DOI: 10.1007/s11099-016-0205-y PHOTOSYNTHETICA 54 (4): 491-501, 2016

Photosynthetic and growth responses of two mustard cultivars differing in phytocystatin activity under cadmium stress

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Abstract

Cadmium inhibits photosynthetic capacity of plants by disturbing protein conformations, whereas phytocystatins prevent degradation of target proteins and are involved in abiotic stress tolerance. Two mustard (*Brassica juncea* L.) cultivars, Ro Agro 4001 and Amruta, were grown with Cd (50 μM) in order to study physiological and biochemical basis of differences in Cd tolerance. Amruta accumulated higher Cd and H_2O_2 concentrations in leaves than that of Ro Agro 4001. Cd significantly decreased photosynthesis and growth of plants in both cultivars by reducing a chlorophyll content, gas exchange parameters, and activity of Rubisco; the effects were more prominent in Amruta than those in Ro Agro 4001. The greater photosynthesis and growth of Ro Agro 4001 under Cd stress might be attributed to its higher phytocystatin activity together with greater ascorbate peroxidase activity, photosynthetic nitrogen-use efficiency, sulphur assimilation (ATP-sulphurylase activity and S content), and contents of cysteine and reduced glutathione compared to Amruta. In contrast, the activity of superoxide dismutase (SOD) was higher in Amruta than that of Ro Agro 4001 under control conditions, whereas the Cd treatment increased significantly the SOD activity in both cultivars with the greater increase in Ro Agro 4001. The fluorescence spectra of phytocystatin showed a lesser change in Ro Agro 4001 under Cd stress than that in Amruta suggesting higher resistance of Ro Agro 4001 to Cd. The higher phytocystatin activity under Cd stress in Ro Agro 4001 compared to Amruta enabled the plants to protect their proteins more efficiently. This resulted in a greater increase of photosynthetic capacity in Ro Agro 4001 than that of Amruta. Thus, the phytocystatin activity may be considered as a physiological parameter for augmenting photosynthesis and growth of mustard under Cd stress.

Additional key words: chlorophyll fluorescence; fluorescence spectra; leaf area; phytocystatin; plant dry mass.

Introduction

Cadmium is a nonessential heavy metal (HM) and one of the key environmental pollutants producing toxicity to plants, and is widely distributed in natural and agricultural environments (Khan *et al*. 2015). It induces oxidative stress and inhibits photosynthetic and growth responses of plants even at low concentrations (Mobin and Khan 2007). The accumulation of Cd in plants results in reduction of photosynthesis by impairment of chlorophyll (Chl) synthesis, and the inhibition of efficiency of PSII and activity of Rubisco (Burzyński and Kłobus 2004, Masood *et al*. 2012, Li *et al*. 2015). In addition, Cd toxicity negatively influences photosynthesis by decreasing stomatal conductance (Asgher *et al*. 2014) and stomatal frequency (Barceló *et al*. 1988), which results in lowering of CO_2 uptake and intercellular CO_2 concentration for assimilation.

Plants adopt different strategies for protection against the adverse effects of excessive reactive oxygen species (ROS) generated under Cd contamination to maintain homeostasis. These adaptive mechanisms include the induction of enzymatic and nonenzymatic antioxidants (Choppala *et al*. 2014, Ahmad *et al*. 2015, Jozefczak *et al*.

Received 15 October 2015, *accepted* 20 January 2016, *published as online-first* 13 February 2016.
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Abbreviations: *ANOVA* – analysis of variance; APX – ascorbate peroxidase; ATPS – ATP-sulphurylase; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DM – dry mass; ETR – electron transport rate; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; F_v'/F_m' – intrinsic efficiency of PSII; g_s – stomatal conductance; GSH – reduced glutathione; LSD – least significant difference; q_N – nonphotochemical quenching; q_p – photochemical quenching; P_N – net photosynthesis; PDM – plant dry mass; PhyCys – phytocystatin; PNUE – photosynthetic nitrogen-use efficiency; PVP – polyvinyl pyrrolidone; ROS – reactive oxygen species; SOD – superoxide dismutase; TPI – proteinase inhibitor; Φ_{PSII} – effective quantum yield of PSII photochemistry.
Acknowledgements: Financial assistance by the Department of Biotechnology (DBT), Government of India, New Delhi under the DBT-BUILDER programe (No. BT/PR4872/INF/22/150/2012) is gratefully acknowledged.

2015) and increase in sulphur assimilation. S plays a significant role in mitigation of Cd-induced toxicity through its involvement in an antioxidant system (Bashir *et al*. 2015). The supplementation of S to Cd-stressed plants increases production of thiol compounds through increased S-assimilation pathway that helps in the alleviation of Cd stress and protection of photosynthesis and growth (Asgher *et al*. 2014). The availability of S and N impacts carboxylation efficiency and stomatal movement to influence photosynthesis of plants (Iqbal *et al*. 2011). The reduction in photosynthetic nitrogen-use efficiency (PNUE) has been found to reduce photosynthesis (Iqbal *et al*. 2012, Khan and Khan 2014). These nutrients also influence reduced glutathione (GSH) production.

A large complex group of proteins known as thiol proteinase inhibitors (TPIs) regulate proteolytic activity of their target proteinases (Leung *et al*. 2000). Phytocystatins (PhyCys) are plant TPIs that function by preventing the catalysis of papain-like cysteine proteases (Kunert *et al*. 2015). They form a subfamily within the cystatin superfamily (Martínez *et al*. 2012). These have been isolated from various monocot and dicot plants. Papain-like cysteine proteases are involved in many physiological processes in vascular plants (Martínez and Díaz 2008). PhyCys plays a major role in the intracellular protein turnover during seed development and germination (Arai *et al*. 2002) and during programmed cell death (Belenghi *et al*. 2003). They are also involved in responses to stress factors, such as salt, drought, oxidation, and cold in rice (*Oryza sativa*) and *Arabidopsis thaliana* (Huang *et al*. 2007, Zhang *et al*. 2008). Study of Pernas *et al*. (2000) has shown that plantlets of chestnut (*Castanea sativa*) induced PhyCys gene *CsC* when subjected to cold and saline shocks in roots and leaves after heat stress. Similarly, the expression of *AtCYS1* in *Arabidopsis* was induced under conditions of cold, drought, heat, and wounding stress, while *AtCYS2* was not induced by cold stress (Hwang *et al*. 2010). The study on transgenic plants expressing PhyCys also consolidates the role of PhyCys in defense mechanisms against stress. The overexpression of *A. thaliana* cysteine proteinase inhibitor gene (*AtCYS1*) in transgenic tobacco (*Nicotiana tabacum*) plants resulted in inhibition of cell death activated by oxidative stress and nitric oxide (Belenghi *et al*. 2003). Ectopic expression of oryzacystatin I (OCI) not only altered the growth and development of tobacco (van der Vyver *et al*. 2003, Prins *et al*. 2008), soybean (*Glycine max.*), and *A. thaliana* plants (Quain *et al*. 2014), but also enhanced tolerance to low temperature and drought (Prins *et al*. 2008, Quain *et al*. 2014).

