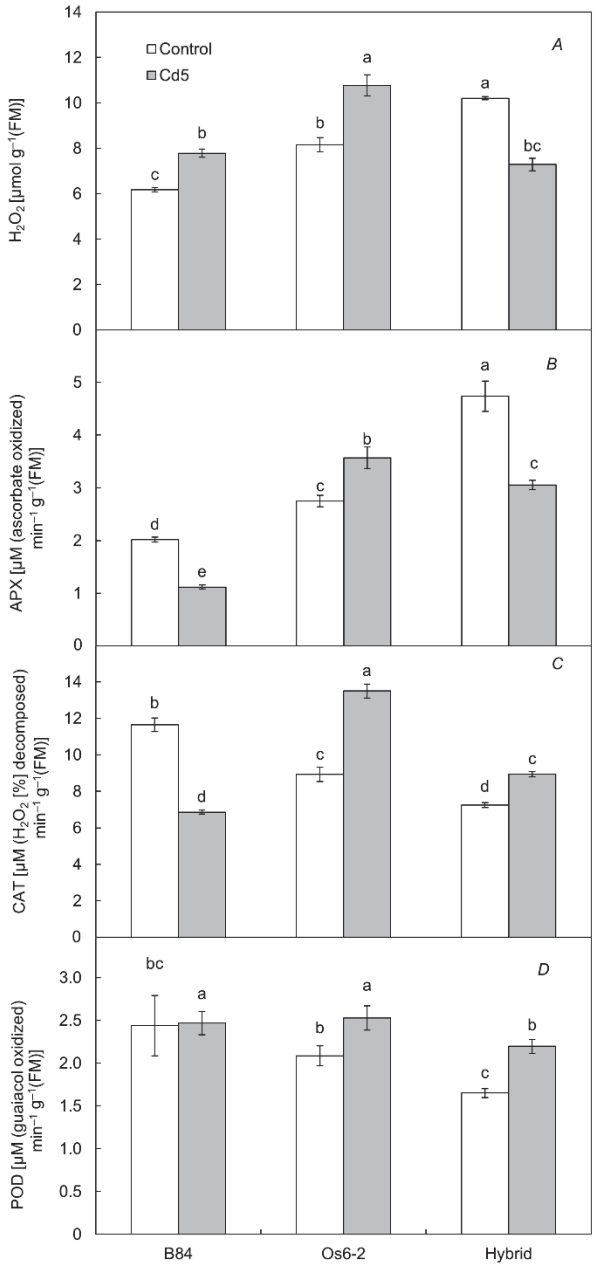

Fig. 4. Effects of elevated cadmium concentration in soil [5 mg(Cd) kg^{-1}(soil)] on hydrogen peroxide (H_2O_2) (*A*), ascorbate peroxidase (APX) (*B*), catalase (CAT) (*C*), and guaiacol peroxidase (POD) (*D*). Means (± SE, n = 9) with *the same letters* are not significantly different (*Tukey´*s $HSD_{0.05}$).

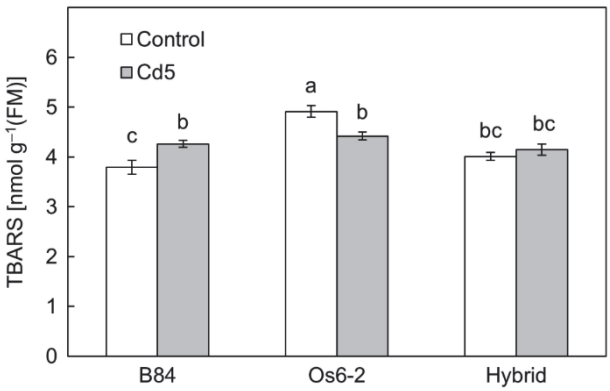

Fig. 5 Effects of elevated cadmium concentration in soil [5 mg(Cd) kg^{-1}(soil)] on thiobarbituric acid-reactive substances (TBARS). Means (± SE, n = 9) with *the same letters* are not significantly different (*Tukey´*s $HSD_{0.05}$).

treatment, B84 line had the highest values of measured pigments (Fig. 3*B–D*). The Cd5 treatment caused an increase in Chl *a* and *b* in B84 line, while in Os6-2, the increase was evident only in Chl *b*, and in hybrid only in Chl *a*. The Car content was the highest in hybrid during the CK treatment. In Cd5 treatment, Car concentration increased in B84 line, while it decreased in Os6-2 and hybrid (Fig. 3*D*). *ANOVA* for Chl *a*, Chl *b*, and Car proved significant effects for main sources of variation and their interactions (Table 1).

