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Seed priming with calcium chloride improves the photosynthesis performance of faba bean plants subjected to cadmium stress

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Abstract

Faba bean (*Vicia faba* L.) seeds were treated with H_2O [nonprimed (NP)] or 2% $CaCl_2$ [primed (P)] before germination for 6 h. After seven days, seedlings were exposed to 0 or 50 µM $CdCl_2$ concentrations for three weeks. Under Cd treatment, P plants showed an improvement of gas-exchange characteristics, chlorophyll (Chl) and carotenoids contents as compared to NP plants subjected to Cd stress. Additionally, the values of Chl fluorescence were relatively similar to those of control, implying that no photodamage occurred. Moreover, under 50 µM Cd, the P plants exhibited lesser accumulation of hydrogen peroxide and superoxide radicals in leaves as compared to NP plants. Likewise, results showed that $CaCl_2$-seed pretreatment alleviated adverse effects of Cd on electrolyte leakage. In conclusion, $CaCl_2$ improved photosynthesis attributes of faba bean plants subjected to Cd stress by mitigating the adverse effects of Cd toxicity through a reduced generation of reactive oxygen species.

Additional key words: nonphotochemical quenching; photosynthetic apparatus; seed priming; water-use efficiency.

Introduction

Cadmium pollution is increasing day by day due to industrialization and anthropogenic activities, such as traffic, smelting, and mining (Karadaş and Kara 2011). Similarly, excessive use of phosphate fertilizers is a major cause of the increase in Cd concentrations in soils (Qadir *et al.* 2000). The main symptoms of Cd-induced toxicity in plants are stunted growth, yellowing of leaves, damage to chloroplast ultrastructure, and ultimately plant death (Daud *et al.* 2009). Photosynthetic processes are directly affected by Cd ions, which disturb the carbon utilization and respiration mechanisms (Prasad 1995, Sanità di Toppi *et al.* 2003). Cd toxicity retards the photosynthetic rate by disturbing plant water balance, stomatal conductance, CO_2 availability (Shi *et al.* 2010, Agami and Mohamed 2013), chloroplast organization (Miller *et al.* 2008, Najeeb *et al.* 2011), membrane structure integrity, and photosynthetic apparatus (Ekmekçi *et al.* 2008, Shi *et al.* 2010). This is due to the generation of reactive oxygen species (ROS), which deteriorate the physiological functions in plants (Dixit *et al.* 2001, Schützendübel *et al.* 2001). Many studies showed that Cd can increase the production of ROS, such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Prasad 1995, Schützendübel *et al.* 2001, Zhang *et al.* 2009). These ROS are able to react with lipids, proteins, and pigments, resulting in membrane damage and enzyme inactivation (Agami and Mohamed 2013).

Various physiological practices have been applied to alleviate the adverse effects of biotic and abiotic stress on normal plant functioning. Seed priming is one of the most effective and cost-efficient methods for seed quality improvement and stress tolerance in plants (Paparella *et al.* 2015). This technique is based on controlled seed hydration that induces a particular physiological state in plants (initial steps of germination *sensu stricto*). This process allows the application of natural and synthetic compounds into the seeds before their germination. Selection of some exogenous chemical priming such as calcium ions can regulate plant metabolism and improve plant resistance (Jiang *et al.* 2005, Delian *et al.* 2014). Calcium (Ca^{2+}) is involved in the regulation mechanisms that plants activate to adjust the adverse environmental conditions such as heavy metals (Antosiewicz and Hennig 2004, Siddiqui *et al.* 2011). Several authors have reported the alleviation of cadmium toxicity by Ca^{2+} in many plants, such as beans

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Abbreviations: Car – carotenoids; Chl – chlorophyll; C_i – internal CO_2 concentration; E – transpiration rate; EL – electrolyte leakage; ETR – photosynthetic electron transport rate; F_0 – minimal chlorophyll fluorescence; FM – fresh mass; F_m – maximal chlorophyll fluorescence; F_m' – maximal fluorescence yield of the light-adapted state; F_s – steady-state fluorescence yield; F_v – variable chlorophyll fluorescence; F_v/F_m – maximum photochemical quantum efficiency of PSII; g_s − stomatal conductance; NPQ – nonphotochemical quenching; P_N – net CO_2 assimilation rate; ROS – reactive oxygen species; SE – standard error; WUE – intrinsic water-use efficiency (P_N/g_s); Φ_{PSII} – actual PSII efficiency.

