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Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes

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ABSTRACT

The effects of cadmium (Cd) on the accumulation of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) in leaves of *Phaseolus aureus* and *Vicia sativa* were investigated. Cadmium at 100 μM significantly increased the production of $O_2^{\bullet-}$ and H_2O_2, as well as the activities of plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and the symplastic and apoplastic activities of superoxide dismutase and ascorbate peroxidase in the leaves of both species. Apoplastic guaiacol peroxidase activity was significantly induced in the leaves of both species, particularly in *P. aureus* exposed to 100 μM Cd. Experiments with diphenylene iodonium as an inhibitor of NADPH oxidase and NaN_3 as an inhibitor of peroxidase showed that the majority of Cd-induced reactive oxygen species production in the leaves of both species may involve plasma membrane-bound NADPH oxidase and apoplastic peroxidase. Compared to *V. sativa*, increases in Cd-induced production of $O_2^{\bullet-}$ and H_2O_2 and activities of NADPH oxidase and apoplastic peroxidase were more pronounced in *P. aureus*. In contrast, *V. sativa* had higher leaf symplastic superoxide dismutase and ascorbate peroxidase activities than *P. aureus*. The results indicated that *V. sativa* was more tolerant to Cd than *P. aureus*.

1. Introduction

Heavy metal contamination is a serious environmental problem that limits crop production and threatens human health through the food chain. Cadmium (Cd) is one of the most toxic environmental pollutants for plants. Cd can interfere with numerous biochemical and physiological processes including photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake [1,2]. However, the mechanisms involved in its toxicity are still not completely understood. Cd toxicity is mediated by the formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) even though Cd is not a redox-active metal [3]. ROS are highly toxic and can oxidize biological macromolecules such as lipids, proteins, and nucleic acids, thus causing lipid peroxidation, membrane damage, and inactivation of enzymes.

To avoid the deleterious effects of ROS, plants have evolved antioxidant defense mechanisms. These include enzymatic components such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), and glutathione reductase (GR, EC 1.6.4.2); as well as non-enzymatic components, such as ascorbate (ASC) and glutathione (GSH; [4]). Superoxide anions generated in plants are dismutated to H_2O_2 by the action of SOD. H_2O_2 is scavenged by CAT and the ascorbate–glutathione cycle where APX reduces it to H_2O. GR also plays a key role in the antioxidant defense processes by reducing oxidized glutathione to GSH. These defensive mechanisms against oxidative damage have been specifically observed in plants subjected to Cd stress [5–12]. However, the response to Cd of antioxidant enzymes remains controversial and greatly depends on the plant species, age, duration of treatment, and experimental conditions [3].

Accumulation of ROS may be the consequence of a disruption in the balance between their production and the antioxidant system activity. In plant cells and tissues, ROS can be produced by several enzymatic systems [4]. Plasma membrane (PM)-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are believed to be responsible for $O_2^{\bullet-}$ production under biotic and abiotic stress conditions [13–18]. These enzymes can use cytosolic NADPH to produce $O_2^{\bullet-}$, which is rapidly dismutated to H_2O_2 by SOD. The involvement of NADPH oxidases in Cd-induced production of ROS has also been demonstrated in tobacco cells [19], pea leaves [20], pea roots [21], rice leaves [22], and rice roots [23].

In previous studies, we investigated the effect of different Cd concentrations on growth and the antioxidative metabolism of two plant species, *Phaseolus aureus* and *Vicia sativa* [24,25]. Under these conditions, Cd promoted oxidative stress in the two species by

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increasing the accumulation of $O_2^{\bullet-}$ and H_2O_2. During the 12-day Cd treatment, the activities of SOD, CAT, and APX initially increased and then decreased in the leaves of these plants. Compared to *V. sativa*, production of ROS was higher, while activity of the antioxidant enzymes was lower, in *P. aureus* leaves.

Although many reports have investigated Cd-induced oxidative stress and antioxidant response, relatively little information is available about the ability of heavy metal stress to induce the production of ROS in the apoplast and the roles of apoplastic antioxidant enzymes [18,26,27]. This information is important since in plant cells subjected to heavy metal toxicity, initial events most likely occur externally in the apoplast-cell membrane space. Here we investigated the effect of Cd on the production of ROS in the leaves of *P. aureus* and *V. sativa* using both histochemical and cytochemical methods. We also studied the roles of different antioxidant enzymes in the symplast and apoplast of plant leaves in the defense against harmful effects of Cd stress.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *P. aureus* and *V. sativa* were surface-sterilized with 0.1% $HgCl_2$ and soaked in distilled water at room temperature for 12 (*P. aureus*) or 24 h (*V. sativa*). The seeds were germinated in plastic dishes filled with vermiculite. After 3 d, the plants were transferred to vessels containing Hoagland nutrient solution (1 mM KH_2PO_4, 1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 20 μM Fe–EDTA, 46 μM H_3BO_3, 9 μM $MnCl_2$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.11 μM H_2MoO_4) and grown under controlled environmental conditions (14 h day length with photosynthetically active radiation of 400 μmol m^{-2} s^{-1} and 25/20 °C day/night temperatures).