Antioxidant enzymes activity, TBARS content and H_2O_2 concentration: *ANOVA* showed significant effects for main sources of variation and their interactions in all measured parameters except for interaction in POD and treatment in TBARS (Table 1). APX activity in control treatment was the highest in hybrid and the lowest in B84 line, while in Cd5 treatment it was the highest in Os6-2 line and the lowest in B84 line. Cd treatment resulted in a decrease of APX activity in B84 line and hybrid, while its activity increased in Os6-2 line (Fig. 4*B*). CAT activity in CK treatment was the highest in B84 line and the lowest one in hybrid. In Cd5 treatment, Os6-2 had the highest values, while B84 had the lowest values. Cd treatment caused decrease in CAT activity in B84 line, while it caused an increase in CAT activity in other two genotypes (Fig. 4*C*). POD activity in CK treatment was the highest in Os6-2 line and the lowest one in hybrid. Hybrid had the

lowest value in Cd5 treatment, while there was no difference between B84 and Os6-2. The Cd5 treatment caused the same increasing POD activity trend for all three genotypes (Fig. 4*D*). TBARS content in CK was the highest in Os6-2 line, while after Cd treatment, no differences were found between three genotypes. In general, Cd treatment caused increase of TBARS content only in B84, while it caused a decrease in TBARS content in Os6-2. In hybrid, there was no difference between treatments (Fig. 5). H_2O_2 concentration in CK treatment was the highest one in hybrid, while it was the highest in Os6-2 line after the Cd5 treatment. B84 line had the lowest value in CK, while hybrid had the lowest value after the Cd5 treatment. The Cd treatment caused and increase in H_2O_2 concentration in both lines (B84, Os6-2), while it caused a decrease in H_2O_2 concentration in hybrid (Fig. 4*A*).

