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Original article

Novel mode of resistance to *Fusarium* infection by a mild dose pre-exposure of cadmium in wheat

B. Mittra [a,*], P. Ghosh [b], S.L. Henry [c], J. Mishra [d], T.K. Das [e], S. Ghosh [b], C.R. Babu [a], P. Mohanty [f,g]

[a] *Center for Environmental Management of Degraded Ecosystems (CEMDE), School of Environmental Studies, North Campus, University of Delhi, Delhi 110 007, India*
[b] *Department of Biophysics, University of Delhi, South Campus, New Delhi 110021, India*
[c] *Department of Plant Sciences, Institute of Bio-medical and Life Sciences, University of Glasgow, Glasgow, UK*
[d] *NAM S&T Center, New Delhi 110003, India*
[e] *Electron Microscopic Facility, All India Institute of Medical Sciences, New Delhi 110019, India*
[f] *Jawaharlal Nehru University, New Delhi 110067, India*
[g] *D.A. University, Indore 452001, India*

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Abstract

Exposure of healthy wheat seeds (*Triticum aestivum* var Sonalika) to mild dose of cadmium (Cd^{2+}) given as 50 µM $CdCl_2$ for 48 h and then washed off Cd^{2+} offered resistance to the subsequent infection by *Fusarium oxysporum* inoculum. Seven days old seedlings having two primary leaves were aseptically inoculated with fungus, *F. oxysporum* (1×10^6) spores. The seedlings pre-exposed to low level of Cd^{2+} survived the *Fusarium* infection, while plantlets without Cd^{2+} stress wilted and then perished due to *Fusarium* infection. The stress associated proteins induced by Cd^{2+} (50 µM), *F. oxysporum* and by the co-stress (50 µM Cd^{2+} and then with *F. oxysporum*) treatments were observed to be of same molecular weight (51 kDa). Antibody was raised against the purified Cd^{2+}-stress associated protein (CSAP). Immuno-gold labeling of wheat seedling root tissue showed the presence of this CSAP in Cd^{2+} pre-exposed and in co-stressed tissues and to be located predominantly on the inner linings of the cell membranes. We also observed that the anti-CSAP-antibody also labeled the root tissue of only *Fusarium* inoculated seedlings and the gold labeling was intensely located on the membrane. This cross-reaction of anti-CSAP suggests that *Fusarium*-induced stress protein (FISP) possibly has close homology to CSAP. We thus show for the first time the over expression of a high molecular mass protein by mild dose of Cd^{2+} pre-exposure to wheat seeds which subsequently provided protection against *Fusarium* infection. This mode of resistance developed by an abiotic stress-causing agent against pathogen infection is novel.
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Keywords: Co-stress protein; CSAP; ELISA; FISP; *F. oxysporum*; Immunolabeling; *Triticum aestivum*

1. Introduction

Abiotic stresses like high light, salinity, drought, elevated temperature, heavy metals and biotic stresses mainly fungal and viral infections cause colossal loss to crop productivity as well as crop quality. Responses to stress are important aspect of environmental adaptation in plants. These responses allow plants to survive in diverse adverse environmental conditions. Over the past decades, considerable research has been directed to elucidate the biochemical and molecular basis of heavy metal toxicity and on the nature of resistance (or tolerance) in plants [6,12,19]. Plants do not have a defined immune system directly comparable to that of animals. However, plants respond to pathogen attack by a variety of biochemical means known as defense responses. Particularly, during fungal infection, plants synthesize low molecular mass inhibitory compounds such as phytoalexins and accumulate pathogen-related (PR) proteins [2,17]. In many plants, enhanced disease resistance is accompanied by the activation of genes encoding PR proteins [29]. Recently, the defense-role of sugarcane glycoproteins against the smut

* Corresponding author.
E-mail address: bhaba_mit@yahoo.co.uk (B. Mittra).