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(Ismail 2008), radish (Siddiqui *et al.* 2013), and cabbage (Chen *et al.* 2002).

Moreover, in a previous study, we found that seed priming with $CaCl_2$ was effective in reducing the adverse effects of Cd on *V. faba* seedlings (Nouairi *et al.* 2012). Calcium appears to play a central role in many defense mechanisms that are induced by environmental stresses (Delian *et al*. 2014, Hironari and Takashi 2014), and Ca^{2+} signalling is required for the acquisition of plant tolerance (Delian *et al*. 2014).

The objective of the present study was to assess ameliorative roles of $CaCl_2$-seed priming against the cadmium toxicity in faba bean plants based on the measurement of the photosynthetic parameters, ROS accumulation, and the electrolyte leakage (EL) in leaves.

Materials and methods

Plant growth and treatment: Seeds of faba bean (*Vicia faba* L. cv. Saber 02) were surface-sterilized for 3 to 4 min with 0.1% $HgCl_2$ and washed thrice with deionized water and then divided into two parts. For priming, one part was soaked in 2% $CaCl_2$ solution for 6 h (P), the other part of a nonprimed (NP) seeds was soaked in H_2O. Sterilized seeds of both parts (P and NP) were germinated in 15 cm in diameter plastic pots filled with acid-washed and sterilized sand used as a growth support and supplied with half strength Hoagland solution (Hoagland and Arnon 1938). After 7 d from sowing, the medium was changed to full-strength Hoagland solution (containing 0 or 50 µM Cd). Pots were saturated daily with 300 mL of solution. The seedlings were grown for 3 weeks (21 d) in a greenhouse at a day/night cycle of 12/12 h, at 23/18°C, respectively; relative humidity was approximately 70%.

Gas exchange and Chl fluorescence: After three weeks of Cd treatment (0 or 50 µM Cd), gas-exchange attributes, such as net CO_2 assimilation rate (P_N), stomatal conductance (g_s), intracellular CO_2 concentration (C_i), transpiration rate (E), and water-use efficiency [WUE, the ratio between carbon gain in photosynthesis (P_N) and water loss in transpiration (E)] were determined on the youngest and fully expanded top leaves by using an open type and portable photosynthesis system (*LCA-4*, *Bio-Scientific*, Great Amwell, Herts, UK) under the following conditions: saturating light intensity of about 1,350 µmol(photon) m^{-2} s^{-1}, CO_2 concentration of 380 µmol mol^{-1}, leaf temperature of 27 ± 2°C, and relative humidity was 65 ± 5%.

Chl fluorescence was monitored using a modulated Chl fluorimeter (*OS1-FL*, *Opti-Sciences*, Tyngsboro, MA, USA). Leaves previously selected for the measurement of photosynthetic gas exchange were used for Chl fluorescence measurements following the procedure described by Genty *et al.* (1989). After a dark-adaptation period of 30 min, the minimal Chl fluorescence (F_0) was determined by a weak red light pulse (6 s). Maximum fluorescence of dark-adapted state (F_m) was measured during a subsequent saturating pulse of white light [8,000 µmol(photon) m^{-2} s^{-1} for 0.8 s].

The maximal photochemical efficiency of PSII (F_v/F_m) was expressed as: $F_v/F_m = (F_m - F_0)/F_m$. The relative quantum yield of PSII at steady state was calculated as $\Phi_{PSII} = (F_m' - F_s)/F_m'$, where F_s and F_m' are fluorescence at steady state and maximum fluorescence in the light, respectively. The nonphotochemical quenching coefficient (NPQ), describing regulated dissipation of excess energy, was estimated as $(F_m - F_m')/F_m'$ (Bilger *et al.* 2001). The linear electron transport rate (ETR) was calculated using the equation: $ETR = PAR \times 0.5 \times \Phi_{PSII} \times 0.84$, where PAR is the photosynthetic active radiation, 0.5 is distribution of energy between the two photosystems, and 0.84 is a fraction of light energy absorbed by a leaf (Genty *et al.* 1989). All Chl fluorescence measurements were taken from 11:00 to 13:00 h. The mean of the six measurements was calculated and used for each treatment.

