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RESEARCH ARTICLE

Calcium invigorates the cadmium-stressed *Brassica napus* L. plants by strengthening their photosynthetic system

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Abstract
Introduction Cadmium (Cd) in plants interrupts numerous metabolic processes and reduces the water and nutrient uptake that cause chlorosis, growth retardation, and ultimately plant death. Response of *Brassica napus* L. to calcium (Ca) enrichment in growth medium for reducing Cd toxicity stress by strengthening the photosynthesis organelles and their functionality was explored in this study.
Materials and methods B. napus seedlings of two cultivars (ZS 758 and ZS 72) were exposed to Cd toxicity at 500 μM in hydroponics, and it was ameliorated with Ca at 2.0 mM. The study included determinations and evaluations pertaining to physiological attributes of plant growth, chlorophyll, and photosynthesis.
Results and discussion Cadmium stress significantly depressed the seedling growth and reduced photosynthetic rate (Pn), stomatal conductivity (Gs), and transpiration rate (Tr). Further, Cd toxicity markedly decreased the electron transport rate of PSII, effective quantum yield of photochemical energy conversion in PSII [Y(II)], photosynthetic active radiation, coefficient of photochemical quenching (qP), and chlorophyll fluorescence decrease ratio (R_{Fd}). Addition of Ca in Cd-stressed plants antagonized the toxicity effects on all the above-mentioned attributes. Calcium amendment also reversed the Cd stress-induced increase in intercellular CO_2 concentration (Ci) and non-photochemical quenching, and countered the Cd accumulation in seedlings.
Conclusion This study suggests that Ca^{2+} in the proximity of plasma membrane is proficient in alleviating Cd toxicity by reducing the cell-surface negativity and competing for Cd^{2+} ion influx. Consequently, both the plant growth and activity of diurnal photosynthetic system remain the least altered under Cd-provoked toxicity stress.

Keywords Rapeseed · Cadmium stress · Photosynthetic gas exchange · Chlorophyll fluorescence · Tolerance

Abbreviations

Ca	Calcium
Cd	Cadmium
Ci	Intercellular CO_2 concentration
ETR	Electron transport rate of PSII
Gs	Stomatal conductivity
NPQ	Non-photochemical quenching
PAR	Photosynthetic active radiation
Pn	Photosynthetic rate
PSII	Photosystem II
qP	Coefficient of photochemical quenching
R_{Fd}	Chlorophyll fluorescence decrease ratio
Tr	Transpiration rate
Y(II)	Effective quantum yield of photochemical energy conversion in PSII

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1 Introduction

Escalating industrialization has resulted into the deposition of excessive amounts of metals, e.g., Pb, Zn, Cu, Mn, and Cd, into soils and water (Wong 2003) becoming a serious threat to environmental process. Cadmium (Cd) is among the extremely toxic heavy metals to plants and animals due to its high water solubility and neurotoxic, mutagenic, and carcinogenic effects (Sanita and Gabbrielli 1999). Enhanced Cd pollution in agricultural lands is mostly through human interventions, e.g., use of phosphatic fertilizers, sewage sludge, untreated wastewater and pesticides (Qadir et al. 2000), mining and smelting, and traffic (Karadaş and Kara 2010). Cadmium interferes different metabolic processes and causes diminished water and nutrient uptake in plants (Najeeb et al. 2011), resulting in chlorosis, growth inhibition, browning of root tips, ultra-structural damage, and ultimately plant death (Daud et al. 2009). Reduced plant biomass, chlorophyll fluorescence, and photosynthesis attributes under Cd stress (Küpper et al. 2007) are related to effective index for evaluating Cd toxicity in plants (Sun and Shen 2007).

Calcium (Ca) is an essential nutrient that participates in many biological response systems of plant (Zhou et al. 1999). It counters for uptake of Cd in plants (Ismai 2008) and alleviates metal toxicity stress by restoring plant metabolism and chlorophyll (Zhang et al. 1998). Plasma membrane is the first target of heavy metal toxicity in plants (Hall 2002), and its destruction alters normal Ca signal transferring system, which serves as a stress indicator (Lynch 1989). Therefore, application of moderate amount of exogenous Ca can alleviate plants from environmental stresses by enhancing plasma membrane stability and restoring calcium signal transferring system (Cramer 1985). There have been reports about the role of exogenous Ca application for alleviating Cd toxicity stress in beans (Ismai 2008), tobacco (Zheng 2005), and cabbage (Chen et al. 2002).