Discussion

Inbred lines used in this study have been shown to differ in their respective ionomic profiles (Brkić *et al.* 2003, Sorić *et al.* 2011, Šimić *et al.* 2012) as well as in leaf Cd accumulation affinity (Sorić *et al.* 2009), B84 being a low, and Os6-2 high Cd accumulator, and their respective hybrid being intermediate (Franić *et al*. 2013). Mo17 was also designated as a high Cd accumulator elsewhere (Zhang *et al.* 2012) and shown similar in accumulation to Os6-2 (Franić *et al.* 2013). Soil Cd content of 5 mg kg^{-1}(soil) was used as the middle value from da Silva *et al*. (2012) which caused visual symptoms of phytotoxicity. If calculated per volume of substrate, the content of applied Cd equals 2 mg L^{-1}. The Cd5 treatment caused a decrease in F_m in all three genotypes. Decrease in F_m (Fig. 2), increase in F_0 (although our results showed increase in F_0, *ANOVA* showed it was not significant between treatments, data not shown) and the accompanying decrease in F_v/F_m have been previously observed in maize cultivars exposed to Cd (Ekmekçi *et al.* 2008). Increase of F_0 with increased Cd content in soil can be attributed to a reduction in energy transfer from antennae to the reaction center (Ralph and Burchett 1998) and the decrease of F_m with increased Cd content in soil indicates changes in ultrastructure of thylakoid membranes that affects electron transport rate (Ekmekçi *et al.* 2008). The t_{max} was reduced in Cd5 treatment in all three genotypes. The t_{max} is used to indicate the time at which F_m is reached, and the reduction in t_{max} indicates stress. Since it is accepted that F_m expresses the state of PSII at which all Q_A are reduced (Mallick and Mohn 2003), the decrease in t_{max} suggests that the pool of Q_A available for reduction decreased. Increase in V_J parameter in B84 and hybrid suggests the decrease in efficiency of Q_A^- reoxidation (accumulation of reduced Q_A^-), since V_J is a measure of the fraction of reduced Q_A^- (Strasser *et al.* 2000, Havaux and Strasser 1992, Kalaji *et al.* 2014). S_m is proportional to the number of electrons that pass through the electron transport chain (Stirbet and Govindjee 2011). Decrease in S_m in B84 and Os6-2 lines suggests that maximum fluorescence could be reached quicker because fewer electrons are needed to reduce PSII electron acceptors which can be also seen through a decrease of t_{max} parameter. Initial slope of the relative variable fluorescence (M_o) corresponds to relative rate of Q_A reduction; it increased in B84 and hybrid (and was reduced in Os6-2). It has been shown previously that M_o is increased in stressed plants (Christen *et al.* 2007). Negative effects of Cd5 treatment were evident in the decrease of quantum yields of PSII electron transport and the efficiency of excitation energy capture by open PSII reaction centers (TR_o/ABS, ET_o/ABS, and ET_o/TR_o). Decreases in acceptor side-dependent yields (ET_o/ABS, ET_o/TR_o), which describe the efficiency of electron transport, suggested photoinhibitory damage to PSII caused by excess Cd which has been observed in earlier studies (Pagliano *et al.* 2006). Decrease in TR_o/ABS caused by heavy metal exposure has been reported previously (Jiang *et al.* 2008, Turnau *et al.* 2008) and has been attributed to photoinhibition caused by excess of heavy metals. Increase in ABS/RC, which was observed in B84 and hybrid in Cd5 treatment, suggests that a fraction of active reaction centers was inactivated or that the apparent antenna size increased (Lichtenthaler *et al.* 1982). These changes were confirmed by decreases in RC/CS_o and RC/ABS. Inactivation of reaction centers can be an indication of susceptibility to photoinhibition, and the inactivation is a downregulation mechanism of dealing with excess of absorbed light. Increase in trapping per RC (TR_o/RC) can indicate impairment of the oxygen evolving complex (Kalaji *et al.* 2014) and was observed under Cd treatment in the same genotypes. Most notable deviation from the control values was observed in the energy dissipation (DI_o/RC). Energy dissipation is enhanced to protect leaves from photo-oxidative damage; increased dissipation suggests that absorbed energy was dissipated instead of utilized to reduce Q_A^-. Dissipation of excess absorbed light by heat has been previously shown in heavy metal treated plants, including Cd (Zhou *et al.* 2005, Begović *et al.* 2016). PI_{ABS} is calculated as (RC/ABS) $(TR_o/ABS - TR_o)(ET_o/TR_o - ET_o)$. Higher PI_{ABS} (or plant "vitality") under Cd5 treatment in Os6-2 genotype occurred due to the increase in all three parameters but the highest impact on PI_{ABS} was from $ET_o/(TR_o - ET_o)$ suggesting Cd treatment might have caused an increase in CO_2 assimilation in Os6-2 line since a relationship between photosynthetic electron transport and CO_2 assimilation has been established (Krall *et al.* 1992), although the increase of electron transport can be also related to other biochemical pathways (Kalaji *et al.* 2016). Higher PI_{ABS} also suggests better overall photosynthetic performance. Decrease in PI_{ABS} at Cd5 treatment in B84 and hybrid was caused by a decrease of all three components of PI_{ABS}, but it can be seen that lower PI_{ABS}

value in B84 is the result of lower RC/ABS value suggesting that a larger proportion of reaction centers was inactivated in B84 line than in hybrid. Decrease in PI_{ABS} has been previously shown to be negatively affected by heavy metals (Begović *et al.* 2016, Żurek *et al.* 2014). Although PI_{ABS} and most of other fluorescence parameters did not show Cd-induced stress in Os6-2 inbred, some other parameters revealed stress, such as decrease in t_{max} and S_m which suggested that Os6-2 is more tolerant to Cd-induced stress at this stage probably as a result of higher resistance to Cd (or due to lower Cd uptake) as proposed by Kalaji *et al.* (2007, 2010).