Chl determination: Leaf Chl *a*, *b*, and carotenoid (Car) concentrations of the NP or P faba bean plants were determined three weeks after cadmium application by the method of Lichtenthaler and Wellburn (1983). Fresh leaf tissues (0.3 g) from fully expanded healthy leaves were ground and extracted with 10 mL of 80% (v/v) acetone. The suspension was centrifuged at 4°C for 5 min at 5,000 × *g* and absorbance was measured at 470, 646, and 663 nm, using a UV-visible spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd.*, UK). The pigment content [expressed as mg g^{-1}(FM)] was calculated by using the following equations:

$\text{Chl } a = 13.95 \times A_{665} - 6.88 \times A_{649}$
$\text{Chl } b = 24.96 \times A_{649} - 7.32 \times A_{665}$
$\text{Car} = (1{,}000 \times A_{470} - 2.05 \times \text{Chl } a - 114.8 \times \text{Chl } b)/245$

Electrolyte leakage (EL) determination: The total inorganic ions leaked out in the leaves were estimated by the method of Dionisio-Sese and Tobita (1998). Twenty leaf discs were taken in a boiling tube containing 10 mL of deionized water and electrical conductivity (EC_a) was measured (*Conductivity Model Consort K912*, *CONSORT-PARKLAAN*, Belgium). The contents were heated at 45°C and 55°C for 30 min in a water bath and electrical conductivity (EC_b) was measured. Later, the content was again boiled at 100°C for 10 min and electrical conductivity (EC_c) was again recorded. The EL was calculated by using the formula: $EL\ [\%] = (EC_b - EC_a)/(EC_c - EC_a) \times 100$.

Histochemical detection of H_2O_2 and $O_2^{\bullet-}$: *In situ* accumulations of superoxide radical and hydrogen peroxide were examined with histochemical staining protocols. Location of $O_2^{\bullet-}$ in faba bean leaf discs was determined using the nitroblue tetrazolium (NBT) reduction test described by Dutilleul *et al.* (2003). Leaf discs were sampled and immediately vacuum-infiltrated in 0.5 mg(NBT) mL^{-1} and 10 mM potassium phosphate buffer at pH 7.8. After being incubated in dark at room temperature for 1 h, samples were cleared in 90% ethanol at 70°C to remove Chl. $O_2^{\bullet-}$ was visualized as a blue color at the site of NBT precipitation. The stained discs were transferred into Petri dishes, analysed, and photographed under a binocular microscope (*Leica* 56E, *Leica Microsystems*, Wetzlar, Germany) coupled with a digital camera. Histochemical changes in H_2O_2 were studied by using starch-potassium iodide (KI) method (Olson and Varner 1993). Leaf discs were incubated on a solid medium (4% starch, 0.1 M KI, 1.0 M glucose, and 0.8% agarose) for 4 h at room temperature for color development. To quantify the stained areas, digital images of leaf discs were converted into grayscale images and the percentage of stained spot areas to the total leaf disc areas were determined with *Image J* software (*Image J, ver. 1.51p*, Wayne Rasband, National Institutes of Health, USA).

Statistical analysis: All data are presented as means ± SD of at least five replicate seedlings. One-way analysis of variance (*ANOVA*) was performed in *SPSS* program *version 20.0* (*SPSS*, Chicago, USA). Comparisons between the mean values were accomplished by the least significant difference (*LSD*) test at the level of $p \leq 0.05$. All graphs were made using *SigmaPlot version 11.0* (*Systat*).

Results

Chlorophylls and gas-exchange attributes: In NP plants, results showed that the P_N, E, g_s, C_i, and WUE values significantly decreased under 50 µM Cd treatment by 64, 42, 69, 55, and 37%, respectively, as compared with controls (Fig. 1). In contrast, $CaCl_2$ seed priming improved the photosynthetic parameters under Cd stress conditions and increased P_N, E, g_s, C_i, and WUE by 88, 48, 95, 68, and 27%, respectively, compared to Cd-stressed plants alone (Fig. 1). Thus, these results revealed that $CaCl_2$ seed priming could improve photosynthetic activity and alleviate Cd-induced photosynthetic changes in faba bean plants.