Nine-day-old plants were treated with various regents. In Treatment 1, the plants were pretreated with the full nutrient solution containing 20 μM diphenylene iodonium (DPI, a NADPH oxidase inhibitor), 1 mM NaN_3 (sodium azide, a peroxidase inhibitor), 1 mM $LaCl_3$ (a Ca^{2+} channel inhibitor), 5 μM cantharidin (CANT, a protein-phosphatase inhibitor), or 200 μM 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, a guanylate cyclase inhibitor) for 24 h. The plants were then exposed to a nutrient solution containing 100 μM $CdCl_2$ for 6 d. Twenty leaves (the second youngest) in each treatment were collected for localization of H_2O_2 and $O_2^{\bullet-}$ production *in situ* and cytochemical detection of H_2O_2 and $O_2^{\bullet-}$. Control plants were grown in full nutrient solution without any pretreatment or Cd. In Treatment 2, the plants were grown in full nutrient solution with 100 μM $CdCl_2$ for 6 d, and were then treated with 0, 5, 10, or 20 μM DPI for 24 h. The second youngest leaves were collected for analysis of plasma-membrane NADPH oxidase activities by native polyacrylamide gel electrophoresis (PAGE). Plants grown in full nutrient solution without Cd and DPI served as controls. In Treatment 3, the plants were grown in full nutrient solution without (control) or with 100 μM $CdCl_2$ for 6 d. The second youngest leaves were collected for isolation of apoplastic fluids and the symplast.

The experiment was a completely randomized design with six replicate vessels for each treatment. The pH of the nutrient solution was adjusted to 5.5 with NaOH or HCl, and the nutrient solution was renewed every other day and aerated continuously.

2.2. Superoxide anion and hydrogen peroxide localization in situ

Hydrogen peroxide was visually detected in the leaves using 3,3-diaminobenzidine (DAB) as the substrate [28]. The $O_2^{\bullet-}$ formation in the leaves was visually detected by infiltration with nitroblue tetrazolium (NBT), resulting in visible stains as reported by Romero-Puertas et al. [20].

To check the specificity of DAB-staining for H_2O_2 and NBT-staining for $O_2^{\bullet-}$, the leaves from plants treated with 100 μM $CdCl_2$ for 6 d were immersed in 1 mM ASC (an H_2O_2 scavenger) or 1 mM tetramethylpyrazine (TMP, an $O_2^{\bullet-}$ scavenger) solution for 8 h before the infiltration of DAB or NBT.

H_2O_2 and $O_2^{\bullet-}$ deposits were quantified by measuring the number of pixel of spots by PHOTOSHOP 7.0. Results are expressed as percentage of spot area versus total leaf area in pixels.