There are reports available on the various mechanisms adopted by plants to survive under stressful environment, but no information is available on the role of PhyCys in resistance to Cd stress and protection of photosynthetic activity. The present study was undertaken in order to examine the response of two cultivars of mustard to Cd stress, and find if there is any role of PhyCys in protection of photosynthesis under Cd stress.

Materials and methods

Plant material and growth conditions: Two cultivars of mustard (*Brassica juncea* L.), Ro Agro 4001 (Cv_1) and Amruta (Cv_2), which exhibited the greatest and lowest photosynthetic capacity and growth, respectively, among the four cultivars tested under normal condition, were chosen for our study. Seeds of mustard cultivars Cv_1 and Cv_2 were surface sterilized with 0.01% $HgCl_2$ solution followed by repeated washings with double distilled water and were sown in 23-cm diameter earthen pots filled with acid-washed sand purified according to Hewitt (1966). Pots were kept in a net house of the Botany Department, Aligarh Muslim University, Aligarh, India. The plants were grown under natural day/night conditions with an average day and night temperatures of 20 ± 3 and 12 ± 2°C, respectively, relative humidity of 60 ± 5%, PAR was 900 ± 25 μmol(photon) m^{-2} s^{-1}, and a critical photoperiod of 10−12 h**.** The plants were supplied with Hoagland's nutrient solution (Hewitt 1966) containing 0 or 50 μM Cd ($CdCl_2$) after 10 d from sowing. Three plants per pot were maintained. Each pot was supplied with 250 mL of nutrient solution on alternate days. A control group of plants receiving 250 mL of the nutrient solution without Cd was maintained. Four replicates for each treatment were maintained and the experiment followed a completely randomized block design. After 30 d from sowing, the intact second fully expanded leaf of the plant for each treatment and its replicates were used for photosynthetic parameter measurements. Then the leaf was excised and used for measuring content of Cd, S-assimilation, activity of leaf superoxide dismutase (SOD), ascorbate peroxidase (APX), and PhyCys.

Cd content: Leaf samples were dried for 48 h at 80°C in an oven and the dried tissue was weighed, ground to fine powder and digested with concentrated HNO_3/$HClO_4$ (3:1, v/v). The content of Cd was determined by the atomic absorption spectrophotometer (*GBC, 932 plus; GBC Scientific Instruments,* Braeside, Australia).

H_2O_2 content: Content of H_2O_2 was determined by peroxidase assay following the method of Okuda *et al*. (1991). Fresh leaf tissue (0.2 g) was ground in an ice-cold 200 mL of perchloric acid. After centrifugation at 1,200 × *g* for 10 min, perchloric acid of the supernatant was neutralized with 4 M KOH. The insoluble potassium perchlorate was removed by centrifugation at 500 × *g* for 3 min. In a final volume of 1.5 mL, 1 mL of the super-natant, 400 μL of 12.5 mM 3-(dimethylamino) benzoic

acid in 0.375 M phosphate buffer (pH 6.5), 80 µL of 3-methyl-2-benzothiazoline hydrazone, and 20 µL of peroxidase (0.25 Unit) were added. The reaction was started by the addition of peroxidase, and an increase in the absorbance was recorded at 590 nm for 3 min on UV-VIS spectrophotometer (*2700, Analytical Technologies Ltd., Labtronics*, India).

Photosynthetic parameters and Rubisco activity: The content of Chl was determined using *SPAD* Chl meter (*502 DL PLUS, Spectrum Technologies,* USA). Gas-exchange parameters (net photosynthesis, P_N; stomatal conductance, g_s; intercellular CO_2 concentration, C_i; and transpiration rate, E) were measured in fully expanded uppermost leaves of plants from each treatment using infrared gas analyzer (*CID-340, Photosynthesis System, Bio-Science*, USA). The measurements were done between 11.00 and 12.00 h at light saturating intensity, PAR of 720 µmol(photon) m^{-2} s^{-1}, and at atmospheric CO_2 concentration of 370 ± 5 µmol mol^{-1}.

Activity of Rubisco (EC 4.1.1.39) was determined spectrophotometrically by the method of Usuda (1985) by monitoring the oxidation of NADH at 30°C at 340 nm during the conversion of 3-phosphoglycerate to glycerol-3-phosphate after the addition of enzyme extract to the assay medium. Leaf tissue was homogenized with ice-cold extraction buffer containing 0.25 M Tris–HCl (pH 7.8), 0.05 M $MgCl_2$, 0.0025 M EDTA, and 37.5 mg DTT. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The resulting supernatant was used to assay the enzyme. The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 40 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA, 5 mM DTT, 1 U of glyceraldehyde-3-phosphodehydrogenase and 1 U of 3-phosphoglycerate kinase, and 0.2 mM ribulose 1,5-bisphosphate (RuBP). The activity of Rubisco was expressed in µmol(CO_2) mg^{-1}(protein) s^{-1}.

Chl fluorescence parameters: Fully expanded leaves were dark adapted for 30 min for Chl fluorescence measurements using *Junior-PAM* chlorophyll fluorometer (*Heinz Walz*, Germany). Minimal (F_o) and maximal (F_m) fluorescence of a dark-adapted leaf were measured at specified intervals after a 30 min of dark adaptation followed by a flash of weak modulated saturating light [125 µmol(photon) m^{-2} s^{-1}] for 0.2 s. Maximal quantum yield of PSII photochemistry (F_v/F_m) was then calculated. The leaf was then illuminated by an actinic light of 1,500 µmol(photon) m^{-2} s^{-1} for 15–20 min. Then the maximum Chl fluorescence in the light (F_m') was determined by applying saturating light pulse [720 µmol(photon) m^{-2} s^{-1}] for 0.8 s together with steady-state fluorescence (F_s). After the actinic light had been switched off, a far-red light was exerted to determine the minimal level of fluorescence (F_o'). Efficiency of energy harvesting in the light (F_v'/F_m'), the values of the effective quantum yield of PSII (Φ_{PSII}) photochemistry, photochemical quenching (q_P), nonphotochemical quenching (q_N), and electron transport rate (ETR) were calculated.