The growth retardation of faba bean plants (Fig. 2) by the Cd application (50 µM), was found to be associated with a significant decrease in Chl *a* and Chl *b* contents (by 53 and 59%, respectively, as compared with the Cd-free control plants) (Fig. 3). However, $CaCl_2$ pretreatment (P) significantly alleviated this Cd-induced inhibition; Chl *a*, Chl *b*, and Car increased by 30, 67, and 73%, respectively, compared with a Cd-stressed plants (NP with 50 µM Cd). Moreover, results indicated that $CaCl_2$-pretreated plants (P) exhibited significant increases in Chl *a*, Chl *b*, and Car values (by 17, 4, and 54%, respectively) compared with the Cd-free control (NP at 0 µM Cd) (Fig. 3).

Chl fluorescence: Under Cd treatment (50 µM), Chl fluorescence parameters (F_v/F_m, Φ_{PSII}, and ETR) declined by 14, 7, and 23%, respectively, as compared with control

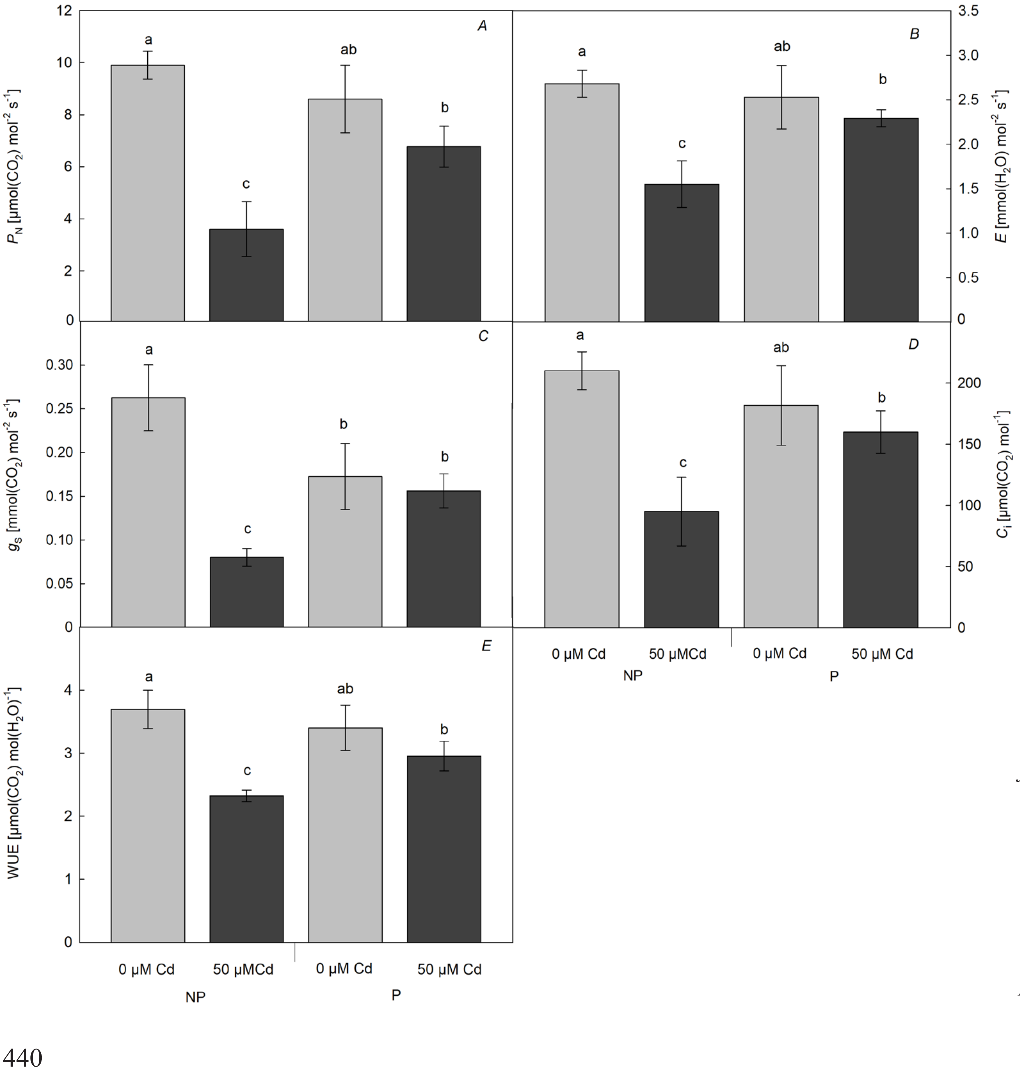

Fig. 1. Cadmium effect on net photosynthetic rate, P_N (*A*), transpiration rate, E (*B*), stomatal conductance, g_s (*C*), intracellular CO_2 content, C_i (*D*), and water-use efficiency, WUE (*E*) in *Vicia faba* plants pretreated with 2% $CaCl_2$ (P) or H_2O (NP) for 6 h and growing for 21 days under 0 or 50 µM Cd. Values are the means of five replications ± SD. The data followed by *different letters* are significantly different at $p \leq 0.05$.