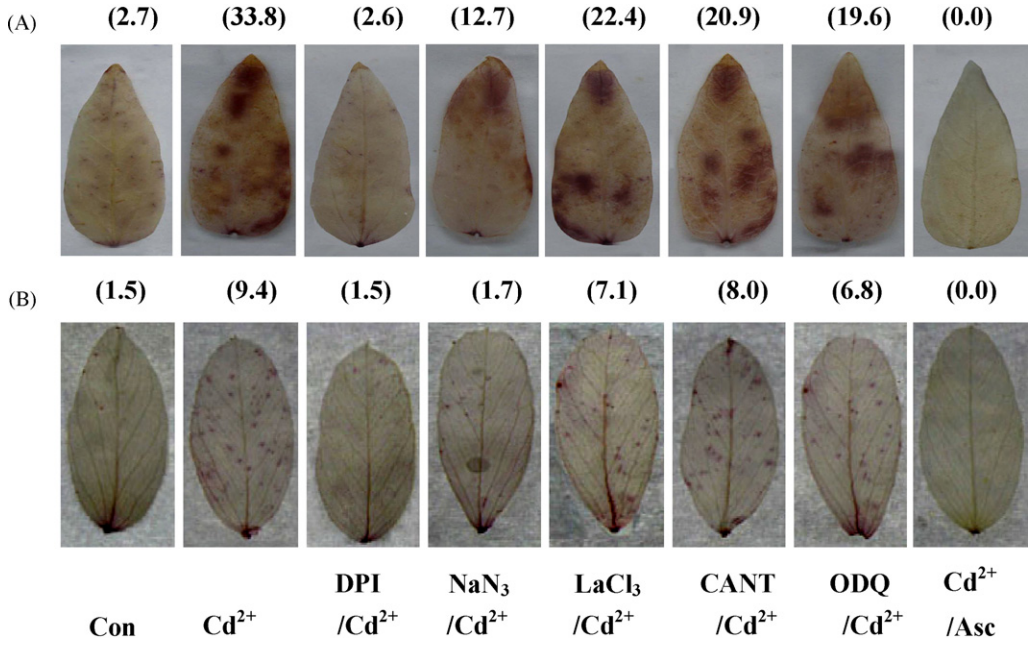

Fig. 1. Effects of inhibitor (DPI, NaN_3, $LaCl_3$, CANT, and ODQ) pretreatment and the H_2O_2 scavenger ASC on the production of Cd-induced H_2O_2 in leaves of *P. aureus* (A) and *V. sativa* (B). The plants were pretreated with 20 μM DPI, 1 mM NaN_3, 1 mM $LaCl_3$, 5 μM CANT, or 200 μM ODQ for 24 h, and then treated with 100 μM $CdCl_2$ for 6 d before infiltrating with DAB. The control plants were grown in full nutrient solution with no pretreatment or Cd. For ASC treatment, the leaves from plants treated with 100 μM $CdCl_2$ for 6 d were immersed in 1 mM ASC solution for 8 h before infiltrating with DAB. H_2O_2 deposits were quantified by measuring the number of pixel of spots by PHOTOSHOP 7.0. Results are expressed as percentage of spot area versus total leaf area in pixels. Experiments were repeated at least three times with similar results.

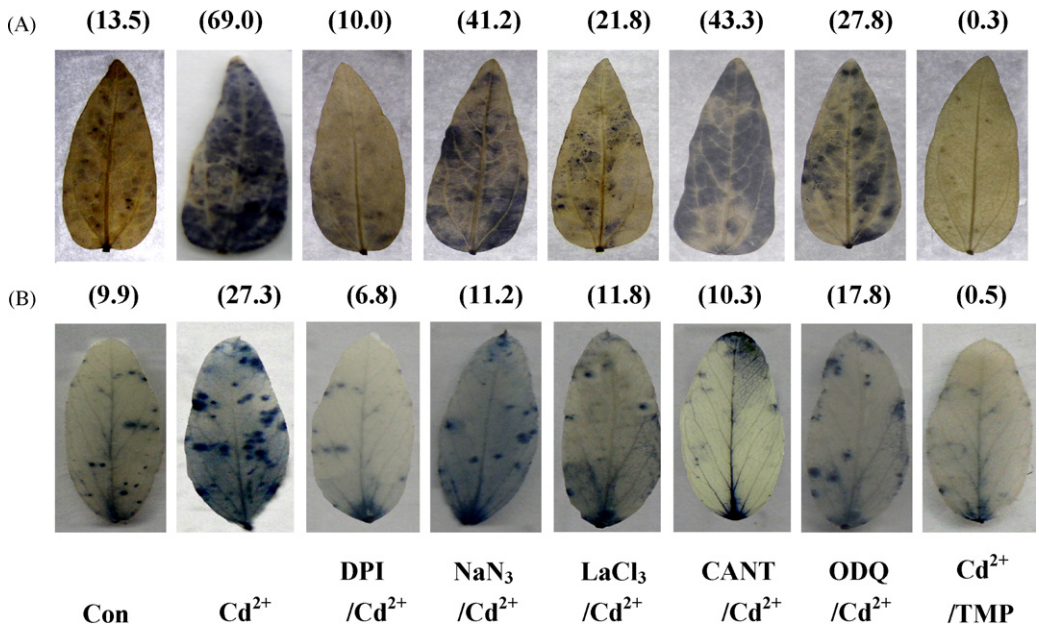

Fig. 2. Effects of inhibitor (DPI, NaN_3, $LaCl_3$, CANT, and ODQ) pretreatment and the H_2O_2 scavenger ASC on the production of Cd-induced $O_2^{\bullet-}$ in leaves of *P. aureus* (A) and *V. sativa* (B). The plants were pretreated with 20 μM DPI, 1 mM NaN_3, 1 mM $LaCl_3$, 5 μM CANT, or 200 μM ODQ for 24 h, and then treated with 100 μM $CdCl_2$ for 6 d before infiltrating with NBT. The control plants were grown in full nutrient solution with no pretreatment or Cd. For TMP treatment, the leaves from plants treated with 100 μM $CdCl_2$ for 6 d were immersed in 1 mM TMP solution for 8 h before infiltrating with NBT. $O_2^{\bullet-}$ deposits were quantified by measuring the number of pixel of spots by PHOTOSHOP 7.0. Results are expressed as percentage of spot area versus total leaf area in pixels. Experiments were repeated at least three times with similar results.