Leaf N content and PNUE: Leaf N content was determined in acid-peroxide digested material using the method of Lindner (1944). A 10-mL aliquot of digested material was taken in a 50 mL volumetric flask to which 2 mL of 2.5 N sodium hydroxide and 1 mL of 10% sodium silicate solutions were added to neutralise the excess of acid and to prevent turbidity, respectively. In a 10 mL graduated test tube, 5-mL aliquot of this solution was taken and 0.5 mL Nessler's reagent was added. The final volume was maintained with deionised water. The contents of the test tubes were allowed to stand for 5 min for maximum colour development. The optical density of the solution was read at 525 nm on UV-VIS spectrophotometer (*2700, Analytical Technologies Ltd., Labtronics*, India). The standard curve was plotted using different concentrations of ammonium sulphate solution *vs.* optical density, and with the help of the standard curve the amount of nitrogen present in the sample was determined. PNUE was calculated by the ratio of net photosynthesis to N content per unit of leaf area.

Growth: After measuring photosynthesis, plants were uprooted carefully from the pots, washed to remove dust, and dry mass of leaves and shoot was taken separately after drying them in a hot air oven at 80°C for 72 h till constant mass was obtained. The dried leaves were used for determining other parameters and the dry mass of leaves and shoots was taken together to record plant dry mass (PDM). Leaf area was measured using a leaf area meter (*LA211, Systronics,* New Delhi*,* India).

Activity of ATP-sulphurylase, contents of S, cysteine and GSH: Activity of ATP-sulphurylase (ATP-S; EC 2.7.7.4) in leaves was estimated adopting the method of Lappartient and Touraine (1996) by grinding one g of fresh leaf tissue at 4°C in a buffer consisting of 10 mM Na_2EDTA, 20 mM Tris–HCl (pH 8.0), 2 mM DTT, and 0.01 g mL^{-1} polyvinylpyrrolidone (PVP), using 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was used for *in vitro* ATP-S assay. The enzyme activity was measured using a molybdate-dependent formation of pyrophosphate. The reaction was initiated by adding 100 µL of the extract to 500 µL of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2-ATP, and 0.032 units mL^{-1} of sulphate-free inorganic pyrophosphate in 80 mM Tris–HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture except that Na_2MoO_4 was absent. Incubations were carried out at 37°C for 15 min, after which phosphate was measured at 660 nm. The ATP-S dependent formation of pyrophosphate was estimated from the difference between the two figures. The activity of

ATP-S was expressed in U mg^{-1}(protein) min^{-1}.

For the determination of S content dried leaf material (0.1 g) was digested in a mixture of concentrated HNO_3 and 60% strength $HClO_4$ (85:15, v/v) and determined using turbidimetric method. Total S content in leaf samples was estimated using the turbidimetric method of Chesnin and Yien (1950). A 5-mL aliquot was used for turbidity development in 25-mL volumetric flask. Turbidity in 5-mL aliquot was developed by adding 2.5 mL gum acacia (0.25%) solution, 1.0 g $BaCl_2$ sieved through 40-60 mm mesh and the volume was made to 25 mL with deionized water. The contents of 25 mL volumetric flask were thoroughly shaken till $BaCl_2$ completely dissolved. The values were recorded at 415 nm on UV-VIS spectrophotometer (*2700, Analytical Technologies Ltd., Labtronics*, India) within 10 min after the turbidity development. A blank was run simultaneously after each set of determination. The content of S was expressed in mg g^{-1}(DM).

The content of cysteine in leaves was determined spectrophotometrically using UV-VIS spectrophotometer (*2700, Analytical Technologies Ltd., Labtronics*, India) (Giatonde 1967). Fresh leaf tissue (0.5 g) was homogenized in 5% (w/v) ice-cold perchloric acid. For the final volume of 4 mL one g of plant tissue was used. The suspension was centrifuged at 2,800 × *g* for 1 h at 5°C, and the supernatant was filtered. The filtrate (1 mL) was treated with acid ninhydrin reagent. The absorption was read at 580 nm, and the amount of cysteine was calculated with reference to a calibration curve obtained under similar conditions for standard cysteine. The content of cysteine was expressed in nmol g^{-1}(FM).

GSH content was measured using the method of Anderson (1985) by an enzymic recycling procedure in which it was sequentially oxidized by 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of glutathione reductase (GR; EC 1.8.1.7). Fresh leaf tissue (0.5 g) was ground in liquid nitrogen using mortar and pestle. The ground tissue was suspended in 0.5 mL of 5% sulfosalicylic acid and centrifuged at 12,000 × *g* for 10 min. A 300-µL aliquot of supernatant was removed and neutralized by addition of 18 µL 7.5 M triethanolamine. Aliquot (50 µL) of the sample was mixed with 700 µL 0.3 mM NADPH, 100 µL DTNB, and 150 µL buffer containing 125 mM sodium phosphate, and 6.3 mM EDTA (pH 6.5). A 10 µL aliquot of GR (5 U mL^{-1}) was then added and the change in absorbance at 412 nm was monitored at 30°C. The content of GSS was expressed in nmol g^{-1}(FM).

Activity of SOD and APX: Activity of SOD (EC 1.15.1.1) and APX (EC 1.11.1.11) was determined following the method described earlier (Asgher *et al*. 2014). Fresh leaf tissue (0.5 g) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) PVP in potassium-phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant obtained after centrifugation was used for the assay of SOD. For the assay of APX, extraction buffer was supplemented with 2 mM ascorbate.

Activity of SOD was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) as determined by Beyer and Fridovich (1987) and Giannopolitis and Ries (1977). A 5 mL of reaction mixture containing 5 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.0), 13 mM methionine, 0.025% (v/v) Triton X-100, 63 µmol NBT, 1.3 µmol riboflavin, and the enzyme extract was illuminated for 15 min and a control set was not illuminated to correct for background absorbance. A unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reaction of NBT at 560 nm. The activity of SOD was expressed in U mg^{-1}(protein) min^{-1}.