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disease in sugarcane caused by *Ustilago scitaninea* was demonstrated [7]. Similarly, there has been increasing evidence for direct influences of strobilurins, a fungicide, on plant defense physiology [13], which suggest that in addition to their fungicidal activity, strobilurin also enhances the capability of plants to ward off viral and bacterial pathogens. The antifungal activity of a strobilurin compound, F-500 (Pyraclostrobin), has been tested in tobacco plants which enhanced plant resistance against tobacco mosaic virus (TMV) and bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* [10]. Some chemicals and metal ions are antifungal in that the treatment of seeds prevent or arrests fungal infection to plants. Cadmium salts are usually used as antifungal [20]. However, cadmium is also phytotoxic, at high concentration it retards plant growth and development [3]. Exposure of plants to heavy metals like Cd^{2+} induces production of phytochelatin (PC) and some stress associated proteins, SAP [2,4,5,22,24,34]. However, no report has been found on Cd^{2+} pre-exposure imparting resistance to fungal specification.

In this communication, we report, for the first time, on a novel mode of antifungal activity of $CdCl_2$ in that a pre-exposure of wheat seedlings to a mild dose of $CdCl_2$ imparts immunity to plants against *Fusarium* infection.

2. Results

2.1. Plant materials and treatments

Wheat seedlings grown after 48 h of 50 µM Cd^{2+} pre-exposure to seeds reduced the percentage of seed germination marginally, but did not affect the seedling growth. Exposure to 100 µM Cd^{2+}-exposure did not affect the plant severely during the experimental period. Thus, 50 µM Cd^{2+} is considered by us to be mild and nontoxic. Control seedlings inoculated with *Fusarium oxysporum* (1×10^6) spores wilted after 7 d of *Fusarium* infection while the untreated (control) plants, the Cd^{2+} pre-exposed plants and the co-stressed (50 µM Cd^{2+} pre-exposed and then infected with *F. oxysporum*) seedlings grown under the same conditions did not show wilting of leaves (Fig. 1). The seedlings only inoculated with *Fusarium* spores subsequently wilted while the co-stressed seedlings survived. Repeated experiments yielded similar results.

2.2. Identification of stress-induced proteins

In case of control seedlings, the cadmium stress associated protein (CSAP) band of 51 kDa was faintly seen in a 10% SDS-PAGE profile of soluble protein, but it was quite intense in the Cd^{2+}-exposed samples (Fig. 2, lane 3). Besides this 51 kDa protein which was over expressed by Cd^{2+}, we also observed a new band at 100 kDa to have been induced by Cd^{2+}. This band was also observed in the *Fusarium* infected seedlings and not in co-stressed samples. This observation of a new high molecular weight protein by Cd^{2+}-exposure to

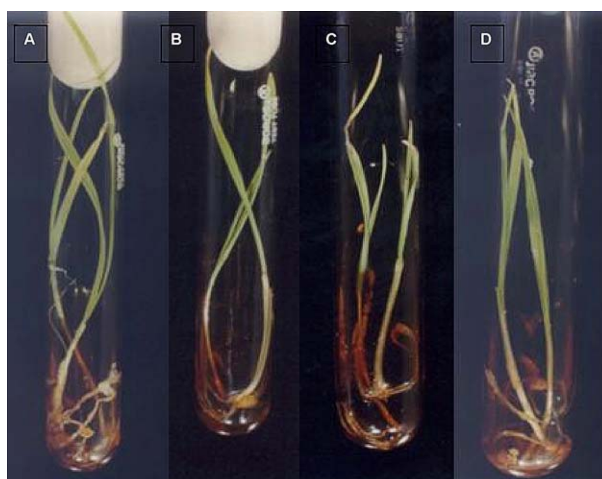


Fig. 1. Photograph showing the growth of Cd^{2+} (50 µM) pre-exposed/untreated 7-day-old germinated wheat seedlings inoculated with *F. oxysporum* for another 7 d. **A**, untreated (control) wheat seedlings grown in MS liquid medium; **B**, wheat seedlings pre-exposed to Cd^{2+} (50 µM) for 48 h grown in MS liquid medium; **C**, untreated (control) wheat seedlings grown in MS liquid medium infected with *F. oxysporum*; **D**, wheat seedlings grown in MS liquid medium pre-exposed to Cd^{2+} (50 µM) for 48 h and then infected with *F. oxysporum*.