2.3. Cytochemical detection of hydrogen peroxide and superoxide anion

For the subcellular location of H_2O_2, the cytochemical method based on the generation of $CeCl_3$ precipitates developed by Bestwick et al. [29]. The method used for the localization of the superoxide anion $O_2^{\bullet-}$ was based on the generation of DAB precipitates in the presence of Mn^{2+} ions and $O_2^{\bullet-}$ [30]. Briefly, leaf pieces (approximately 0.5 mm × 0.5 mm) were cut from the treated and untreated plants and incubated for 1 h in freshly prepared 5 mM $CeCl_3$ in 50 mM MOPS buffer (pH 7.2) for detection of H_2O_2, or incubated for 1 h in freshly prepared 0.1 M HEPES buffer (pH 7.2) containing 2.5 mM DAB, 0.5 mM $MnCl_2$ and 1 mM Na-azide for detection of $O_2^{\bullet-}$ [20]. As controls, leaf pieces were incubated in the MOPS buffer without $CeCl_3$ or in the HEPES buffer without DAB or $MnCl_2$. Then the leaves sections were fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 1 h. The tissues were washed with the same buffer without fixatives three times for 10 min, and post-fixed for 45 min in 1% (v/v) OsO_4, dehydrated in a graded ethanol series (30–100%; v/v) and then embedded in Eponaraldite (Agar Aids, Bishop's, UK). After 12 h in pure resin, followed by a change of fresh resin for 4 h, the samples were polymerized at 60 °C for 48 h. Blocks were sectioned (70–90 nm) on a Reichert-Ultracut E microtome and mounted on uncoated copper grids (300 mesh). Sections were examined using a transmission electron microscope at an accelerating voltage of 75 kV.

2.4. Isolation of the plasma membrane (PM)

PM-enriched fractions from leaves were isolated immediately after leaf harvesting as described by Buckout et al. [31] with some modification. Briefly, the leaves were homogenized in a pre-cooled mortar with 2.5 volumes of freshly prepared medium containing 250 mM sucrose, 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)–Tris buffer (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.5% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth and then centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was recovered and centrifuged at 100,000 × *g* for 50 min at 4 °C to obtain a microsomal membrane pellet. The pellet was gently resuspended in medium with 5 mM phosphate buffer (pH 7.8) containing 0.25 mM sucrose and 2 mM dithiothreitol (DTT), then layered on a discontinuous sucrose gradient consisting of 25% (w/w) sucrose layered over 38% (w/w) sucrose and centrifuged at 13,000 × *g* for 60 min. The PM pellets were quickly frozen in liquid N_2 and stored at −70 °C until used for the enzyme assays. To check the degree of enrichment of the PM in our preparations, vanadate-sensitive (100 mM vanadate), nitrate-sensitive (100 mM KNO_3), and azide-sensitive (1 mM NaN_3) H^+-ATPase activities (PM, tonoplast, and mitochondrial enzyme markers, respectively) were determined in PM vesicles obtained using the above described procedures.

2.5. Determination of plasma-membrane NADPH oxidase activity

The plasma-membrane NADPH oxidase activity was assayed by measuring the superoxide dismutase-inhibitable and NADPH-dependent reduction of sodium 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) by $O_2^{\bullet-}$. The procedure was performed according to the methods of Sagi and Fluhr [32].

2.6. In-gel assays of plasma-membrane NADPH oxidase activity

30 μg of PM proteins were subjected to native PAGE in 7.5% (w/v) polyacrylamide separating gels and 4% (w/v) stacking gels. The PM NADPH oxidase activities were assayed in the gels using the NBT method [32].