Activity of APX was determined following the method of Nakano and Asada (1981) by recording the decrease in absorbance of ascorbate at 290 nm on UV-VIS spectrophotometer (*2700, Analytical Technologies Ltd., Labtronics*, India). The assay mixture contained phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and the enzyme extract. Activity of APX was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One unit of the enzyme was the amount necessary to decompose 1 µmol of substrate per min at 25°C. The activity of APX was expressed in U mg^{-1}(protein) min^{-1}.

Activity and purification of leaf PhyCys: Activity of leaf PhyCys was determined in homogenate by its ability to inhibit the caseinolytic activity of papain following the method of Kunitz (1947). The purification of PhyCys was done adopting the method described by Sharma *et al*. (2006) with some modifications. Leaves (15 g) of the two cultivars were homogenized in 50 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, 3 mM EDTA, and 2% n-butanol. The extract was centrifuged at 6,800 × *g* for 15 min at 4°C. The supernatant was collected and was then fractionated by 40-60% ammonium sulphate saturation. The precipitate was collected by centrifugation at 10,600 × *g* for 30 min at 4°C and dissolved in minimum amount of 50 mM sodium phosphate buffer (pH 7.5) and dialyzed three times against the same buffer. A column of *Sephacryl S-100 HR* was prepared at room temperature (25°C). The dialyzed sample was subjected to gel filtration chromatography on the *Sephacryl S-100* column (60 × 1.9), equilibrated with 50 mM sodium phosphate buffer (pH 7.5). The flow rate of the column was 15 mL h^{-1}. Fractions of 5 mL were collected and assayed for protein. The activity of PhyCys was expressed in terms of % inhibition of caseinolytic activity of papain.

The protein content of leaf sample was estimated by the method of Lowry *et al*. (1951).

Fluorescence spectra: Fluorescence spectra were taken for the purified leaf PhyCys by a spectrofluorometer (*RF5301PC, Shimadzu*, Japan) in order to understand the PhyCys behaviour under Cd stress. The excitation wavelength was 280 nm and emission range was 300−400 nm and path length was 1.0 cm.

Statistical analysis: The experimental design was completely randomized block design with four replicates (n = 4) for each treatment. The data were analyzed statistically using analysis of variance (*ANOVA*) by *SPSS 17.0* for *Windows*, and presented as treatment mean ± SE (n = 4). Least significant difference (LSD) was calculated for the significant data at P<0.05. Data followed by same letter are not significantly different by LSD test at P<0.05.

Results

Content of Cd: The cultivar Cv_2 showed a higher leaf Cd content than that of Cv_1. The contents of Cd in Cv_1 and Cv_2 were 69 ± 3.5 and 88 ± 4.4 µg g^{-1}(DM), respectively, compared with the control (Fig. 1).

Content of H_2O_2: A significant increase in the H_2O_2 content was found in both the cultivars after the Cd treatment compared with the control. In the Cd-treated plants, the content of H_2O_2 increased by 75% in Cv_1 and 150% in Cv_2 compared with the control plants (Fig. 1).

Photosynthetic parameters: The Cd treatment reduced photosynthetic attributes in both the cultivars compared with the control, but Cv_2 exhibited greater reduction than that of Cv_1. In Cv_1, the Chl content and P_N decreased equally by about 40%, while g_s and C_i decreased by about 14% and E by 25% with Cd compared with the control. In Cv_2, the Chl content and P_N decreased equally by about 50%, and g_s and C_i by about 25%, while E decreased by 32% in comparison with the control (Table 1). Rubisco activity significantly decreased with the Cd treatment in comparison with control in both the cultivars, but to a greater extent in Cv_2. The decrease in Rubisco activity due to Cd was 21% in Cv_1 and 31% in Cv_2 compared with the control (Table 1).

Chl fluorescence was measured under both stress and nonstress conditions. Cd significantly decreased Φ_{PSII}, F_v/F_m, intrinsic efficiency of PSII (F_v'/F_m'), q_P, and ETR compared with the control plants in both the cultivars, but to a greater extent in Cv_2 than that in Cv_1. The decrease in these Chl fluorescence parameters, Φ_{PSII}, F_v/F_m, F_v'/F_m', q_P, and ETR was 28.8, 12.2, 17.3, 20.3, and 28.9% in Cv_1, and 39.6, 24.3, 27.5, 31.7, and 39.6%, respectively, in Cv_2 compared with the control. Contrarily, q_N significantly increased with the Cd treatment in both the cultivars, and the greater increase in q_N (51.4%) was observed in Cv_2 than that in Cv_1 (25.7%) compared with the control. The maximum F_v/F_m in the Cd-stressed plants sharply decreased below those of the control plants in both the cultivars. The Φ_{PSII} exhibited the lowest value in response to the Cd treatment compared with the control. The (F_v'/F_m') was the greatest in the control plants of both the cultivars, but decreased in the Cd-treated plants (Table 1).

Leaf N content and PNUE: Plants treated with Cd showed decrease in the N content by 32% in Cv_1 and 46% in Cv_2 compared with the control plants (Table 1). PNUE decreased more conspicuously in Cv_2 with Cd stress compared to the control plants. The treatment by Cd resulted in a decrease of PNUE by 34% in Cv_1 and 44% in Cv_2 compared with the control plants (Table 1).

Growth characteristics: The Cd treatment decreased a leaf area and PDM in both the cultivars, but more

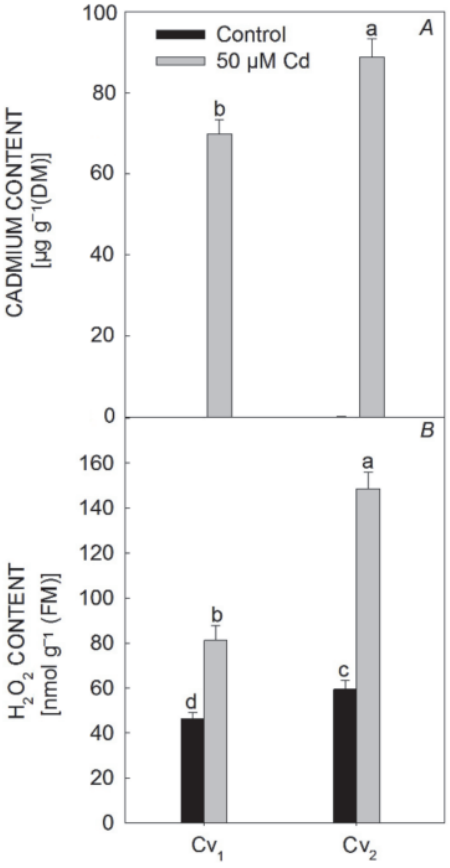

Fig. 1. Content of cadmium (*A*) and H_2O_2 (*B*) in leaves of Ro Agro 4001 (Cv_1) and Amruta (Cv_2) cultivars of mustard (*Brassica juncea* L.) treated with 0 or 50 µM Cd at 30 d after sowing. Values are means ± SE (n = 4). Data followed by *the same letter* are not significantly different by LSD test at P<0.05. DM – dry mass.