Oilseed rape (*Brassica napus* L.) is grown throughout the world as a major oilseed crop for edible oil production. *Brassica* species are generally regarded as heavy metal tolerant due to their fast growth, higher biomass production, and ability to absorb heavy metals (Momoh and Zhou 2001; Meng et al. 2009; Vangronsveld et al. 2009). In heavy metal contaminated environments, *Brassica* plants employ several strategies to avoid or tolerate metal toxicity through specific physiological mechanisms (Papazoglou et al. 2005). Photosynthesis improves the conditions for active absorption of metals by providing required energy and oxygen to the plants. In many plant species, Cd toxicity inhibited photosynthesis by disturbing plant water balance, stomatal conductance, and CO_2 availability (Vrettos et al. 2001), or chloroplast organization (Najeeb et al. 2011) and chlorophyll biosynthesis (Küpper et al. 2007).

In the present research, we probed into the toxicity impact of Cd stress on growth and photosynthetic attributes of two *B. napus* L. cultivars. We also investigated the role of Ca in protecting photosynthetic system in rapeseed seedlings. These findings would contribute in understanding the mechanisms for Ca-induced increase in heavy metal tolerance of *Brassica* plants.

2 Materials and methods

2.1 Plant material and treatments

Seeds of two leading commercial cultivars of oilseed rape (*B. napus* L. cv. ZS 758 and ZS 72) were provided by the College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China. Initially, we grew the seedlings in plastic pots (170×220 mm) filled with peat soil in the glasshouse at Zhejiang University. Then, five-leaf stage seedlings were transferred to the pots containing half strength Hoagland's nutrient solution. After 1 week acclimatization period, these plants were shifted to full strength Hoagland's nutrient solution containing $CdCl_2$ (500 μM Cd) with or without Ca $(NO_3)_2$ (2.0 mM Ca) as amendment. Control plants were also grown in the nutrient solution containing no Cd and Ca ions to compare the seedling growth and physiological response against treated ones. The treatment concentrations were based on pre-experimental studies, in which several lower and higher levels of two metals used, i.e., 100, 200, 300, 400, 500, and 1,000 μM of Cd and 1, 2, 3, 4, and 5 mM of Ca. The Cd concentrations lower than 500 μM had no or negligible effects, while those higher were too toxic for plant growth. It was similar in the case of Ca application, where plants exhibited optimum response under treatment of 2 mM concentration.

The experiment was performed in a completely randomized design with six replications. The pH of nutrient solution was maintained at 6.0 throughout the study period for all the treatments. We collected morphologically uniform seedlings at the seventh day to record data on biomass, and from the middle region of the topmost fully expanded leaf, various physiological parameters were measured, e.g., chlorophyll contents, gas exchange parameters, and chlorophyll fluorescence. This experiment was performed to estimate short duration effects of heavy metal stress on plant growth and physiological traits. Since heavy metal stress (Cd) changes the parameters related to energy fluxes within PSII in a very short time, they can be used as indicators for monitoring of Cd stress at early stages of plant development (Kalaji and Loboda 2007).

2.2 Determination of chlorophyll contents

Chlorophyll was eluted from two leaf discs of the topmost fully expanded leaf by submerging discs in 2 mL of mixture

of absolute alcohol and acetone (*v*:*v*=5:5) in the dark for 72 h. Absorbance of extract solutions was read at λ=647 nm and λ=664 nm with UV–Vis spectrophotometer (UV-2450, serial No. A10834133413 CS, Shimadzu Corporation, Japan). Leaf chlorophyll contents were determined using the equations of Welburn and Lichtenthaler (1984).

2.3 Photosynthetic gas exchange

Photosynthetic gas exchange parameters were measured using LI-6400 portable photosynthesis system (Li-Cor, Inc., USA). Photosynthetic rate (Pn), stomatal conductivity (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were measured from the middle region of the topmost fully expanded leaf at 25°C under a light intensity of 1,000 μmol m^{-2} s^{-1}, relative humidity of 45%, and CO_2 concentration of 350 μmol mol^{-1} during 8:00 to 11:00 a.m.

2.4 Chlorophyll fluorescence

Chlorophyll fluorescence parameters were determined by the method described by Lichtenthaler et al. (2005) using portable fluorometer (Monitoring-PAM, Walz, Germany). The topmost fully expanded leaves of treated and control plants were first light- and dark-adapted for 20 min to obtain F and F_0 (minimum fluorescence yield of light-adapted and dark-adapted leaves, respectively). The Fm′ and Fm values (maximum fluorescence yield of light- and dark-adapted leaves, respectively) were calculated with a saturation pulse, and then the maximum photosystem II quantum yield was calculated by the formula [$(Fm-F_0)/Fm=Fv/Fm$]. The effective quantum yield of PSII, $Y(II)=(Fm'-F)/Fm'$, was determined according to Genty et al. (1989). Electron transport rate ($ETR=Y(II)\times PAR\times 0.5\times 0.84$) was recorded automatically by monitoring PAM (Walz), with the coefficient 0.5 as a factor assuming an equal distribution of absorbed photons between PSII and PSI, and the homogeneous absorption factor (0.84; Björkman and Demmig 1987). Non-photochemical quenching coefficient value was calculated as $NPQ=(Fm-Fm')/Fm'$, and photochemical quenching coefficient was estimated as $qP=(Fm'-F)/(Fm'-Fo')$. All measurements were taken from six plants of each replication during 8:00 to 11:00 a.m.