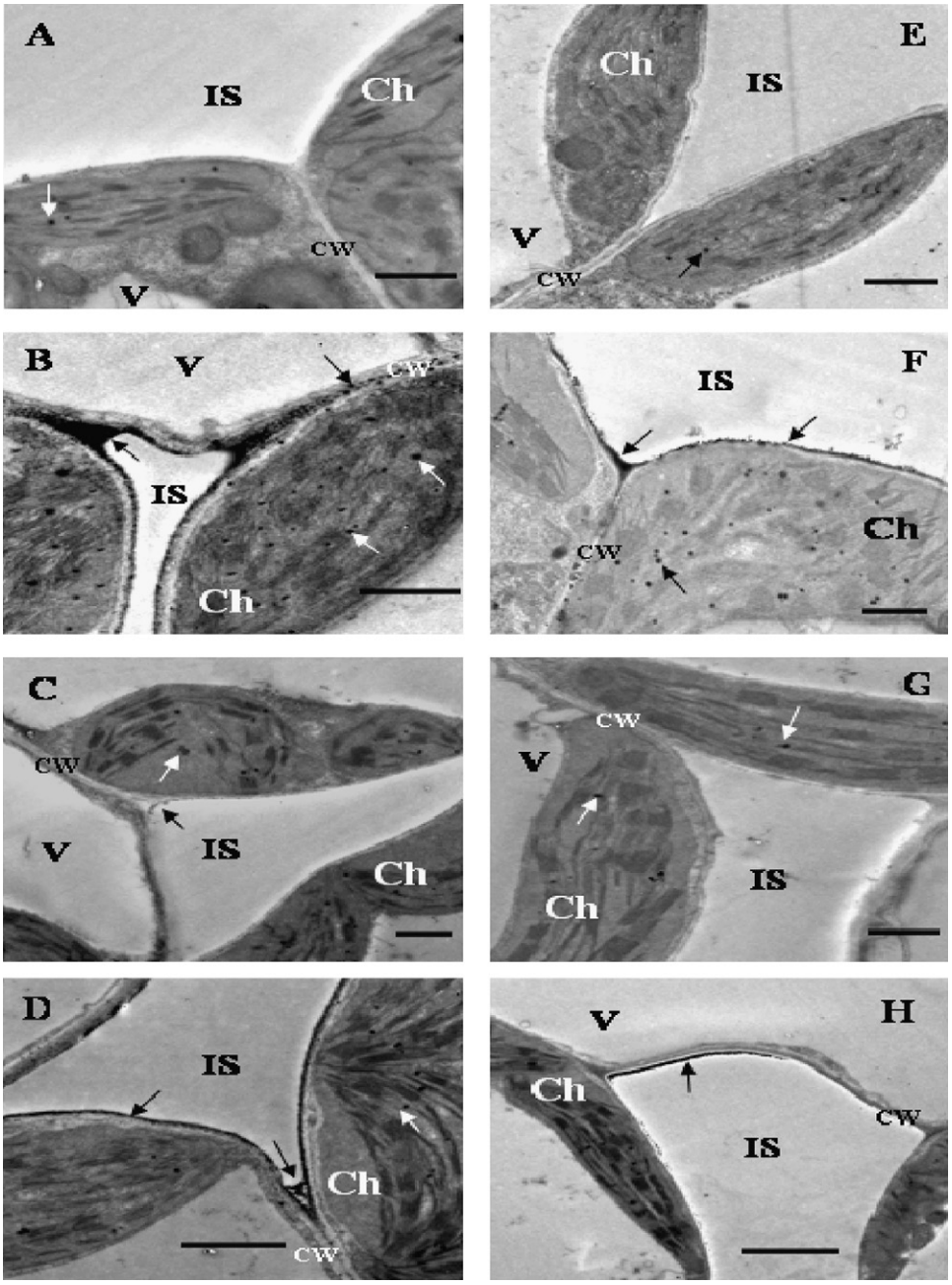

Fig. 3. Cytochemical localization of hydrogen peroxide accumulation in leaf mesophyll cells of *P. aureus* (A–D) and *V. sativa* using $CeCl_3$-staining (E–H). Plants were pretreated with various inhibitors for 24 h and subsequently exposed to 100 μM $CdCl_2$ for 6 d. (A and E) Control plants with no pretreatment or Cd. (B and F) Cd-treated plants with no pretreatment. (C and G) Cd-treated plants pretreated with 20 μM DPI. (D and H) Cd-treated plants pretreated with 1 mM NaN_3. Experiments were repeated at least three times with similar results. Ch, chloroplast; CW, cell wall; V, vacuole; IS, intercellular space. Bar = 1 μm.

2.7. Isolation of apoplastic fluids and the symplast

Apoplastic fluid was extracted by a vacuum infiltration/centrifugation technique similar to that reported by Vanacker et al. [33]. About 2 g of fresh tissues were washed quickly in distilled water, placed in a Petri dish filled with a solution of 10 mM sodium phosphate buffer, pH 6, 1% (w/v) PVPP, 1 mM EDTA, and 0.5 mM PMSF, and then submitted to vacuum for 10 min at 4 °C. Afterwards, the tissues were blotted dry with filter paper and placed in syringes, which were then placed in centrifugation tubes. Tissues were centrifuged at 1500 × *g* for 5 min and the apoplastic fluid recovered at the bottom of the tubes. Tissues remaining in the syringes were the symplast. According to the activity of the cellular marker enzyme glucose-6-phosphate dehydrogenase (G6PDH) in the apoplastic fluid, the contamination of the apoplastic fluid by cytosolic protein was less than 1%.