The Chl *a* content increase was followed by increase in some corresponding fluorescence parameters, such as F_0, F_{150}, and F_{300}, but stress in B84 line was revealed through malfunction of photosystem components detected by increase in dissipation of energy per RC, as well as with the decrease in TR_o/DI_o (Strasser 2000). Elevated contents of Chl *a* in B84 after the Cd5 treatment might be a result of stress adaptation, reaction of young leaves to Cd stress (Drążkiewicz *et al*. 2003, Chaneva *et al.* 2010) or Cd content in substrate was simply too low to induce stress for all the physiological traits (Chaneva *et al*. 2010). Same authors (Chaneva *et al.* 2010) obtained comparable results in young maize plants also for Chl *a*/*b* ratio which decreased in both inbreds, as well as for F_v/F_m and F_v/F_0 values (results not shown) which were not affected in hybrid. Similar results for Chl contents were shown in dill plants (Aghaz and Bandehagh 2013). Hydrogen peroxide contents increased in both inbreds after the Cd5 treatment, while the treatment caused its decline in hybrid, although hybrid had significantly the highest concentration of peroxide in CK which is in accordance with results of De Gara *et al*. (2000) and is probably related to heterosis effect. We obtained comparable results in CK for APX and CAT activities as well. High production of ROS is related to Cd accumulation and since Cd cannot take a part in Fenton-type reactions, its effects on peroxide-generating mechanisms are yet to be elucidated (Rodríguez-Serrano *et al*. 2009). Effect of the Cd5 treatment on hydrogen peroxide enhancement was observed in both inbreds, although it was more pronounced in Os6-2, which was designated as an accumulator, while peroxide contents in hybrid were lowered by the treatment. Similar results were shown by Anjum *et al*. (2015) in young maize plants of two cultivars differing in Cd uptake. As hydrogen peroxide causes lipid peroxidation in maize leaves (Procházková *et al*. 2001), CAT (Ci *et al*. 2009) and APX (Anjum *et al*. 2015) activities are related to its accelerated production and cultivars showing their lower activities can be considered Cd-sensitive. Contents of TBARS, a direct product of lipid peroxidation, were significantly elevated in B84 line, which is in agreement with lower APX and CAT activities alleviated by the Cd5 treatment, as these two enzymes are most important plant cellular mechanism for hydrogen peroxide detoxification (Asada 1992, Cakmak *et al*. 1993). Decrease in TBARS in Os6-2 inbred could be attributed to positive effect of the Cd5 treatment on all measured antioxidant enzyme activities. Elevated activities of enzymes probably lowered the deleterious effects of peroxide for lipids and proteins in Os6-2. There were no observed differences for TBARS in hybrid, although activity of APX was lowered by the Cd5 treatment. CAT and POD activities were slightly elevated but we suggest that Cd dose was too low to induce stress in hybrid because its high vigor due to heterosis caused a dilution effect (Chien and Menon 1996). Elevated POD activities caused by the Cd5 treatment in all genotypes generally agreed with results of Ekmekçi *et al*. (2008). Car are a group of pigments that act as nonenzymatic agent of ROS detoxification (Krinsky 1989). In our study, the increase of Car content was observed after the Cd5 treatment in B84 inbred line, while there was a significant decrease detected in Os6-2 and hybrid. Chaneva *et al*. (2010) obtained similar results with ascending trend for three Cd concentrations in young maize plants, similarly as B84 in our research, while Chaudhary and Sharma (2009) showed that the Car response to Cd was concentration-dependent.

According to examined parameters, inbred B84 was shown to be Cd sensitive at this particular growth stage, while Os 6-2 did not appear to be negatively affected by Cd treatment. In B84 line, the Cd treatment negatively affected most of measured parameters indicating problems during all stages of photosynthesis and overall oxidative status impairment. Although there was no stress detected in Os6-2 through decreasing antioxidant enzyme activity, declines in Chl *a* fluorescence parameters such as t_{max} and S_m indicated stress. Probably due to a dilution effect, there was no or very weak Cd stress detectable through antioxidant enzyme activities or hydrogen peroxide content in hybrid. Although, stress was detected through Chl *a* fluorescence, where the response of hybrid was similar to B84 but of lesser intensity. In this study, chlorophyll *a* fluorescence was shown to be more sensitive method for Cd-stress detection than biochemical parameters examined and our results showed these methods as complementary to each other.

References

Aebi H.: Catalase *in vitro*. – Methods Enzymol. **105**: 121-126, 1984.

Aghaz M., Bandehagh A.: Phytotoxic effects of cadmium on photosynthesis pigments in dill (*Anethum graveolens*). – Int. J. Farm. Alli. Sci. **2**: 544-548, 2013.

Anjum S.A., Tanveer M., Hussain S. *et al.*: Cadmium toxicity in maize (*Zea mays* L.): consequences on antioxidative systems, reactive oxygen species and cadmium accumulation. – Environ. Sci. Pollut. R. **22**: 17022-17030, 2015.