Fig. 2. *Vicia faba* L. plants pretreated with 2% $CaCl_2$ (P) or with H_2O (NP) for 6 h, and growing for 21 days under 0 or 50 µM Cd.

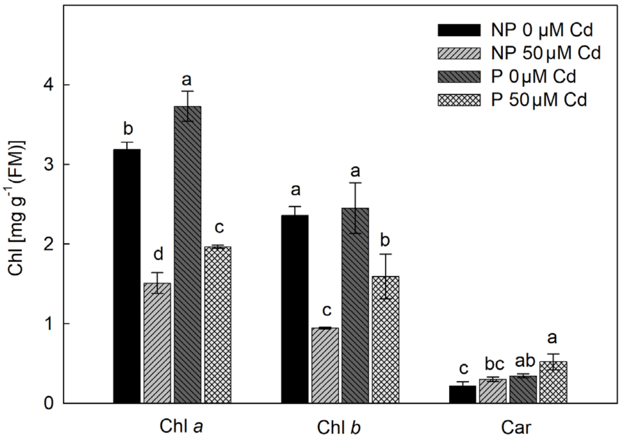

Fig. 3. Chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) in leaves of *Vicia faba* plants pretreated with 2% $CaCl_2$ (P) or H_2O (NP) for 6 h and growing for 21 days under 0 or 50 µM Cd. Values are the means of 12 replications ± SD. The data followed by *different letters* are significantly different at $p \leq 0.05$.

(Fig. 4*A*,*B*,*D*). The value of NPQ under Cd treatment exhibited an increase of 20% in comparison with control (Fig. 4*C*). Furthermore, in Cd-treated faba bean plants, $CaCl_2$ pretreatment showed significant improvement in the values of F_v/F_m, Φ_{PSII}, and ETR compared to Cd-stressed plants alone. In contrast to this finding, the value of NPQ declined significantly in $CaCl_2$-pretreated plants subjected to Cd stress compared to Cd-stressed plants alone (Fig. 4*C*).

H_2O_2 and $O_2^{\bullet-}$ production: Endogenous $O_2^{\bullet-}$ in leaf tissues was stained with NBT to form a dark blue insoluble formazan compound. As expected, the generation of $O_2^{\bullet-}$ increased in leaves considerably in response to the Cd treatment. However, $CaCl_2$ seed pretreatment reduced $O_2^{\bullet-}$ generation in leaves of Cd-treated plants (Fig. 5*A*). To verify *in situ* the accumulation of H_2O_2 in faba bean leaves, a histochemical method with starch-KI, based on the formation of local brown spots by H_2O_2 in leaves, was used (Fig. 5*B*). Under 50 µM $CdCl_2$ treatment, H_2O_2 accumulation was high in leaves of faba bean seedlings. However, $CaCl_2$ seed pretreatment decreased intensity of brown deposit in leaves of Cd-treated seedlings indicating lower concentration of H_2O_2 (Fig. 5*B*).

EL: Membrane stability decreased significantly with Cd stress (50 µM) in control plants (NP). An increase (by 61%) in the EL under 50 µM Cd treatment was observed (Fig. 6). However, in Cd-treated faba bean plants, $CaCl_2$ pretreatment showed significant improvement of the values of EL compared with a Cd-stressed plants.