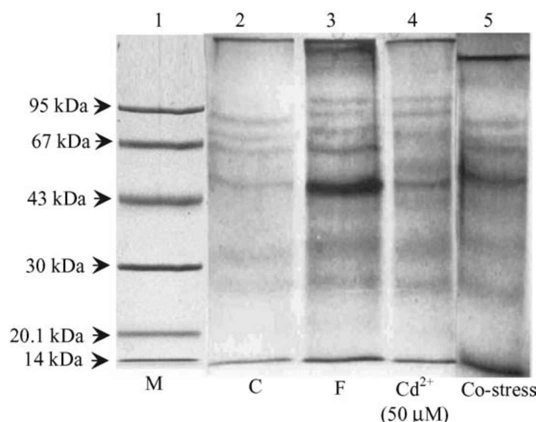


Fig. 2. Molecular characterization of untreated, *Fusarium* infected, Cd^{2+} (50 µM) and co-stressed (Cd^{2+}/*Fusarium*) protein on 10% SDS-PAGE. Lanes 2–4 over expression of a 51 kDa protein. M, Marker protein. Lanes 1: C, Untreated (control) wheat seedlings; 2: F, *Fusarium* infected wheat seedlings; 3: Cd^{2+}-50 µM, cadmium (50 µM)-exposed wheat seedlings; 4: Co-stress, wheat seedlings pre-exposed to 50 µM Cd^{2+} and then infected with *F. oxysporum*.

seeds is novel. However, the 100 kDa band is less prominent than the 51 kDa stress protein and this 100 kDa band would be representative of heat-shock type of protein [30]. Since the biosynthesis of 51 kDa protein upon Cd^{2+} stress appeared to be highly over expressed, we have characterized this 51 kDa CSAP further in this investigation. Surprisingly, similar 51 kDa protein band appeared upon *Fusarium* infection of wheat seedlings. The *Fusarium*-induced stress protein (FISP) and the CSAP, both were over expressed by the respective stresses and *Fusarium* infection induced protein appeared to be same or similar proteins because of their apparent molecular mass (Fig. 2, lanes 2, 3). The soluble protein profile of the wheat seedlings pre-exposed to 50 µM

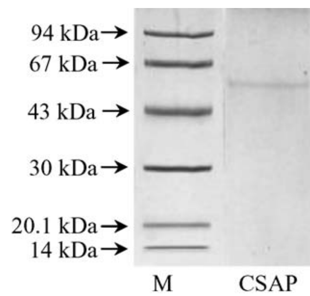


Fig. 3. Purified CSAP showing single band on 10% SDS-PAGE.

$CdCl_2$ and later inoculated with *Fusarium* spores also exhibited an intense band at 51 kDa range (Fig. 2, lane 4).

2.3. Purification of CSAP

To further characterize the CSAP, the protein was isolated on a 10% preparative gel and electro-eluted. The purified protein showed a single band of same moiety on a 10% SDS-PAGE (Fig. 3) suggesting it to be a monomeric protein.

2.4. Specificity of anti-CSAP-antibody

Anti-CSAP polyclonal antibody was developed in rabbit using CSAP and the specificity was checked by ELISA (Fig. 4) as well as by Western-blot (Fig. 5). The titre value of polyclonal antibody by ELISA was observed to be 1:100.

2.5. Localization of CSAP and FISP in respective root and leaf sections

Both the Cd^{2+} pre-exposed and *Fusarium*-infected wheat root tissues showed positive gold labeling under the transmission electron microscopy (TEM) when challenged with the anti-CSAP-antibody (Fig. 6C,E). The same anti-CSAP-antibody also could weakly label the untreated (control) root tissue (Fig. 6A). The number of labeled gold particles found incase of Cd^{2+} pre-exposed, the co-stressed and untreated control seedlings to be 105.75 ± 26.69, 82.75 ± 37.73 and 6.15 ± 1.62, respectively (Fig. 6A,C,D), whereas gold particles were abundant incase of *Fusarium* infected root tissues