2.5 Elemental analysis

Plants were thoroughly washed, and roots were soaked in 20 mM EDTA-Na_2 for 15 min to remove excess of metal ions from root surface. Samples of oilseed rape leaf and root were dried at 70°C for 48 h, and then 0.1 g of the dried samples was digested with 5 mL HNO_3 and 1 mL $HClO_4$ in closed Teflon. The flask was successively shaken at a rate of 200 rpm at 25°C for 24 h. The extracted solution was filtered through a non-ash filter paper. All solutions were prepared with de-ionized water. The Cd and Ca concentrations in plant samples were determined with inductively coupled plasma mass spectrophotometer (Agilent 7500A).

2.6 Statistical analysis

Two-way analysis of variance (ANOVA) was performed for plant growth and physiological parameters. Data were analyzed using the SAS v.9 statistical software, whereas *F* test was employed to find significant difference among means at the 0.05 level of probability (Steel et al. 1997).

3 Results

3.1 Seedling growth

Cadmium stress negatively influenced plant growth of the two studied cultivars (Fig. 1) causing significant reduction in fresh and dry biomass (Fig. 2a–d). The Cd-stressed plants of both the cultivars responded variably towards Ca amendment, viz. cv. ZS 758 was more responsive to Ca amendment, and showed substantial recovery in plant fresh and dry biomass. There was no significant improvement (except for root dry weight) in cv. ZS 72 with Ca addition to the nutrient media. Application of Ca alone to the nutrient medium showed no visible effect on plant growth attributes (fresh and dry biomass) of both *B. napus* cultivars. Figure 1a–d clearly elucidates the response of ZS 758 cultivar seedlings to both Cd and Ca application. Plants treated only with Cd became yellow showing restricted growth, while Ca amendment to the Cd-treated seedling helped them retain their vigor and growth.

3.2 Chlorophyll contents

Effects of Cd and Ca treatments on chlorophyll *a*, *b* and total chlorophyll contents of *B. napus* L. leaves are presented in Fig. 3a–c. The Cd-induced stress appreciably reduced the chlorophyll contents (*a*, *b* and total) in both cultivars. Application of Ca alone had no effect on chlorophyll contents of the two cultivars; however, it significantly improved the chlorophyll contents of Cd-stressed plants.

3.3 Photosynthesis attributes

Cadmium-induced stress drastically decreased the photosynthetic rate (Pn) in the leaves of both tested cultivars