Discussion

The reported research was undertaken to improve our understanding of physiological processes determining heavy metal tolerance and the induction of such processes by $CaCl_2$ seed priming for the alleviation of Cd-induced decreases in photosynthesis. In the present study, plant growth and photosynthetic parameters decreased under Cd stress (Figs. 1,2). Cadmium-induced inhibition in plant growth has already been reported in many plant species, such as wheat (Rizwan *et al.* 2012), rice (Cao *et al.* 2013), maize (Vaculík *et al.* 2015), *Brassica napus* (Nouairi *et al.* 2006), tomato (Ammar *et al.* 2015), and bean plants (Saidi *et al.* 2013). It has been shown that photosynthesis was closely related to plant growth and biomass production. Therefore, the decrease in plant growth might be due to Cd-induced toxicity on photosynthetic apparatus (Bashir *et al.* 2015, Moradi and Ehsanzadeh 2015) and/or structural alterations in plants (Nouairi *et al.* 2006, Belkhadi *et al.* 2010). Decrease in plant growth and biomass might also be due to oxidative damage and reduction in antioxidant enzymes activities (Ahmad *et al.* 2009) and/or reduction in mineral nutrients uptake by plants (Ben Ghnaya *et al.* 2009). It might be assumed that this decrease in plant growth could be due to the reduced cell expansion (Daud *et al.* 2013), decrease of Calvin-cycle enzymes, inhibition of the photosynthetic electron transport chain, and might also be due to Cd-induced inhibition in gas-exchange characteristics and Chl contents (Ali *et al.* 2014, Per *et al.* 2016).

In the present investigation, it was found that $CaCl_2$ seed priming markedly alleviated Cd-induced reduction in growth and photosynthetic parameters in faba bean plants (Figs. 1,2). Indeed, under Cd stress, results indicated that $CaCl_2$ pretreatment caused significant increases in g_s, C_i, and E values. Thus, these results suggested that $CaCl_2$ could alleviate the stomata closure caused by Cd and promote the photosynthesis by ensuring the transport of abundant CO_2 to the chloroplast. Moreover, the Cd-induced Chl content reduction was significantly reversed when faba bean seeds were treated with 2% $CaCl_2$ (Fig. 3). This indicated that Ca^{2+} pretreatment mediated improvement

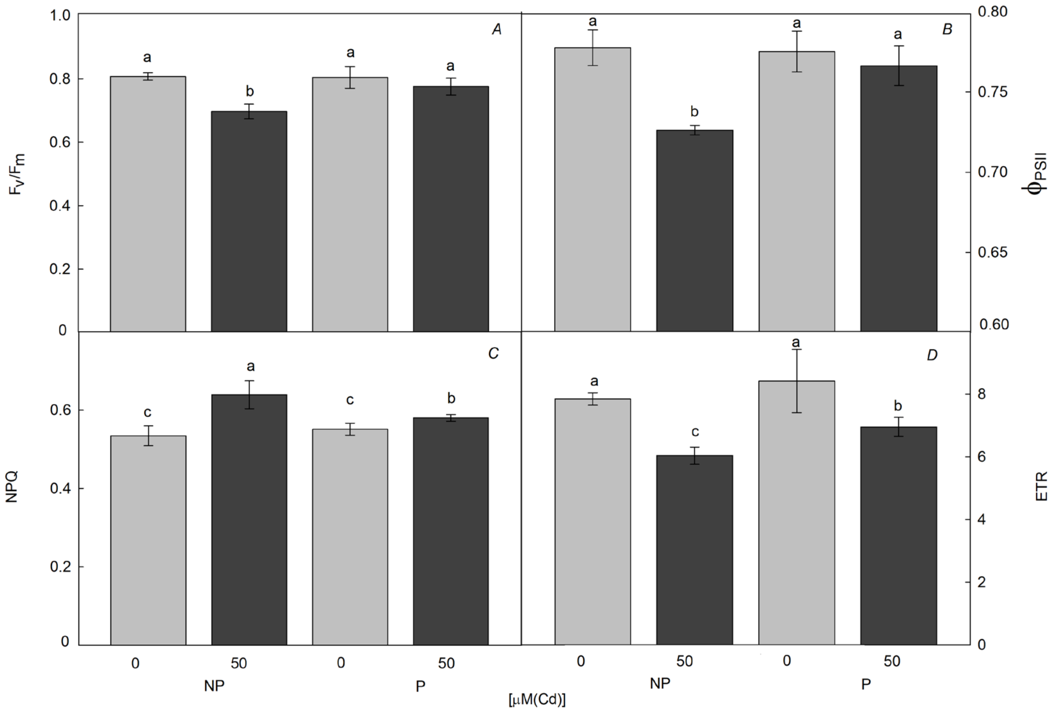

Fig. 4. Cadmium effect on the maximum quantum yield of PSII photochemistry, F_v/F_m (*A*), quantum efficiency of PSII photochemistry, Φ_{PSII} (*B*), nonphotochemical quenching, NPQ (*C*), and the relative PSII electron transport rate, ETR (*D*) in *Vicia faba* plants pretreated with 2% $CaCl_2$ (P) or H_2O (NP) for 6 h and growing for 21 days under 0 or 50 µM Cd. Values are the means of 6 replications ± SD. The data followed by *different letters* are significantly different at $p \leq 0.05$.