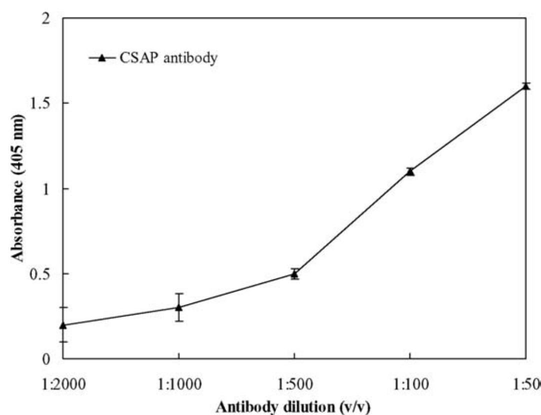


Fig. 4. ELISA for CSAP-antibody in sequential dilution.

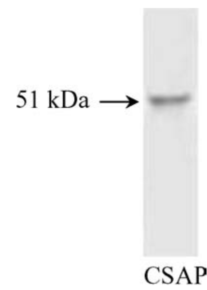


Fig. 5. Western-blot analysis of CSAP.

(Fig. 6E). The localization of CSAP was found primarily at the plasma membrane close to cell wall (Fig. 6C). However, in the *Fusarium* infected root tissues, the stress proteins were primarily located in the plasma membranes as well as vacuolar membranes (Fig. 6E). In co-stressed root tissue, the localization of the stress protein was observed mostly at vacuolar membranes (Fig. 6D), besides plasma membrane.