Table 1. Effect of cadmium (Cd) on chlorophyll (Chl) content, net photosynthetic rate (P_N), stomatal conductance (g_s) , intercellular CO_2 concentration (C_i), transpiration rate (E), Rubisco activity, plant dry mass, quantum yield of PSII, maximum PSII efficiency, intrinsic PSII efficiency, photochemical quenching, nonphotochemical quenching, electron transport rate, nitrogen content, and photosynthetic nitrogen-use efficiency (PNUE) in Ro Agro 4001 and Amruta cultivars of mustard (*Brassica juncea* L.) at 30 d after sowing. Data are presented as treatments mean ± SE (n = 4). Data followed by *the same letter* are not significantly different by LSD test at P<0.05.

Parameter	Ro Agro 4001		Amruta	
	Control	50 μM Cd	Control	50 μM Cd
Chl [SPAD value]	36.4 ± 1.82^a	21.4 ± 1.07^c	29.1 ± 1.46^b	14.1 ± 0.71^d
P_N [μmol(CO_2) m^{-2} s^{-1}]	16.6 ± 0.83^a	9.1 ± 0.50^c	13.5 ± 0.68^b	5.9 ± 0.30^e
g_s [mmol m^{-2} s^{-1}]	320 ± 16.1^a	275 ± 13.8^b	282 ± 14.1^b	224 ± 11.2^c
C_i [μmol mol^{-1}]	213 ± 10.7^a	183 ± 9.1^b	185 ± 9.3^b	143 ± 7.2^c
E [mmol m^{-2} s^{-1}]	4.4 ± 0.22^a	3.3 ± 0.18^c	3.7 ± 0.19^b	2.5 ± 0.13^d
Rubisco activity [μmol(CO_2) mg^{-1}(protein) s^{-1}]	45.9 ±2.29^a	36.3 ± 1.82^c	39.1 ± 1.96^b	26.9 ± 1.35^d
Quantum yield of PSII	0.52 ± 0.026^a	0.37 ± 0.02^c	0.48 ± 0.025^b	0.29 ± 0.02^d
Maximum PSII efficiency	0.809 ± 0.04^a	0.710 ± 0.04^c	0.780 ± 0.04^b	0.590 ± 0.03^d
Intrinsic PSII efficiency	0.75 ± 0.04^a	0.62 ± 0.03^c	0.69 ± 0.03^b	0.50 ± 0.03^d
Photochemical quenching	0.69 ± 0.03^a	0.55 ± 0.03^c	0.63 ± 0.03^b	0.43 ± 0.02^d
Nonphotochemical quenching	0.35 ± 0.02^b	0.44 ± 0.02^e	0.35 ± 0.02^b	0.53 ± 0.03^a
Electron transport rate	162.5 ± 8.0^a	115.6 ± 5.9^b	150.0 ±7.9^a	90.6 ±4.9^c
Leaf area [cm^2]	90.4 ± 4.52^a	71.3 ± 3.58^b	78.7 ± 3.95^b	54.6 ± 2.81^c
Plant dry mass [g per plant]	2.08 ± 0.10^a	1.76 ± 0.09^c	1.87 ± 0.01^b	1.41 ± 0.07^d
Leaf nitrogen content [mg g^{-1}(DM)]	33.2 ± 1.66^a	22.5 ± 1.13^b	24.6 ± 1.23^b	13.4 ± 0.59^c
PNUE [g m^{-2}]	27.8 ± 1.34	18.4 ± 1.01	22.3 ± 1.19	12.5 ± 0.69

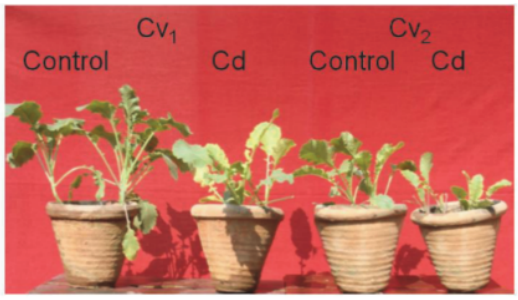

Fig. 2. Cadmium induced changes in phenotype of Ro Agro 4001 (Cv_1) and Amruta (Cv_2) cultivars of mustard (*Brassica juncea* L.) treated with 0 or 50 μM Cd at 30 d after sowing.

prominently in Cv_2 compared with the control. Leaf area and PDM were reduced by 21 and 15% in Cv_1, and 31 and 25% in Cv_2 compared with the controls (Table 1). Phenotypical difference between these cultivars under normal and Cd stress conditions are shown in Fig. 2.

Sulphur assimilation (ATP-S activity and content of S): To assess S-assimilation in response to the Cd treatment in the two cultivars, we measured ATP-S activity and a content of S and cysteine. The increase in ATP-S activity was significant after the Cd treatment and was greater in Cv_1 than that in Cv_2; ATP-S activity in Cv_1 was two times higher than in Cv_2 compared with the control (Fig. 3). On the contrary, the S content decreased significantly with Cd treatment in both cultivars, but more conspicuously in Cv_1. The content of S decreased by 27.6 and 21.5% in Cv_1 and Cv_2, respectively, in the Cd-treated plants compared with the control (Fig. 3).

Content of cysteine and GSH: The content of cysteine increased significantly with Cd compared with the control in both the cultivars. The content of Cys increased by 58% in Cv_1 and 44% in Cv_2 with the Cd treatment compared with the control (Fig. 3). The GSH content also increased significantly in both cultivars after the Cd treatment compared with the control, but a greater increase of 25% was found in Cv_1 than that of 15% in Cv_2 (Fig. 3).

Activity of SOD and APX: Activity of SOD and APX significantly increased with the Cd treatment in comparison with the control. SOD activity was higher in Cv_2 than that in Cv_1 in the control plants. However, the Cd treatment resulted in a greater increase in SOD activity in Cv_1 (50%) than that of Cv_2 (37%) in comparison with the control (Fig. 4). On the other hand, APX activity was higher in Cv_1 than that in Cv_2 in the control plants, and the Cd treatment further increased the activity by 136.8% and 109.6%, respectively, compared with the control (Fig. 4).