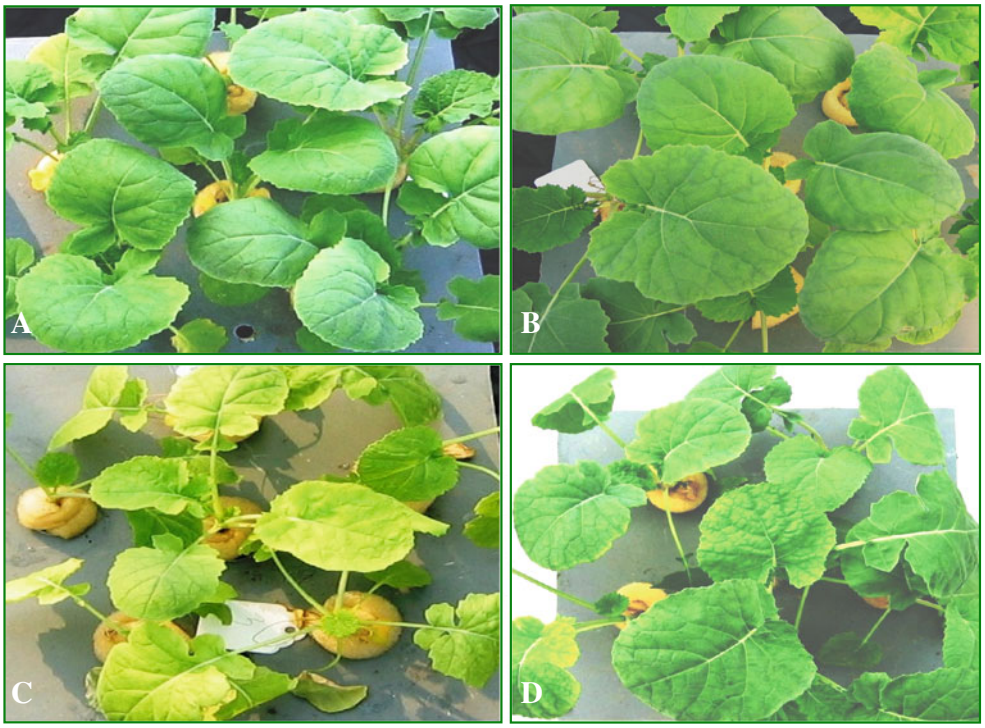

Fig. 1 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) treatments on seedling growth and morphology of *Brassica napus* L. cv. ZS 758. **a** *CK*, Control without Cd or Ca treatment. **b** Plants applied with 2.0 mM $Ca(NO_3)_2$. **c** Plants treated with 500 μM $CdCl_2$. **d** Plants treated with 500 μM $CdCl_2$ and 2.0 mM $Ca(NO_3)_2$

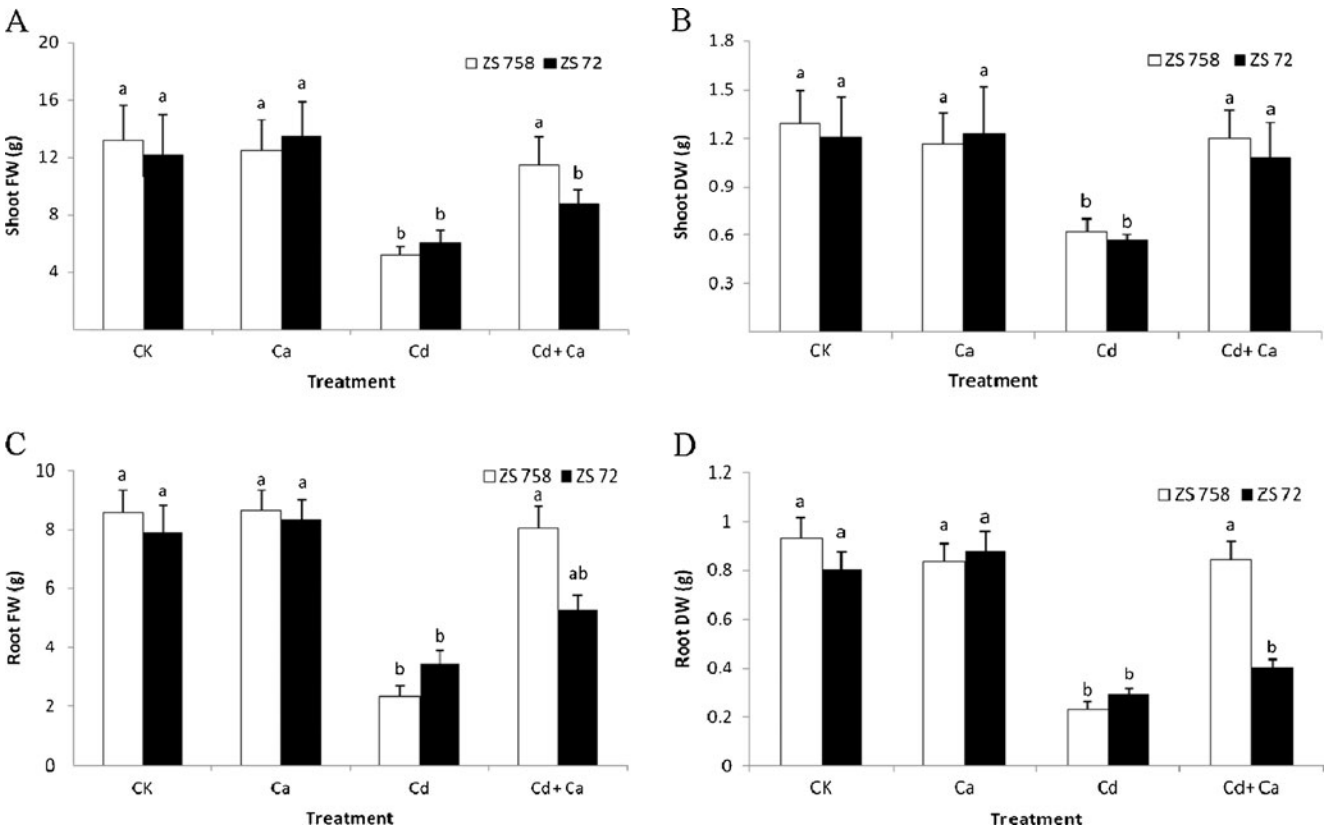

Fig. 2 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) on biomass (gram per seedling) of *Brassica napus* L. seedlings (n=6). **a** Shoot fresh weight. **b** Shoot dry weight. **c** Root fresh weight. **d** Root dry weight. *Different letter(s)* after data for each cultivar represent significant difference at 95% probability. *CK* control