Asada K.: Ascorbate peroxidase – a hydrogen peroxide-scaven-

ging enzyme in plants. – Physiol. Plantarum **85**: 235-241, 1992.
Begović L., Mlilnarić S., Antunović Dunić J. *et al*.: Response of *Lemna minor* L. to short-term cobalt exposure: The effect on photosynthetic electron transport chain and induction of oxidative damage. – Aquat. Toxicol. **175**: 117-126, 2016.
Brkić I., Šimić D., Zdunić Z. *et al.*: Combining abilities of corn-belt inbred lines of maize for mineral content in grain. – Maydica **48**: 293-297, 2003.
Burzyński M., Żurek A.: Effects of copper and cadmium on photosynthesis in cucumber cotyledons. – Photosynthetica **45**: 239-244, 2007.
Cakmak I., Štrbac D., Marchner H.: Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. – J. Exp. Bot. **44**: 127-132, 1993.
Chaneva G., Parvanova P., Tzvetkova N., Uzunova A.: Photosynthetic response of maize plants against cadmium and paraquat impact. – Water Air Soil Pollut. **208**: 287-293, 2010.
Chaoui A., Mazhoudi S., Habib Ghorbal M., El Ferjani E.: Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). – Plant Sci. **127**: 139-147, 1997.
Chaudhary S., Sharma Y.K.: Interactive studies of potassium and copper with cadmium on seed germination and early seedling growth in maize (*Zea mays* L.). – J. Environ. Biol. **30**: 427-432, 2009.
Chien S.H., Menon R.G.: Dilution effect of plant biomass on plant cadmium concentration ad induced by application of phosphate fertilizers. – In: Rodriguez-Barrueco C. (ed.): Fertilizers and Environment. – Development in Plant and Soil Sciences. Pp. 437-442. Kluwer Academic Publishers, Dordrecht 1996.
Cho U.H., Seo N.H.: Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. – Plant Sci. **168**: 113-120, 2005.
Christen D., Schönmann S., Jermini M. *et al*.: Characterization and early detection of grapevine (*Vitis vinifera*) stress responses to esca disease by in situ chlorophyll fluorescence and comparison with drought stress. – Environ. Exp. Bot. **60**: 504-514, 2007.
Ci D., Jiang D., Dai T. *et al*.: Effects of cadmium on plant growth and physiological traits in contrast wheat recombinant inbred lines differing in cadmium tolerance. – Chemosphere **77**: 1620-1625, 2009.
Da Silva A.J., Nascimento C.W.A., Gouveia-Neto A.S., da Silva-Jr E.A.: LED induced chlorophyll fluorescence spectral analysis for the early detection and monitoring of cadmium toxicity in maize plants. – Water Air Soil Pollut. **223**: 3527-3533, 2012.
Das P., Samantaray S., Rout R.: Studies on cadmium toxicity in plants: a review. – Environ. Pollut. **98**: 29-36, 1998.
De Gara L., Paciolla C., De Tullio M. *et al.*: Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: Evidence of an improved detoxification mechanism against reactive oxygen species. – Physiol. Plantarum **109**: 7-13, 2000.
Di Cagno R., Guidi L., Stefani A., Soldatini G.F.: Effects of cadmium on growth of *Heliantus annus* seedlings: physiological aspects. – New Phytol. **144**: 65-71, 1999.
Di Toppi L.S., Gabbrielli R.: Response to cadmium in higher plants. – Environ. Exp. Bot. **41**: 105-130, 1999.
Drążkiewicz M., Tukendorf A., Baszyński T.: Age dependent response of maize leaf segments to cadmium treatment: Effect on chlorophyll fluorescence and phytochelatin accumulation. – J. Plant Physiol. **160**: 247-254, 2003.
Ekmekçi Y., Tanyolaç D., Ayhan B.: Effects of cadmium on antioxidant enzyme and photosynthetic activities in leaves of two maize cultivars. – J. Plant Physiol. **165**: 600-611, 2008
Florijn P.J., van Beusichem M.L.: Uptake and distribution of cadmium in maize inbred lines. – Plant Soil **150**: 25-32, 1993.
Franić M., Sorić R., Lončarić Z. *et al.*: Genotype variations in maize on cadmium contaminated soil. – In: Jug I., Đurđević B. (ed.): Proceedings of 6th Conference Agriculture in Nature and Environment Protection. Pp. 113-117. Glas Slavonije d.d., Osijek 2013 .
Gallego S.M., Pena L.B., Barcia R.A. *et al.*: Unraveling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. – Environ. Exp. Bot. **83**: 33-46, 2012.
Grant C.A., Buckley W.T., Bailey L.D., Selles F.: Cadmium accumulation in crops. – Can. J. Plant Sci. **78**: 1-17, 1998.
Havaux M., Strasser R.J.: Dynamics of electron transfer within and between PS II reaction center complexes indicated by the light-saturation curve of *in vivo* variable chlorophyll fluorescence emission. – Photosynth. Res. **31**: 149-156, 1992.
Jiang H.-X., Chen L.-S., Zheng J.-G. *et al*.: Aluminium-induced effects on photosystem II photochemistry in citrus leaves assessed by chlorophyll *a* fluorescence transient. – Tree Physiol. **28**: 1863-1871.
Krall J.P., Edwards G.E.: Relationship between photosystem II activity and CO2 fixation in leaves. – Physiol. Plantarum **86**: 180-187, 1992.
Kalaji H.M.; Loboda T.: Photosystem II of barley seedlings under cadmium and lead stress. – Plant Soil Environ. **53**: 511-516, 2007.
Kalaji H M, Oukarroum A, Alexandrov V *et al*.: Identification of nutrient deficiency in maize and tomato plants by *in vivo* chlorophyll *a* fluorescence measurements. – Plant. Physiol. Bioch. **81**: 16-25, 2014.
Kalaji H.M., Schansker G., Breštič M. *et al*.: Frequently asked questions about chlorophyll fluorescence, the sequel. – Photosynth. Res.: doi: 10.1007/s11120-016-0318-y, 2016.
Krantev A., Yordanova R., Janda T. *et al*.: Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. – J. Plant Physiol. **165**: 920-931, 2008.
Krinsky N.: Antioxidant functions of carotenoids. – Free Radical Bio. Med. **7**: 617-635, 1989.
Küpper H., Küpper F., Spiller M.: Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. – J. Exp. Bot. **47**: 259-266, 1996.
Larsson E.H., Bornman J.F., Asp H.: Influence of UV-B radiation and Cd^{2+} on chlorophyll fluorescence, growth and nutrient content in *Brassica napus*. – J. Exp. Bot. **49**: 1031-1039, 1998
Lee E.A., Tracy W. F.: Modern maize breeding. – In: Bennetzen J., Hake, S. (ed.): Handbook of Maize: Genetics and Genomics. Pp. 141-160. Springer, New York 2009.
Lichtenthaler H.K., Kuhn G., Prenzel U. *et al*.: Adaptation of chloroplast-ultrastructure and of chlorophyll-protein levels to high-light and low-light growth conditions. – Z. Naturforsch. **37**: 464-475, 1982.
Lichtenthaler H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. – Methods Enzymol. **148**: 350-382, 1987.
Mallick N., Mohn F.H.: Use of chlorophyll fluorescence in metal-stress research: a case study with green microalga *Scenedesmus*. – Ecotox. Environ. Safe. **55**: 64-69, 2003.
Nakano Y., Asada K.: Hydrogen peroxide is scavenged by