Effect of Cd on leaf PhyCys activity: Cd significantly increased leaf PhyCys activity of both the cultivars in comparison with the control. Leaf PhyCys from the control plants of Cv_1 inhibited caseinolytic activity of papain by 25.5%, while the inhibition was 81% in the Cd-treated plants. However, in Cv_2, the inhibition of caseinolytic activity of papain was 14.2% in the control plants and 21.1% in the Cd-treated plants (Table 2).

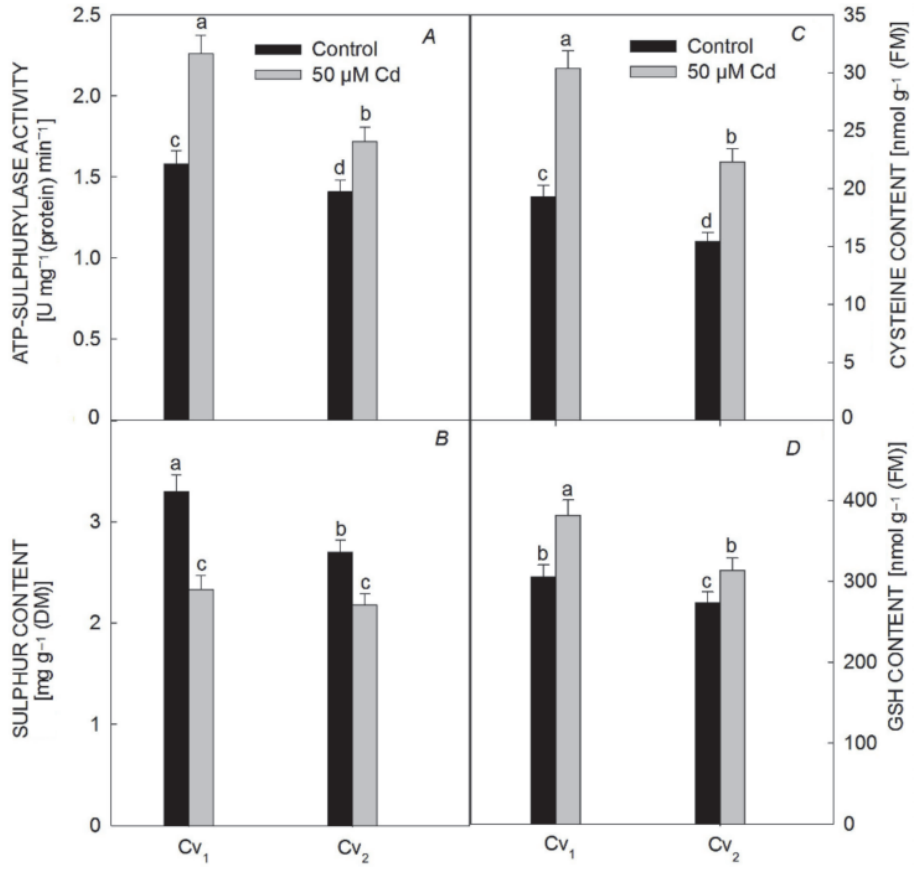

Fig. 3. Activity of ATP-S (*A*), content of S (*B*), content of cysteine (*C*), and GSH (*D*) in leaves of Ro Agro 4001 (Cv_1) and Amruta (Cv_2) cultivars of mustard (*Brassica juncea* L.) treated with 0 or 50 µM Cd at 30 d after sowing. Values are means ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P<0.05$. (ATP-S and S content in dry mass, DM and content of cysteine and GSH in fresh mass, FM).

Fluorescence spectra: Fluorescence spectra of leaf PhyCys for the Cd-treated plants in both the cultivars markedly increased in comparison to the control plants. Fluorescence spectra of leaf PhyCys from Cv_2 plants showed a greater increase than that of Cv_1 in the control and Cd treated plants (Fig. 5). There was, however, no shift in the maxima which remained at 335 nm.

Discussion

The contamination of agricultural soil by Cd is of great concern as it induces oxidative stress, inhibits enzyme activity, photosynthetic capacity, growth and productivity of plants worldwide (Asgher *et al*. 2015, Khan *et al*. 2015). Plants adopt different strategies, such as synthesis of thiol compounds, under metals stress for maintaining photosynthetic capacity. PhyCys are involved in regulating several physiological processes in vascular plants (Martínez *et al.* 2012, Eason *et al*. 2014). In the present study, our attempt was made to find if leaf PhyCys activity corresponds to the changes in photosynthetic activity, S-assimilation, and antioxidant activity. The Cv_1 cultivar with higher photosynthetic capacity showed the greater PhyCys activity than that of Cv_2 under control conditions. Under Cd stress, higher induction in PhyCys activity was parallel to photosynthetic protection more prominently in Cv_1 than that in Cv_2, suggesting that PhyCys played role in determining photosynthetic activity of cultivars under control conditions and also helped in maintaining the photosynthetic activity under Cd stress.

Cd accumulation leads to enhanced ROS production and induction of oxidative stress in plants (Anjum *et al*. 2011, Asgher *et al*. 2014). The higher Cd content in Cv_2 than that in Cv_1 led to greater oxidative stress in Cv_2 than Cv_1 by enhancing H_2O_2 production, resulting in larger reduction in photosynthetic and growth characteristics. The reduction in photosynthesis by Cd could be attributed to the decrease in the Chl content, gas-exchange parameters, Rubisco activity, and efficiency of PSII. Shi and Cai (2008) observed an inhibition in P_N due to the decreased g_s and content of photosynthetic pigments in *Arachis hypogaea*. Liu *et al.* (2011) also observed decrease