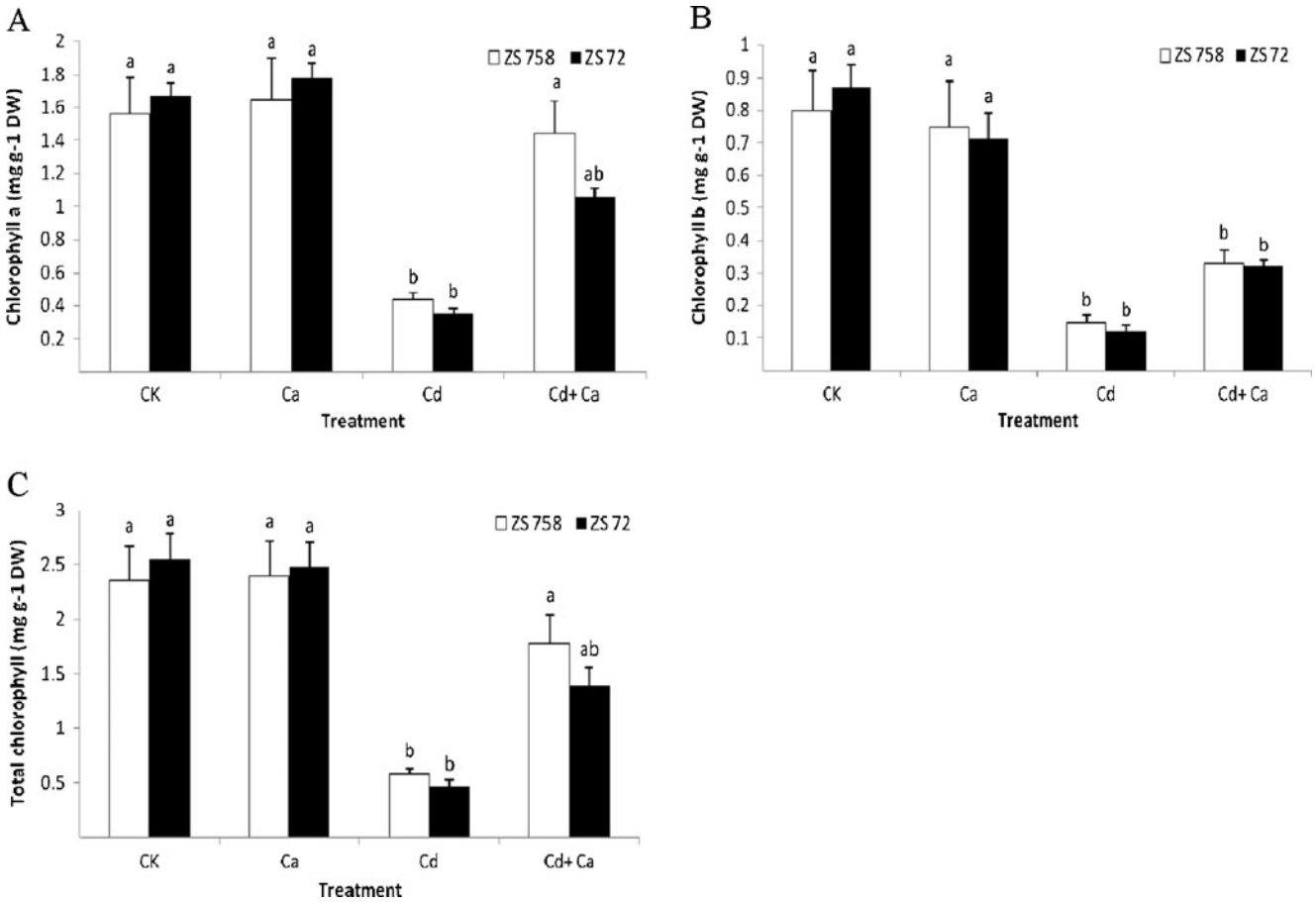

Fig. 3 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) on chlorophyll contents in *Brassica napus* L. leaves (n=6). **a** Chlorophyll *a*. **b** Chlorophyll. **c** Total chlorophyll. *Different letter(s)* after data for each cultivar represent significant difference at 95% probability. *CK* control, *DW* dry weight

(Fig. 4a–d). Although addition of Ca did not alter the Pn level in non-stressed plants, it recovered Cd-stressed plants in cv. ZS 758, showing higher increase in Pn value. Similarly, there was considerable reduction in other photosynthesis parameters like stomatal conductivity (Gs) and transpiration rate (Tr) in both cultivars under Cd stress in both cultivars, which were recovered by Ca application. On the other hand, Cd stress significantly increased intercellular CO_2 concentration (Ci) of the two cultivars.