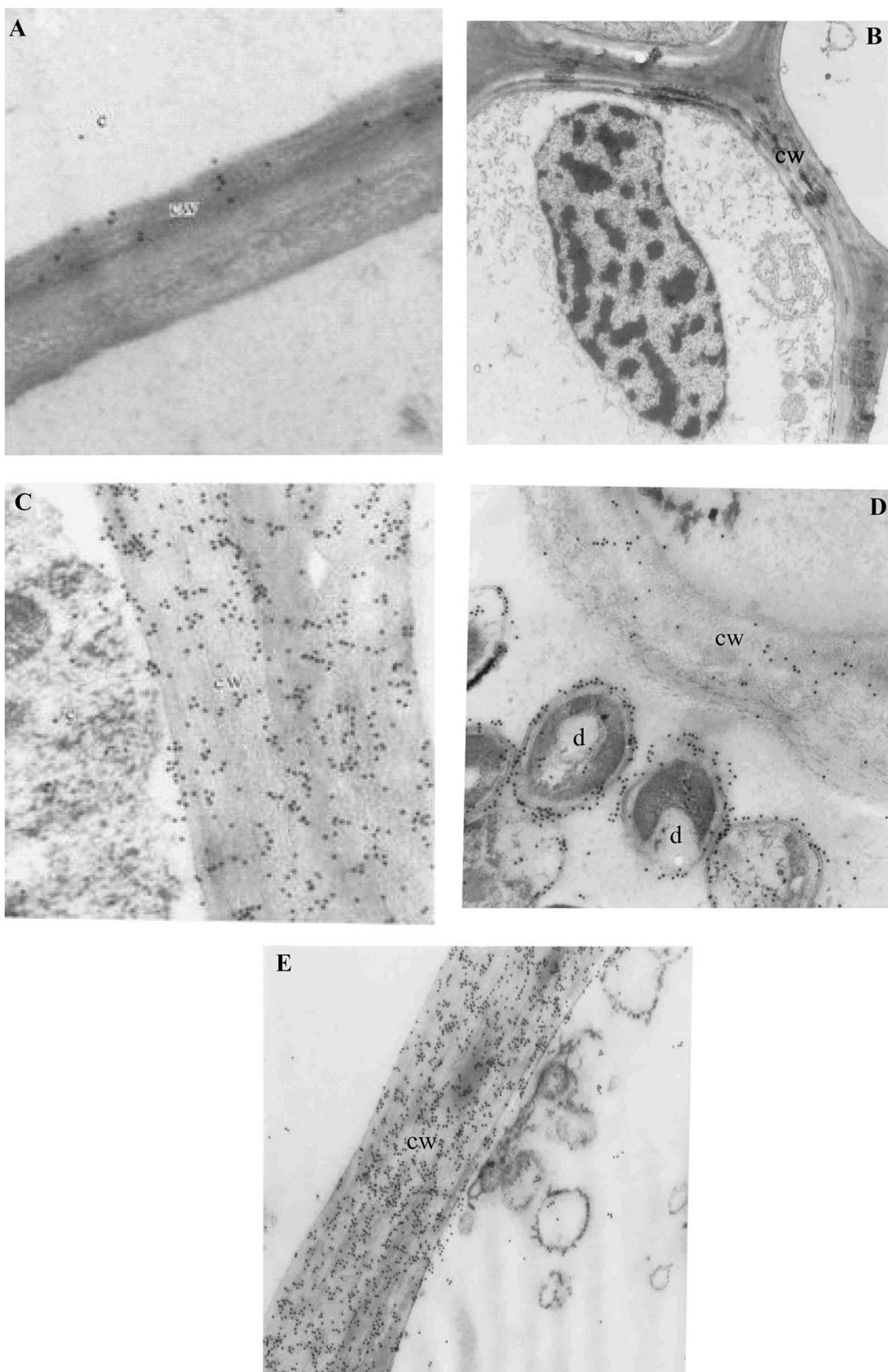
3. Discussion

Wheat seedlings pre-exposed to Cd^{2+} when infected with *Fusarium* spores did not wilt, while the untreated (control) seedlings inoculated with *Fusarium* spores wilted after 7 d of *Fusarium* infection. This clearly suggested that Cd^{2+}-exposure to seeds provided resistance to fungal infection.

Cd^{2+} is known to induce synthesis of PCs, a class of low molecular weight metalchelatin binding proteins that complex Cd^{2+} and reduces the metal toxicity in the cytosol of higher plants [5,27]. However, in the present study, we observed an intense extent of a 51 kDa protein which was over expressed due to cadmium stress besides the induction of a 100 kDa new protein. *Fusarium* infection also produced an intense band at 51 kDa, which appeared to be the same or similar protein alongwith an additional band at 100 kDa. The intense band at 51 kDa induced by *Fusarium* infection, the FISP, was over expressed like that of CSAP. Both CSAP and FISP appeared to be a microsomal protein like that of Al^{3+}-induced protein reported in wheat [1,2]. Interestingly, the SDS-PAGE profile of the co-stressed (50 μM Cd^{2+} pre-exposed and then infected with *Fusarium*) seedlings although showed an induction of similar protein band of same molecular mass (51 kDa), but it was more like CSAP band in appearance on SDS-PAGE. We also noted that the co-stress did not induce the 100 kDa protein band.

As the 51 kDa band in the co-stressed plants looked more to CSAP than FISP, we investigated its cellular localization by using CSAP antibody. The localization pattern indicated that CSAP could have some role in protecting *Fusarium* infection. On a careful examination of transmission electron micrographs, the localization of respective stress proteins was mostly restricted near the plasma membrane and vacuolar membrane of wheat tissues.

This observation on stress protein localization is similar with the results of earlier workers [3,8,11,16,25,31]. The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immunolabeling of the CSAP and FISP suggest a possibility that the same protein may be synthesized in the cytosol then trafficked and finally get deposited at the inner membrane of the cell wall (Fig. 6C–E). However, the most intriguing aspect of observation of the present study is that the anti-CSAP-antibody also could bind to FISP, which suggest that either both (CSAP and FISP) are same proteins or they have same recognizing epitopes. We also note that FISP is unlikely to be host specific fungal toxins, which are usually of low molecular mass but may be a fungus induced plant stress protein. This led us to assume that FISP is a similar stress protein with homologous epitope. However, establishment of similarity of CSAP with FISP would need further investigation.