2.8. Assay of antioxidant enzyme activities

Soluble proteins were extracted with ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% PVPP (w/v). In the case of APX assays, 1 mM ASC was also added to the extraction solution. CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm [34]. CAT activity was expressed as μmol H_2O_2 decomposed min^{-1} mg^{-1} fresh weight (FW). APX activity was measured by monitoring the decrease in the absorbance at 290 nm as ASC was oxidized, as described by Nakano and Asada [35]. The activity of APX was calculated as μmol ASC oxidized

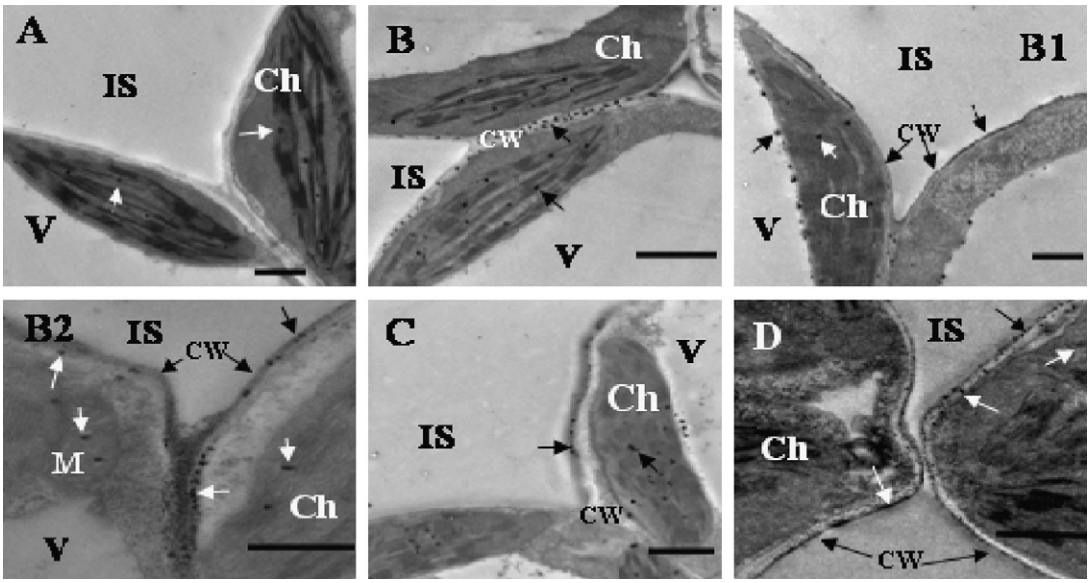

Fig. 4. Cytochemical localization of superoxide anion ($O_2^{\bullet -}$) accumulation in leaf mesophyll cells of *P. aureus* using with DAB-staining. Plants were pretreated with various inhibitors for 24 h and subsequently exposed to 100 μM $CdCl_2$ for 6 d. (A) Control plants with no pretreatment or Cd. (B, B1 and B2) Cd-treated plants with no pretreatment. (C) Cd-treated plants pretreated with 20 μM DPI. (D) Cd-treated plants pretreated with 1 mM NaN_3. Experiments were repeated at least three times with similar results. Ch, chloroplast; CW, cell wall; V, vacuole; IS, intercellular space. Bar = 1 μm.

min^{-1} mg^{-1} FW. POD activity was determined by measuring the change in the absorption at 470 nm according to Zheng and Van Huystee [36]. The activity of POD was calculated as μmol tetraguaiacol min^{-1} mg^{-1} FW. SOD activity was determined by its ability to inhibit the formation of nitroblueformazan from NBT according to the method of Giannopolitis and Ries [37]. In this assay, SOD activity was expressed as units (U) mg^{-1} FW; one unit of SOD was defined as the amount required to inhibit the photoreduction of NBT by 50%.

2.9. Protein assay

Protein content was estimated according to Bradford [38] using bovine serum albumin as the standard.

2.10. Statistical analysis

Data were analyzed using the programs of SPSS 10.0 (the Statistical Package for the Social Science for Windows 10.0). All the values reported in this paper are the means of three replicates. Statistical assays were carried out by analysis of variance (ANOVA) test. Significant differences among means were determined at $p < 0.05$.