3.4 Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters, viz. Fv/Fm, Y(II), qP, ETR, and R_{Fd}, were negatively affected under Cd-induced stress in both cultivars (Table 1). Addition of Ca ameliorated Cd toxicity stress in these plants by significantly improving all these parameters. Under non-stress conditions, Ca application variably influenced the chlorophyll fluorescence parameters of the two cultivars. It greatly improved the R_{Fd} level in the leaves of cv. ZS 758, while reduced in cv. ZS 72. Moreover, Ca addition to the media reduced the value of qP and ETR in cv. ZS 72. Contrary to other chlorophyll fluorescence parameters, value of NPQ significantly increased in Cd-stressed plants. Compared to control, Ca amendment alone had no effect on NPQ value under non-stress condition; however, it considerably reduced the NPQ value of Cd-stressed plants.

3.5 Metals content in seedlings

Concentrations of Cd and Ca in shoot and root of the two cultivars cv. ZS 758 and ZS 72 were shown in Table 2. Addition of Cd in solution radically elevated Cd contents in the shoots and roots of plants. Both of the cultivars retained higher Cd contents in roots as compared to aboveground parts. Application of Ca alone slightly lowered Cd contents, while it greatly reduced Cd uptake in Cd-stressed plants of two cultivars. Compared to CK, Ca amendment alone significantly improved Ca uptake in the roots of both cultivars; however, no significant improvement was seen in its translocation to shoots. Drop in Ca uptake was recorded in shoots of cv. ZS 758 and roots of cv. ZS 72 under Cd stress that was compensated by Ca application to Cd-stressed plants.