ascorbate – specific peroxidase in spinach chloroplasts. – Plant Cell. Physiol. **22**: 867-880, 1981.

Pagliano C., Raviolo M., Dalla Vecchia F. *et al.*: Evidence for PSII donor-side damage and photoinhibition induced by cadmium treatment on rice (*Oryza sativa* L.). – J. Photoch. Photobio. B **84**: 70-78, 2006.

Procházková D., Sairam R.K., Srivastava G.C., Singh D.V.: Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. – Plant Sci. **161**: 765-771, 2001.

R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/, 2012.

Ralph P.J., Burchett M.D.: Photosynthetic response of *Halophila ovalis* to heavy metal stress. – Environ. Pollut. **103**: 91-101, 1998.

Rodríguez-Serrano M., Romero-Puertas M.C., Pazmiño D.M. *et al.*: Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. – Plant Physiol. **150**: 229-243, 2009.

Romero-Puertas M.C., Palm, J.M., Gómez M. *et al.*: Cadmium causes the oxidative modification of proteins in pea plants. – Plant Cell Environ. **25**: 677-686, 2002.

Sandalio L.M., Dalurzo H.C., Gómez M. *et al.*: Cadmium-induced changes in the growth and oxidative metabolism of pea plants. – J. Exp. Bot. 52: 2115-2126, 2001.