3. Results

3.1. Effect of Cd on the production of H_2O_2 and $O_2^{\bullet -}$ in leaves

The accumulation of H_2O_2 in the leaves of *P. aureus* and *V. sativa* was studied using histochemical DAB-staining. Results showed that exposure to 100 μM Cd for 6 d caused a significant accumulation of H_2O_2 in the leaves of both plant species (Fig. 1). Cd-induced H_2O_2 production was more pronounced in *P. aureus* than in *V. sativa*. However, H_2O_2 accumulation in the leaves from plants treated with 100 μM Cd was decreased by pretreatment with 20 μM DPI (an inhibitor of NADPH oxidase), 1 mM NaN_3 (an inhibitor of peroxidase), 1 mM $LaCl_3$ (an inhibitor of Ca^{2+} channel), 5 μM CANT (an inhibitor of protein phosphatase), or 200 μM ODQ (an inhibitor of guanylate cyclase). Moreover, the production of Cd-induced H_2O_2 could be decreased by infiltration with the H_2O_2 scavenger ASC, demonstrating the specificity of the DAB-staining reaction for H_2O_2.

To detect the accumulation of $O_2^{\bullet -}$ and the source of H_2O_2 in leaves of both species exposed to excess Cd, the leaves were stained with NBT. Results revealed that exposure to 100 μM Cd caused a significant accumulation of $O_2^{\bullet -}$ in the leaves (Fig. 2). Furthermore, the Cd-induced accumulation of $O_2^{\bullet -}$ in leaves could be decreased by treatment with DPI, NaN_3, $LaCl_3$, CANT, or ODQ. Infiltration with

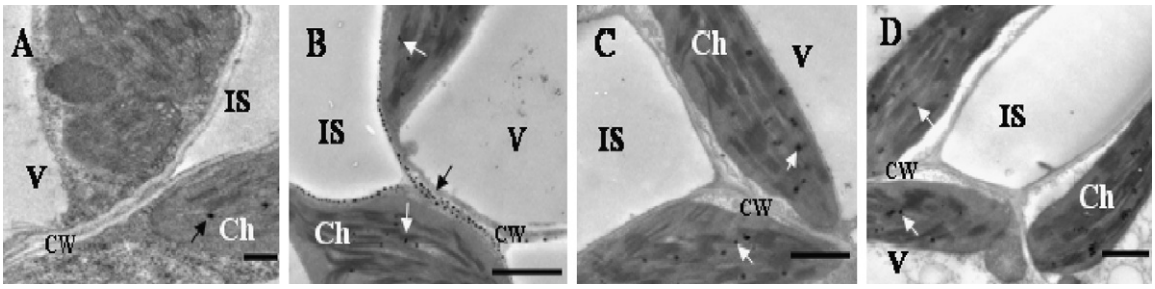

Fig. 5. Cytochemical localization of superoxide anion ($O_2^{\bullet -}$) accumulation in leaf mesophyll cells of *V. sativa* using with DAB-staining. Plants were pretreated with various inhibitor for 24 h and subsequently exposed to 100 μM $CdCl_2$ for 6 d. (A) Control plants with no pretreatment or Cd. (B) Cd-treated plants with no pretreatment. (C) Cd-treated plants pretreated with 20 μM DPI. (D) Cd-treated plants pretreated with 1 mM NaN_3. Experiments were repeated at least three times with similar results. Ch, chloroplast; CW, cell wall; V, vacuole; IS, intercellular space. Bar = 1 μm.

Table 1
Effects of the inhibitors Na_3VO_4, $NaNO_3$, and NaN_3 on the H^+-ATPase activity in plasma membrane fractions from leaves of *P. aureus* and *V. sativa*.

Plant	H^+-ATPase activity (μmol Pi h^{-1} mg $protein^{-1}$)[a]			
	Control	$NaNO_3$ (5.0 mM)	NaN_3 (1.0 mM)	Na_3VO_4 (0.1 mM)
P. aureus	42.07 ± 0.30 a (100%)	41.26 ± 0.12 a (98.07%)	40.83 ± 0.23 a (97.05%)	3.49 ± 0.03 b (8.30%)
V. sativa	43.11 ± 0.15 a (100%)	41.92 ± 0.26 a (97.24%)	42.05 ± 0.37 a (97.54%)	3.33 ± 0.02 b (7.72%)

Different letters indicate a significant difference at the same row at $p < 0.05$ according to the Duncan's test.
[a] Values are means ± SE ($n = 3$) of three different experiments.

the $O_2^{\bullet-}$ scavenger TMP decreased the production of Cd-induced $O_2^{\bullet-}$, demonstrating the specificity of the NBT-staining reaction for $O_2^{\bullet-}$.

The cytochemical assay based on the reaction of H_2O_2 with $CeCl_3$ produced electron-dense insoluble precipitates of cerium perhydroxides at sites where H_2O_2 accumulated. In the leaves of both species treated with 100 μM Cd, $CeCl_3$ was clearly localized as a precipitate in cell walls and the corners of the extracellular space as well as a small amount in chloroplasts of the mesophyll cells (Fig. 3B and F). DPI and NaN_3 treatments inhibited the formation of the $CeCl_3$ precipitates (Fig. 3C, D, F, and G). In the control leaves, electron-dense precipitates of cerium perhydroxides were not detectable in the extracellular space and the cell wall (Fig. 3A and E). It was evident that more H_2O_2 was produced in the chloroplasts and extracellular spaces of *P. aureus* than in those of *V. sativa*.