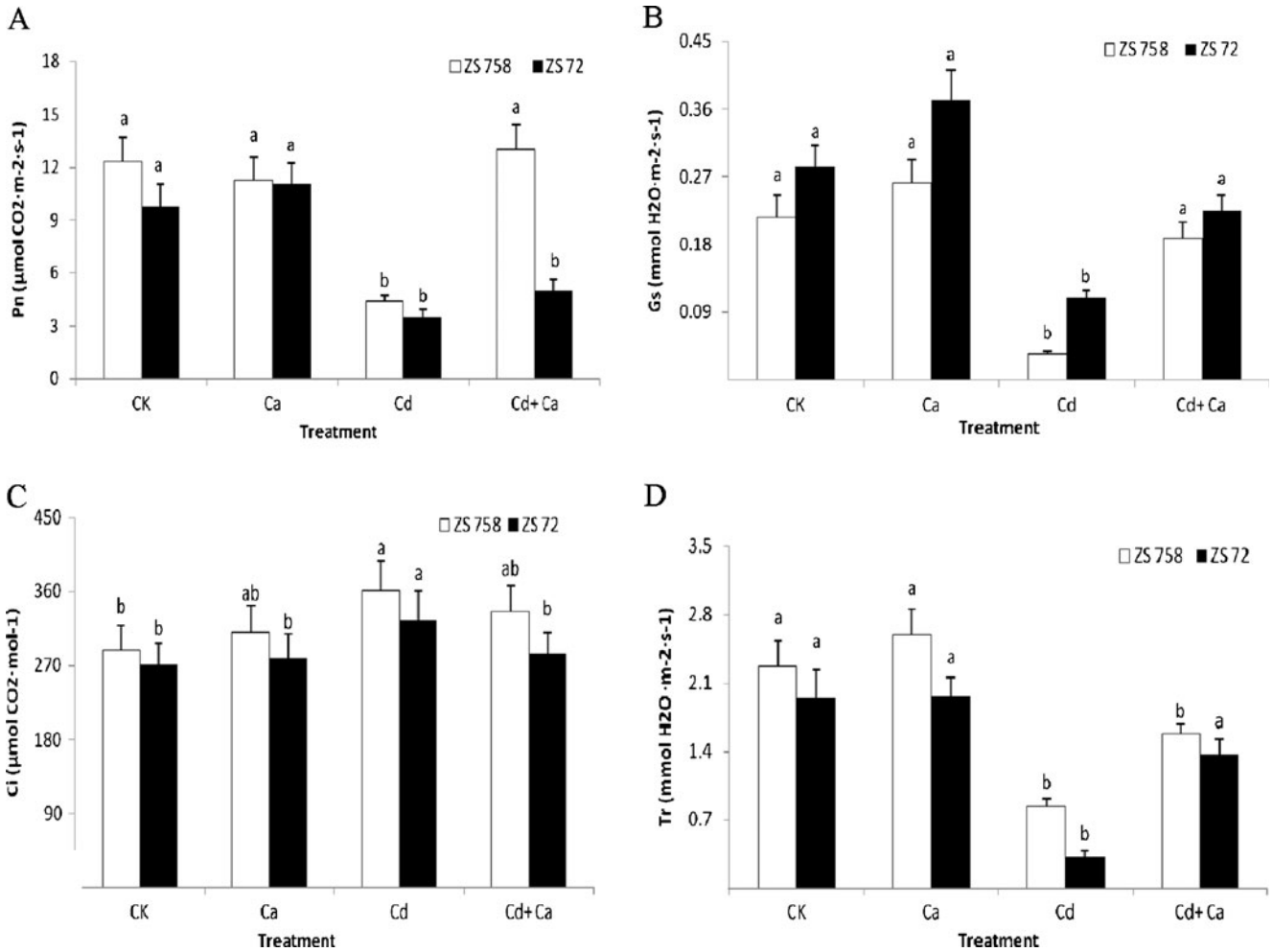

Fig. 4 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) on photosynthesis parameters of *Brassica napus* L. leaves (n=6). **a** Photosynthetic rate. **b** Stomatal conductivity. **c** Intercellular CO_2 concentration. **d** Transpiration rate. *Different letter(s)* after data for each cultivar represent significant difference at 95% probability. *CK* control

4 Discussion

Current studies revealed a significant decline in plant biomass and chlorophyll contents in oilseed rape seedlings under Cd-induced stress. Toxic effects of Cd on plant growth and metabolism were explored by Shamsi et al. (2008) and Daud et al. (2009). The Cd toxicity stress inhibited growth of *B. napus* seedlings that was evident from reduction in plant fresh and dry biomass. Addition of Ca to the growth media restored the growth of Cd-stressed plants. Cadmium stress suppresses plant growth by interfering with various metabolic processes, i.e., inhibition of proton pump (Aidid and Okamoto 1993), reduction of root elongation, and damage to photosynthetic machinery (Najeeb et al. 2011). Rivetta et al

Table 1 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) on chlorophyll fluorescence of *Brassica napus* L. leaves (n=6)

Cultivar	Treatment	Fv/Fm	Y(II)	qP	ETR	NPQ	R_{Fd}
ZS 758	CK	0.84 a[a]	0.81 a	0.54 a	84.3 a	0.51 c	4.97 ab
	Ca	0.82 a	0.83 a	0.57 a	86.2 a	0.51 c	5.21 a
	Cd	0.69 c	0.54 c	0.29 c	44.7 c	0.86 a	2.87 b
	Cd+Ca	0.78 b	0.70 b	0.49 b	66.2 b	0.70 b	4.73 ab
ZS 72	CK	0.85 a[a]	0.81 a	0.60 a	89.8 a	0.47 c	4.52 a
	Ca	0.81 a	0.80 a	0.54 b	82.4 ab	0.47 c	4.01 ab
	Cd	0.74 b	0.63 c	0.31 d	58.1 c	0.75 a	2.24 c
	Cd+Ca	0.79 ab	0.71 b	0.43 c	68.5 b	0.63 b	3.08 b

CK control

[a] Different letter(s) after data within a column for each cultivar represent significant difference at 95% probability

Table 2 Effects of Cd^{2+} (500 μM) and Ca^{2+} (2.0 mM) on Cd and Ca contents of *Brassica napus* L. (n=6)

Cultivar	Treatment	Cd content (μg g^{-1} DW)		Ca content (μg g^{-1} DW)	
		Shoot	Root	Shoot	Root
ZS 758	CK	0.45 c[a]	0.01 c	3.18 a	7.35 b
	Ca	0.33 c	0.00 c	3.52 a	11.04 a
	Cd	572.4 a	6,650.7 a	2.64 b	4.13 c
	Cd+Ca	154.8 b	1,496.5 b	3.16 a	7.52 b
ZS 72	CK	0.38 c[a]	0.12 c	4.53 ab	6.96 b
	Ca	0.35 c	0.06 c	5.62 a	9.87 a
	Cd	561.8 a	6,581.9 a	3.81 b	4.65 c
	Cd+Ca	153.0 b	1,066.3 b	4.27 ab	7.03 b

CK control, *DW* dry weight

[a] Different letter(s) after data within a column for each cultivar represent significant difference at 95% probability

(1997) observed negative effects of Cd on radish growth that were reversed by Ca application.