Although a large number of reports are available on Cd^{2+}-induced stress tolerance in various plants such as, Azuki beans [21], *Arabidopsis ecotypes* [15] and Indian mustard [35], the expression of any high molecular mass protein in response to Cd^{2+}-exposure in wheat has not been reported so far. We also considered if the residual Cd^{2+} contained in the seedlings of Cd^{2+}-exposed seeds to provide resistance when these plants were challenged with *Fusarium*. The residual Cd^{2+} concentration in these plants was far too low to serve as a fungicide. In an earlier work, the inhibitory effect of Cd^{2+} concentration on the growth of potentially pathogenic fungi was reported to be more than 1 µg ml^{-1} [23]. Thus, the survival of the 50 µM Cd^{2+}-exposed plants against *Fusarium* infection would be due to biosynthetic induction of CSAP types of protein and/or changes in the metabolic intermediates, and not by the residual Cd^{2+} present.

Although the exact mechanism of how a CSAP or other similar protein could provide resistance to fungal infection needs further investigation, we would like to propose that Cd^{2+}-triggered pre-development of CSAP across the plasma membrane suppresses the development of *Fusarium*-induced protein which may be the causative reason of the *Fusarium*-induced wilting in wheat. This type of development of plant immunity by mild dose of heavy metal would be new.

4. Methods

4.1. Plant material and treatment with $CdCl_2$

Wheat (*Triticum aestivum* var Sonalika) seeds were surface-sterilized by washing with diluted detergent (Teepol B-300) for 30 min with constant shaking and then they were kept in running water for 5 min, followed by successive five washes in sterile distilled water. Seeds were then treated with 4% sodium hypochlorite for 5 min and washed five times with sterile distilled water for 5 min each. One hundred surface-sterilized seeds were exposed to 50 µM Cd^{2+} as $CdCl_2$ in separate beakers at room temperature (22 °C) for 48 h and equal numbers of seeds were kept in sterile distilled water for same period to serve as untreated control. Subsequently, the Cd^{2+}-exposed seeds were washed off of $CdCl_2$ three times washing with sterile metal free distilled water and were sown along with control seeds on filter paper saturated with sterile distilled water and incubated at 26 °C in the dark. Three days later, the germinated seedlings selected (10 each) for uniform growth were transplanted into sterile glass jars containing MS medium (Sigma) and kept in a growth chamber at 26 °C and 80% relative humidity during the 14 h light period (two 240 W tube light at a height of 60 cm), and at 22 °C and 70% relative humidity during the 10 h dark period for 4 more days. Before transplantation the residual Cd^{2+} concentration was estimated to be 0.6 µM g^{-1} fresh weight of seedlings.

4.2. Seedling growth

Seven days old untreated (control) and $CdCl_2$ pre-exposed seedlings with a well-developed root system were grown in test tubes containing liquid MS (1/10 strength) medium supplemented with 4-day-old freshly cultured *F. oxysporum* (1×10^6) inoculums. The mild dose Cd^{2+} pre-exposed seedlings maintained in tubes containing *F. oxysporum* with MS basal medium were considered as test plants while the only Cd^{2+}-exposed seedlings served as comparative control. Seedlings without Cd^{2+} pre-exposure (control) and seedlings grown in only *F. oxysporum* inoculums with MS basal medium were also maintained for comparison. A total 48 number of seedlings were used per each set of experiment and repeated for three times. All test and control plants were incubated at 26 ± 2 °C. Two seedlings per tubes were considered for better observation. The tolerance of the plant assessed was based on the survival and growth of the seedlings under stressed conditions. Growth and survival data were recorded for a period of another 7 d.

4.3. Soluble protein estimation and dialysis

Fourteen days old germinated seedlings (500 mg) were removed from the seeds and crushed with 10 mM sodium phosphate buffer (pH 7.55). Samples were then centrifuged at 10,000 rpm (2500 g) using a centrifuge for 10 min (Remi). The supernatant containing protein was collected and pellet was washed, recentrifuged and then discarded. Protein was

Fig. 6. Root sections of Cd^{2+} (50 µM)-exposed, *Fusarium* infected, co-stressed and untreated (control) wheat seedlings labeled with anti-CSAP-antibody and detected against GAR-antibody labeled with 15 nm gold particles. **A**, cell membrane of untreated (control) wheat root section showing very little labeling, X 33350; **B**, control section of Cd^{2+}-exposed wheat root in which anti-CSAP-antiserum was replaced with the pre-immune rabbit serum, X 9860; **C**, cell membrane of wheat root section showing labeling, X 45000; **D**, dictyosome-derived vesicles like structure near the cell wall in root section of co-stressed wheat seedlings. Note heavy labeling associated with the vesicle membrane, X 24000; **E**, cell membrane of *Fusarium* infected wheat root section showing heavy labeling (abundant). c, cytoplasm; cw, cell wall; d, dictyosome.

estimated according to Lowry et al. [18]. Protein samples were dialyzed against the same phosphate buffer with two changes for 24 h each and finally concentrated by using poly-ethylene-glycol (PEG-60).