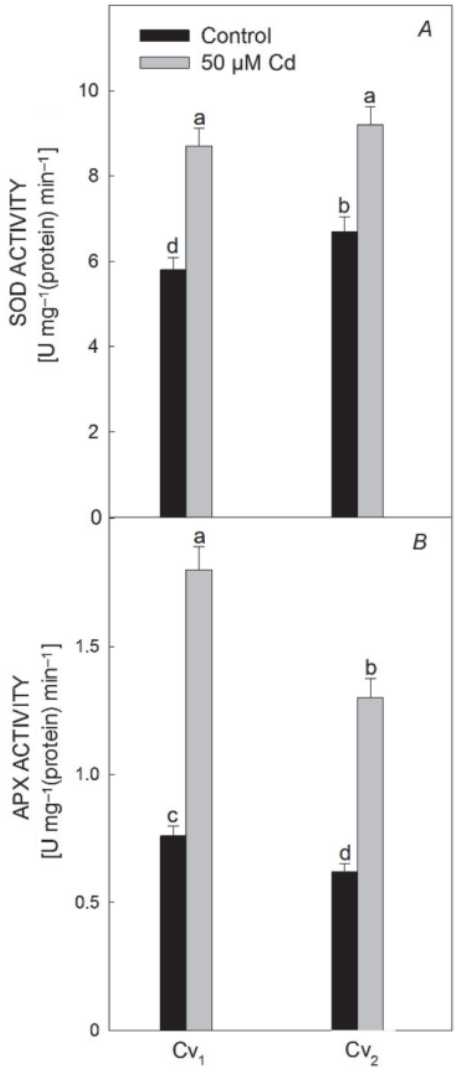

Fig. 4. Activity of SOD (*A*) and APX (*B*) in leaves of Ro Agro 4001 (Cv_1) and Amruta (Cv_2) cultivars of mustard (*Brassica juncea* L.) treated with 0 or 50 µM Cd at 30 d after sowing. Values are means ± SE (n = 4). Data followed by *the same letter* are not significantly different by LSD test at P<0.05. FM – fresh mass.

Table 2. Effect of cadmium (Cd) on the activity of leaf phytocystatin determined as its ability to inhibit activity of papain on casein protein (% inhibition) in Ro Agro 4001 and Amruta cultivars of mustard (*Brassica juncea* L.). Data are presented as means ± SE (n = 4). Data followed by *the same letter* are not significantly different by LSD test at P<0.05.

Cultivars	Control	50 µM Cd
Ro Agro 4001	25.5 ± 1.3^{b}	81.0 ± 4.1^{a}
Amruta	14.2 ± 0.1^{d}	21.1 ± 1.1^{c}

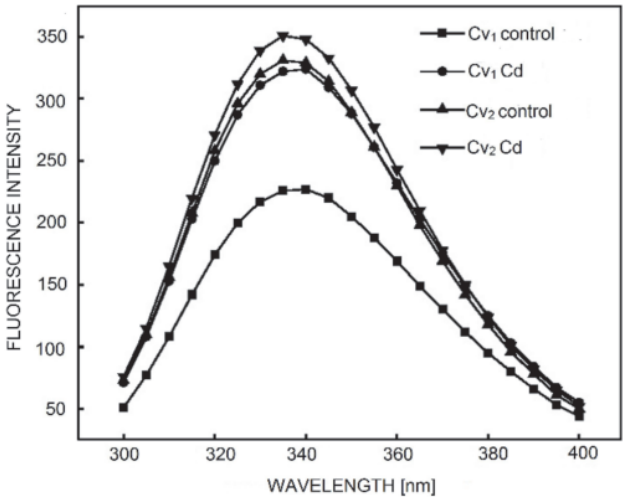

Fig. 5. Fluorescence spectral studies of leaf PhyCys of mustard (*Brassica juncea* L.) cultivars Ro Agro 4001 (Cv_1) and Amruta (Cv_2) treated with 50 µM Cd.

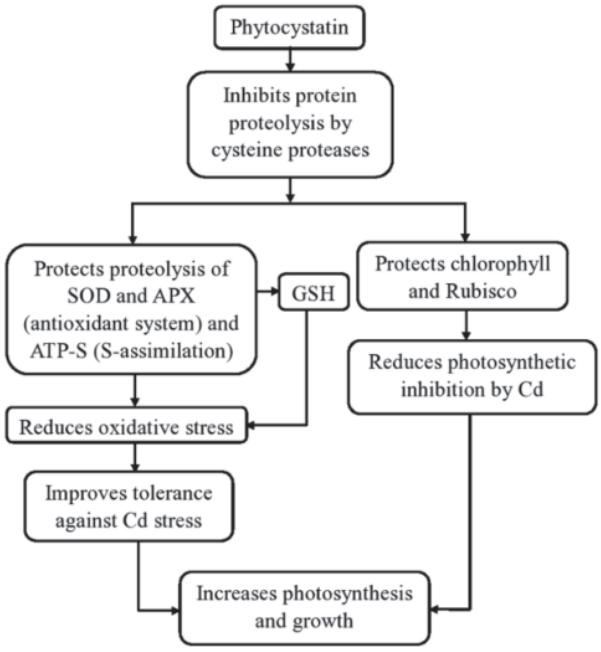

Fig. 6. Schematic representation of mechanism of action of PhyCys in protection of photosynthesis and growth of mustard (*Brassica juncea* L.) plants under Cd stress. PhyCys protects plant proteins by inhibiting the action of cysteine proteases. They can also protect the breakdown of antioxidant enzymes as well as chlorophyll and photosynthetic enzymes including Rubisco, therefore, protecting photosynthesis and growth in mustard under Cd stress.

in gas-exchange parameters including P_N, g_s, C_i, and E in *Ricinus communis* due to Cd stress. The decrease in Chl content with Cd treatment possibly decreased the absorption of light by the chloroplast and thus indirectly

impaired photosynthesis. The measurement of Chl fluorescence parameters has been established as an important indicator of the stress intensity (Baker and Rosenqvist 2004). Among the various Chl fluorescence parameters, Φ_{PSII}, F_v/F_m, and F_v'/F_m' are related to photosynthetic efficiency (Maxwell and Johnson 2000, Shangguan *et al*. 2000). These parameters were found greatly reduced in Cv_2 under Cd stress, indicating that higher inhibition of the PSII activity also contributed to the greater decrease in photosynthesis. The decrease in Φ_{PSII} under Cd stress was an outcome of the enhanced q_N that dissipated light as heat in plants. There are reports confirming that the imbalances between the light absorption and light energy utilization under metal stresses caused excess of light energy leading to the formation of long-lived Chl triplet states, which cause oxidative damage to the photosynthetic thylakoid membrane (Sarvari 2005). Recent report of Liu *et al*. (2014) in cotton (*Gossypium hirsutum*) has also suggested that Cd affects photosynthetic electron transport chain and photosynthetic capacity. The cumulative adverse effect of Cd on metabolism led to decrease in the leaf area in both the cultivars but maximum decrease occurred in Cv_2. The Cv_1 with the larger leaf area efficiently utilized solar radiation resulting in higher photosynthesis and plant dry mass than Cv_2.