Photosynthetic and chlorophyll fluorescence parameters are recognized as powerful tools to study physiological responses of plants against metal-induced stress (Küpper and Kroneck 2005) and suggest the direct method of assessing photosynthetic activity (Küpper et al. 2007). In our study, Cd application negatively influenced various photosynthetic parameters like Pn, Gs, Ci, and Tr. Thylakoid membrane leakage under Cd stress (Najeeb et al. 2011) might be responsible for reduced photosynthetic parameters, and it is the first limiting factor for photosynthesis (Schrader et al. 2004). Results suggested that Cd-induced photosynthetic system impairment was rehabilitated by exogenous Ca fortification possibly through countering the uptake of Cd^{2+} (Ismai 2008).

Diminution of Fv/Fm and Y(II) under Cd stress in this study could be the result of functional disorder of antenna complexes that raised F_0 and thereby reduced Fv/Fm and consequently reduced the plant photosynthesis (Balakhnina et al. 2005). Reduced values of Fv/Fm also indicate that Cd-induced stress damaged the PSII reaction center of photosynthetic system. As oxidative or reducing side of PSII is considered the main target of Cd toxicity stress in plants (Küpper et al. 2007), Cd inhibited the PSII (Küpper and Kroneck 2005) by disturbing water balance (Costa and Morel 1994). In addition, disruption of thylakoid membrane system by Cd-induced stress (Najeeb et al. 2011) inhibited PSII functioning. The NPQ is an excellent indicator of concentration of dissipating complexes because it constitutes an important protective response through dissipating excitation energy in light-harvesting antenna systems (Gilmore 1997). Both cultivars showed a significant increase in NPQ under Cd-induced stress, causing reduced light use efficiency of PSII (Haldimann and Feller 2004).

Calcium is required as a cofactor in the formation of catalytic inorganic core ($Mn_4Ca_1O_xCl_y$) of the photosystem II–water-oxidizing complex (PSII-WOC). Presence of Cd^{2+} in the media inhibits photoxidation yield by blocking Ca^{2+} binding with the PSII-WOC (Bartlett et al. 2008). Exogenously applied Ca^{2+} restored active O_2-evolving centers and thus photosynthetic process by replacing Cd^{2+} in the media. Exogenous application of Ca presents a scope for alleviating Cd^{2+} toxicity stress in plants by restoring growth and photosynthetic system (Zhou et al. 1999). As a matter of fact, Ca^{2+} overturned the toxic effects of Cd^{2+} by increasing the NPQ value of excitation energy (Skórzyńska-Polit et al. 1998). Ameliorative effects of exogenous Ca application on growth and chlorophyll fluorescence parameters have also been reported in salt-stressed wheat (Zhang et al. 1998) and cowpea (Murillo-Amador et al. 2006).

Restoration of fluorescence parameters from Cd stress through Ca enforcement relates directly to the plant survival traits; thus, it provides the basis to study the impacts of environmental factors. The Fv/Fm value around 0.8 or higher shows that there is no effect on the potential efficiency of PSII, and (Björkman and Demmig 1987) application of Ca restored the efficiency of PSII of Cd-stressed *Brassica* plants showing a 0.78 value of Fv/Fm. Drążkiewicz and Baszyński (2008) reported that fortification of Ca reduced F_0 caused by 250–1,000 μM Cd; however, it protected the chlorophyll *a* fluorescence (Fv, Fm, Fv/F_0, and Fv/Fm) only up to 250 μM Cd. Our results showed that Ca amendment significantly increased the ETR of both cultivars, which is consistent with the findings in barley plants (Dai et al. 2007).

Cadmium concentrations in shoots and roots of both cultivars were elevated by Cd application. In the present study, *B. napus* cultivars tend to accumulate more Cd in their roots, which shows their capability to avoid metal-induced stress (Najeeb et al. 2011). Increased Cd concentration inside the plant induces the metal toxicity by replacing some of the essential cations such as Ca^{2+} and Fe^{3+} (Van Engelen et al. 2007). In addition, excess of Cd^{2+} in plant cell can inhibit the activities of various organic compounds by making bonds with side groups (McGrath et al. 2002). Compared to Cd-stressed plants, the plants with

Ca amendments significantly lowered the Cd content. This suggests that Ca enrichment decreases Cd accumulation helping the plants to recover from toxicity stress through displacement of cell surface from toxic cations like Cd^{2+} (Suzuki 2005). 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). Therefore, Ca^{2+} is capable of alleviating Cd toxicity by reducing the cell-surface negativity and competing for the metal ion influx.

5 Conclusions

Exogenous Ca fortification in the growth medium alleviated Cd toxicity stress to oilseed rape by plunging the internal Cd accumulation and escalating the plant biomass. Calcium application could be advantageous to the plants under Cd stress, which enhances the photosynthetic activities to a moderate extent. Therefore, chlorophyll fluorescence parameters can be useful bio-indicators for evaluation of the plant capability to surmount the Cd-induced stress.

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