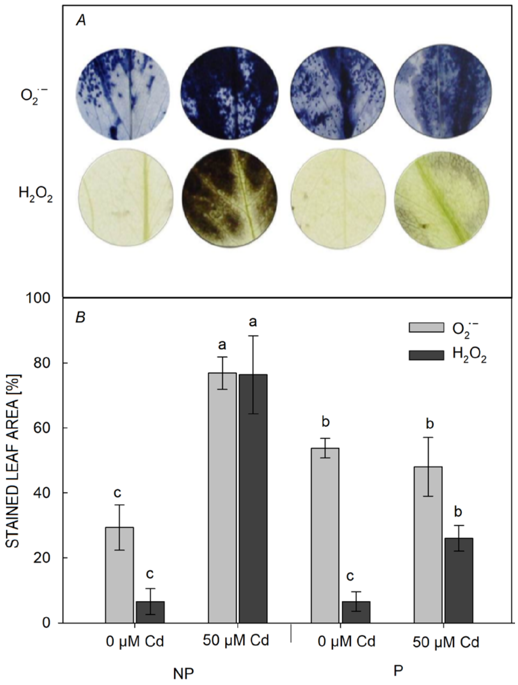

Fig. 5. Histochemical detection of $O_2^{\cdot-}$ and H_2O_2 (*A*) and the percentage of the stained leaf area (*B*) in leaves of *Vicia faba* plants pretreated with 2% $CaCl_2$ (P) or with H_2O (NP) for 6 h and growing for 21 days under 0 or 50 µM Cd. Values are the means of 6 replications ± SD. The data followed by *different letters* are significantly different at $p \leq 0.05$.

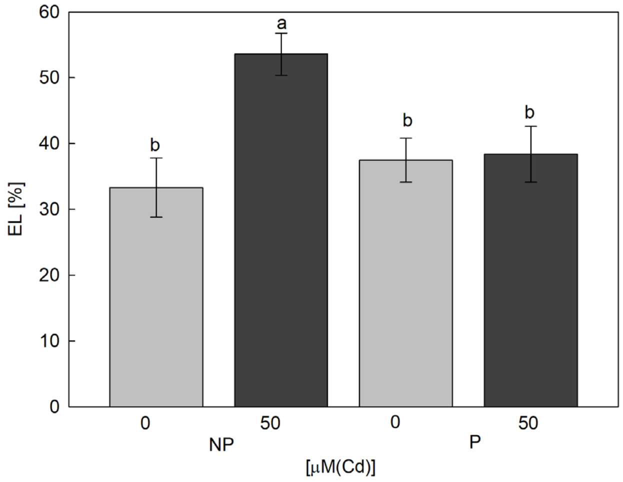

Fig. 6. Electrolyte leakage (EL) in leaves of *Vicia faba* plants pretreated with 2% $CaCl_2$ (P) or with H_2O (NP) for 6 h and growing for 21 days under 0 or 50 µM Cd. Values are the means of 10 replications ± SD. The data followed by *different letters* are significantly different at $p \leq 0.05$.

in photosynthesis, partly due to increasing Chl synthesis. Moreover, it has been shown that Ca^{2+} served as secondary messenger for cytokinin action in improving synthesis of Chl (Lechowski and Bialczyk 1993).