The mechanisms of N and S-assimilation efficiency and antioxidant systems are induced in plants to counteract Cd-caused oxidative stress. The demand for N increased in the presence of Cd stress, signaling plants to absorb more N. The Cv_1 plants absorbed more N that resulted in greater photosynthesis than that of Cv_2 under Cd stress. It has been suggested that N takes part in the regulation of photosynthesis (Marschner 1995). Deprivation of N may cause a significant reduction in the photosynthetic efficiency of plants (Resurreccion *et al*. 2001). The relationship between the N content and photosynthesis has been observed in mustard (Iqbal *et al*. 2011), which is also correlated with the Rubisco content and S assimilation (Iqbal *et al*. 2012). It has been suggested that plants with lower PNUE have a lower ability to allocate N to the photosynthetic machinery (Takashima *et al*. 2004).

The greater capacity of Cv_1 to resist Cd stress may also be attributed to the upregulation of S-assimilation and protection of enzymes of S-assimilation by PhyCys leading to the greater GSH content (Fig. 3) that may sequester Cd^{2+} ions through formation of phytochelatins synthesis. Treatment of the plants with Cd resulted in the higher Cys content in Cv_1 than that of Cv_2. In fact, Cys is the major limiting substrate for GSH synthesis (Noctor *et al*. 1996), and GSH is the substrate for biosynthesis of phytochelatins (Cobbett and Goldsbrough 2002). The increased concentrations of Cd has been reported to stimulate the activity of enzymes of S assimilation for maintaining higher contents of Cys and GSH for the detoxification of metals in *B. juncea* (Heiss *et al*. 1999, Khan *et al*. 2009, Masood *et al*. 2012). The maintenance of low oxidative stress in Cv_1 was possible as a result of balanced tuning between a GSH pool as well as the activity of APX and SOD enzymes. In Cv_1, the accelerated APX activity more efficiently metabolized Cd-mediated H_2O_2 amounts and controlled the H_2O_2-accrued consequences. The greater activity of SOD in Cv_2 than that of Cv_1 without stress resulted in the greater H_2O_2 content. Moreover, higher SOD activity accompanied with lesser APX activity in Cv_2 caused greater H_2O_2 accumulation under both normal and stress conditions, while higher APX activity in Cv_1 was sufficient to detoxify the H_2O_2 formed as a result of SOD activity.

In the present study, Cv_1 exhibiting higher PhyCys activity showed a greater photosynthetic potential, growth, and S-assimilation than those in Cv_2. The higher PhyCys activity in Cv_1 prevented the breakdown of enzymes of S-assimilation and antioxidative metabolism under Cd stress more efficiently than in Cv_2 with low PhyCys activity.This resulted in the increased content of Cys and GSH and protection of photosynthetic capacity in Cv_1 in response to Cd stress. Studies of Huang *et al*. (2007) and Zhang *et al*. (2008) have shown the involvement of PhyCys in response to salt, drought, oxidation, and cold stress tolerance in rice and *A. thaliana*. Recently, Eason *et al*. (2014) showed that overexpression of *BoCPI-1* retarded Chl loss and degreening presumably by protecting the chloroplasts from degradation. The overexpression of rice PhyCys in transgenic tobacco was shown to delay the senescence-related decline in Rubisco activity and photosynthesis and also a large increase in Rubisco protein was found in leaves of transgenic plants compared to control (Prins *et al*. 2008). It has recently been found that ectopic expression of oryzacystatin (OCI) in *A. thaliana* and soybean plants enhanced shoot branching and leaf Chl accumulation (Quain *et al*. 2014). However, the influence of Cd on PhyCys activity and concurrent changes in photosynthetic capacity, S-assimilation and antioxidants has not been explored in detail. The present study showed that the cultivar with high PhyCys activity substantially protected photosynthetic capacity under Cd stress through its protective action on enzymes of photosynthetic machinery, S-assimilation and antioxidant systems, together with lesser capacity of Cd accumulation. The mechanism of PhyCys action in protection of photosynthesis and growth of mustard plants under Cd stress is shown in Fig. 6.

The role of PhyCys activity in augmenting photosynthetic capacity without stress and under Cd is also evident from the different photosythetic potential of the two cultivars showing higher photosynthetic potential in Cv_1 with more PhyCys activity than Cv_2 with lesser photosynthetic potential and PhyCys activity. In order to gain more insight into the influence of Cd on PhyCys activity, fluorescence studies of the PhyCys purified from the leaves of both the cultivars were done. Fluorescence spectra of PhyCys from Cv_1 and Cv_2 leaves increased with Cd stress with a greater increase in Cv_2 showing changes in the conformation of PhyCys. In the presence of Cd, high

fluorescence intensity of Cv_2 showed its lesser tolerance capacity to Cd because of higher conformational changes, while lesser and about equal to control fluorescence intensity in Cv_1 showed its capacity for higher resistance to Cd. Thus, higher PhyCys activity with lesser conformational changes in Cv_1 under Cd stress accounted for greater tolerance to Cd stress.

Conclusions: The cultivar Cv_1 with higher PhyCys activity showed the higher photosynthetic capacity and better tolerance to Cd stress than Cv_2. The higher tolerance of Cv_1 to Cd toxicity was possible due to greater S-assimilation, PNUE, and antioxidant metabolism than that of Cv_2. The differential behaviour of the cultivars to Cd stress showed that PhyCys was related to the protection of enzymes of S-assimilation, antioxidant system (SOD and APX), and Rubisco to maintain photosynthesis under Cd stress. PhyCys is therefore suggested to play a significant role in determining photosynthetic capacity of plants under Cd stress, and could be considered as physiological parameter for augmenting photosynthesis under Cd stress.

The regulation of proteolysis displays positive influence on the acclimation to abiotic stress, and thus PhyCys could play important role in abiotic stress tolerance. However, very little is known about the nature and regulation of protein turnover machinery that fortifies the essential stress-induced cellular restructuring. Cysteine proteases are intrinsic to the genetic programes that support plant development and senescence, but their functions in stress-induced senescence are not well defined. Transgenic plants constitutively expressing PhyCys can be engineered to enhance tolerance to a wide range of different abiotic stresses including heavy metal stress. Thus, modulation of cysteine protease activity by altered expression of PhyCys in plants might be useful to improve resilience and quality against abiotic stress.

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