4.4. Characterization and electro-elution of protein

Extracted protein was characterized on a 10% SDS-PAGE [14] and the molecular weight of the particular protein band of control (without treatment), *Fusarium* treated, Cd^{2+} (50 μM)-exposed and co-stressed (50 μM Cd^{2+} pre-exposed and then infected with *F. oxysporum*) seedling was determined from their Rf values. The particular protein band of interest, which was expressed dominantly due to stress induction was cut out from the particular gels and the protein was electro-eluted in a Bio-rad (Electro-eluter) apparatus, using the electro-elution buffer (Tris 25 mM, glycine 192 mM and SDS 0.1%, pH 8.3) at a constant current of 8–10 mA per glasstube for 5 h. The eluted protein was collected, and concentration was estimated [18] and again ran on a 10% SDS-PAGE [14] to establish its homogeneity.

4.5. Preparation of anti-cadmium stress protein antiserum

Antibodies against CSAP were raised in rabbit following the procedure described by Walzel et al. [32]. The immune serum obtained was used directly as polyclonal antibody without further purification.

4.6. Enzyme-linked immunosorption assay (ELISA)

ELISA was performed following the method of Haines and Patel [9]. Protein A-alkaline phosphatase was used as secondary probe and plates were incubated at 37 °C for 1 h. Plates were then developed using *p*-nitro phenyl phosphate (*p*NPP) as substrate. After incubation at room temperature, 100 µl 3 N NaOH stopping solution was added to each well. Plate was read at 405 nm on an ELISA reader (Bio-rad, model 550 micro plate reader).

4.7. Western-blot analysis

Purified CSAP samples were electro-blotted on to nitrocelluolose membranes using 20 mM Tris–HCl (pH 8.3) containing 150 mM glycine and 20% methanol was used as the electrode buffer in Mini Trans Blotter (Bio-rad, Germany) following the procedure of Towbin et al. [28]. Immunodetection was done according to Zemel and Gepstein [33]; blot was incubated with anti-CSAP-antiserum (1:100 dilution) and peroxidase goat-anti-rabbit (GAR) antibody was used as secondary antibody. Amino-ethylcarbazole was used as substrate.

4.8. Tissue preparation for microscopy

The root tips (0.5 cm from the tip) of wheat seedlings were aseptically excised with a scalpel and fixed in 0.5% glutaraldehyde (Sigma) and 2.0% *p*-formaldehyde in 0.1 M phosphate buffer (Na^+ salt), pH 7.2 ± 0.2, for 12 h at 4 °C. The tissue was then further processed for making blocks following the method described by Roy et al. [26]. Transverse sections (1 µm) from the root tip end were stained with toludine blue for localization of area of interest. For immunolocalization of cadmium stress protein, ultra thin sections (60–90 nm) were cut on UCT ultramicrotome (Leica Mikrosysteme Gmbh, Vienna, Austria) and collected on circular 400 mesh nickel grids.

4.9. Immunolabeling procedure for electron microscopy

Grids were processed for immunolabeling as described by Sinha Roy et al. [26]. GAR-gold conjugated antibody (British Biocell Institute, Cardiff, UK) at 1:50 dilution was used. Images were recorded on a 35 mm Phillips CM-10 camera. To test the specificity of immuno-gold localization, two controls were used. One set was stained with pre-immune rabbit serum instead of anti Cd^{2+}-stress protein-antibody and the other was stained only with GAR-gold conjugate. Transmission electron micrographs were evaluated for the number of gold particles per $µm^2$ in the photograph. Mean (± S.D.) were calculated and compared.

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