Schützendübel A., Polle A.: Plant responses to abiotic stresses: heavy metal induced oxidative stress and protection by mycorrhisation. – J. Exp. Bot. **53**: 1351-1365, 2002.

Siegel B.Z., Galston A.W.: The isoperoxidases of *Pisum sativum*. – Plant Physiol. **42**: 221-226, 1967.

Šimić D., Mladenović Drinić S., Zdunić Z. *et al.*: Quantitative trait loci for biofortification in maize grain. – J. Hered. **103**: 47-54, 2012

Sorić R., Ledenčan T., Zdunić Z. *et al.*: Quantitative trait loci for metal accumulation in maize leaf. – Maydica **56**: 323-329, 2011.

Sorić R., Lončarić Z., Kovačević V. *et al.*: A major gene for leaf cadmium accumulation in maize (*Zea mays* L.). – In: The Proceedings of the International Plant Nutrition Colloquium XVI. http://escholarship. org/uc/item/1q48v6cf. UC Davis, 2009.

STAR, version 2.0.1. Biometrics and Breeding Informatics, PBGB Division, International Rice Research Institute. Los Baños, Laguna 2014.

Stirbet A, Govindjee: On the relation between the Kautsky effect (chlorophyll *a* fluorescence induction) and photosystem II: Basics and applications of the OJIP fluorescence transient. – J. Photoch. Photobio. B. **104**: 236-257, 2011.

Strasser R.J., Srivastava A., Govindjee: Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. – Photochem. Photobiol. **61**: 32-42, 1995.

Strasser R.J., Srivastava A., Tsimilli-Michael M.: Analysis of chlorophyll *a* fluorescence transient. – In: Papageorgiou G.C., Govindjee (ed.): Advances in Photosynthesis and Respiration. Pp. 321-362. Springer, Dodrecht 2004.

Strasser R.J., Srivastava A., Tsimilli-Michael M.: The fluorescent transient as a tool to characterize and screen photosynthetic samples. – In: Yunus M., Pathre, U., Mohanty P. (ed.): Probing Photosynthesis: Mechanisms, Regulation and Adaptation. Pp. 445-483. Taylor and Francis, London 2000.

Strasser R.J., Tsimilli-Michael M., Qiang S., Goltsev V.: Simultaneous *in vivo* recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. – Biochim. Biophys. Acta **1797**: 1313-1326, 2010.

Tuba Z., Saxena D.K., Srivastava K., Kalaji M.H.: Chlorophyll *a* fluorescence measurements for validating the tolerant bryophytes for heavy metal (Pb) biomapping. – Curr. Sci. **98**: 1505-1508, 2010.

Turnau K., Anielska T., Ryszka P. *et al.* Establishment of arbuscular mycorrhizal plants originating from xerothermic grasslands on heavy metal rich industrial wastes – new solution for waste revegetation. – Plant Soil **305**: 267-280, 2008.

Velikova V., Yordanov I., Edreva A.: Oxidative stress and some antioxidant systems in acid-rain treated bean plants. Protective role of exogenous polyamines. – Plant Sci. **151**: 59-66, 2000

Verbruggen N., Hermans C., Schat H.: Mechanisms to cope with arsenic or cadmium excess in plants. – Curr. Opin. Plant Biol. **12**: 364-372, 2009.

Verma S., Dubey R.S.: Leads toxicity induces lipid peroxidation and alters the activities of antioxidant enzxmes in growing rice plants. – Plant Sci. **164**: 645-655, 2003.

Weigel H.J.: Inhibition of photosynthetic reactions of isolated intact chloroplasts by cadmium. – J. Plant Physiol. **119**: 179-189, 1985.

Zhang Z., Jin F., Wang C.: Differences between Pb and Cd accumulation in 19 elite maize inbred lines and application prospects. – J. Biomed. Biotechnol. **2012**: 271485, 2012.

Zhou W., Qiu B.: Effects of cadmium hyperaccumulation on physiological characteristics of *Sedum alfredii* Hance (*Crassulaceae*). – Plant Sci. **169**: 737-745, 2005.

Żurek G., Rybka K., Pogrzeba M. *et al.*: Chlorophyll *a* fluorescence in evaluation of the effect of heavy metal soil contamination on perennial grasses. – PLOS ONE 9: e91475, 2014.