Indeed, it has been reported by many researchers that exogenous application of Ca^{2+} enhanced the plant tolerance to heavy metals (Farzadfar *et al.* 2013, Lwalaba *et al*. 2017) and other stresses (Jiang *et al.* 2001, Xu *et al.* 2013, Methenni *et al.* 2018). Ca^{2+}-mediated biomass enhancement might occur due to an increase in the uptake of nutrients by plants under stressful conditions (Khan *et al.* 2012, Siddiqui *et al.* 2012). Calcium may maintain the photosynthetic capacity of plants by increasing stomatal conductance and maintaining Rubisco activity and chloroplast ultrastructure under abiotic stress (Liang *et al.* 2009, Tan *et al*. 2011, Xu *et al*. 2017). The Chl fluorescence technique has been proven to be a sensitive method for the detection and quantification of changes induced in the photosynthetic apparatus. This analysis permits detection, monitoring, and evaluation of abiotic stresses upon healthy plants (da Silva *et al.* 2012). The F_v/F_m and photochemical quenching coefficient (q_P) are frequently used to measure the maximum photochemical efficiency of PSII and proportion of oxidized (open) reaction centres PSII, respectively, while F_v/F_0 (activity of PSII) and F_m/F_0 (electron transport through PSII) are also used to explore the photosynthetic efficiency of plants in changing environmental conditions (Xing *et al.* 2010). Studies demonstrate that stress factors generally affect functional activity of PSII and thus decrease these ratios (Xing *et al.* 2010). Under Cd stress, the decline in F_v/F_m, Φ_{PSII}, and ETR (Fig. 4) indicated structural and functional alterations in photosynthetic process as evidenced by decreased growth of faba bean plants (Fig. 2). In addition, the NPQ value significantly increased under Cd stress (50 μM), indicating that an antenna pigment could not effectively transform light energy into chemical energy and it was, therefore, released as heat (Zou *et al.* 2015).

On the other hand, $CaCl_2$ seed priming significantly alleviated Cd-induced damaging effects on photosynthesis (Fig. 4), which could be correlated with improved photosynthetic efficiency of faba bean plants. Moreover, under Cd stress, in $CaCl_2$-pretreated plants, the NPQ value remarkably decreased, suggesting that $CaCl_2$ improved the light-utilisation efficiency.

Another protective mechanism of $CaCl_2$ seed pretreatment in alleviating Cd toxicity was *via* eliminating free radical-induced damage caused by Cd. Previous studies have shown that Cd toxicity resulted in the accumulation of ROS and oxidation of lipid membranes (Chaoui *et al.* 1997, Nouairi *et al.* 2006). In the current study, Cd-stressed faba bean plants had the increased $O_2^{\bullet-}$ and H_2O_2 accumulation and EL (Figs. 5,6). However, $CaCl_2$ seed pretreatment significantly lowered the ROS overproduction and decreased EL in leaves of Cd-stressed plants. The Ca-induced inhibition of EL and ROS contents indicates that $CaCl_2$ seed priming could significantly alleviate the harmful effects of Cd stress in faba bean plants. Moreover, our results suggest that calcium alleviated the adverse effect of the oxidative stress by reducing the content of $O_2^{\bullet-}$ and H_2O_2 possibly through stimulation of ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and enhancement of nonprotein thiol (–SH) contents in leaves of faba bean Cd-stressed plants (Nouairi *et al*. 2012). In addition, Ca^{2+} is one of many cellular network parameters orchestrating complex cellular signalling coordinating responses to numerous developmental cues and environmental challenges. It has been shown that Ca^{2+} had the function of preventing cell membrane injury and leakage as well as stabilizing cell membranes under adverse environmental conditions (Guimarães *et al.* 2011, Nouairi *et al.* 2012). Indeed, it has been demonstrated that a high concentration of Ca^{2+} around plasma membrane reduces cell-surface negativity and harmfulness of cationic toxicants (Kinraide 1998) or the uptake of Cd *via* calcium channels to mimic Ca (Suzuki 2005). Thus, seed priming with $CaCl_2$ seems to play a fundamental role in the establishment of a basal resistance to environmental stresses.

Conclusion: Taken together, the overall results from this research suggest that Cd significantly inhibited the growth traits as well as the photosynthetic parameters in faba bean seedlings. It is suggested that Cd acts as the main limiting factors for photosynthesis *via* Chl loss, changes in the photosynthetic apparatus, and damage of PSII reaction centre. However, seed priming with $CaCl_2$ significantly alleviated Cd-induced inhibition of faba bean growth, chlorophyll contents, gas-exchange attributes, and photosynthetic efficiency. $CaCl_2$ pretreatment markedly reduced Cd-induced ROS accumulation and EL, and might be an important technique that enables the plants to tolerate abiotic stress.

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