In the leaves of both species treated with 100 μM Cd, $O_2^{\bullet-}$-dependent DAB precipitates were observed mainly in chloroplasts, cell walls, and extracellular spaces, whereas few precipitates were observed in control plants (Figs. 4 and 5). Cd-induced accumulation of $O_2^{\bullet-}$ in chloroplasts, cell walls, and extracellular spaces could be prevented by treatment with DPI and NaN_3. In chloroplasts, cell walls, and extracellular spaces of *P. aureus*, more $O_2^{\bullet-}$-dependent DAB precipitates were observed than in *V. sativa*.

3.2. Effect of Cd on PM NADPH oxidase in the leaves

Membrane fractions were isolated from the leaves of *P. aureus* and *V. sativa* grown in the control medium or 100 μM Cd solution for 6 d. Characterization of membrane vesicles used in this experiment confirmed that the vesicle preparations were highly enriched in PM vesicles (Table 1). Fig. 6 shows that 100 μM Cd treatment significantly increased the activity of plasma membrane NADPH oxidase in the leaves of both species compared to the control. The increase was more pronounced in *P. aureus* than in *V. sativa* (Fig. 6). We also checked plasma-membrane NADPH oxidase activity using native PAGE. As shown in Fig. 7, a major band of activity was observed in control and Cd-treated leaves of both species. The bands from Cd-treated plants (without DPI) were significantly more intense than those from the controls. For the 100 μM Cd-treated plants, treatment with 5–20 μM DPI for 24 h before isolation of PM decreased NADPH oxidase activity. The activity of NADPH oxidase showed an almost complete inhibition by 20 μM DPI.

Table 2 shows the *in vitro* effect of DPI on the NADPH oxidase activity of the PM-enriched fractions of the leaves from control and 100 μM Cd-treated plants. Membrane vesicles were incubated for 10 min at 25 °C with different concentrations of DPI before starting the reaction. Addition of 5 μM DPI to the reaction medium significantly decreased the activity of NADPH oxidase, and 10 μM DPI almost completely inhibited the activity of NADPH oxidase.

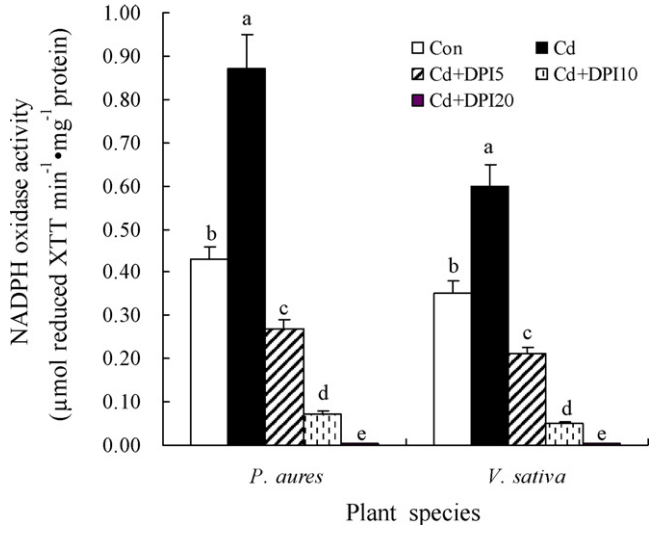

Fig. 6. Effects of Cd and DPI on the activity of the plasma-membrane NADPH oxidase in the leaves of *P. aureus* and *V. sativa*. Plants were grown in full nutrient solution with 100 μM $CdCl_2$ for 6 d and were then treated with 0, 5, 10, or 20 μM DPI for 24 h. Plants grown in the full nutrient solution without Cd and DPI served as controls. Different letters in the same plant species indicate a significant difference at $p < 0.05$ according to the LSD tests.

3.3. Effect of Cd on the activities of antioxidant enzymes in the leaf apoplast and symplast

The total activities of SOD and APX in the leaf symplast of both species were significantly higher than those in the apoplast when calculated on a fresh weight basis (Table 3). In contrast, the total POD activity in the leaf symplast of both species was significantly lower than that in the apoplast. Compared to the control, treatment with 100 μM Cd significantly increased the total activities of SOD and APX in the apoplast and symplast of both species. Cd did not significantly change the POD activity in the leaf symplast of either species, but significantly increased its activity in the apoplast. Leaf apoplastic POD activity increased by 600% and 134%, respectively, in *P. aureus* and *V. sativa* treated with Cd.

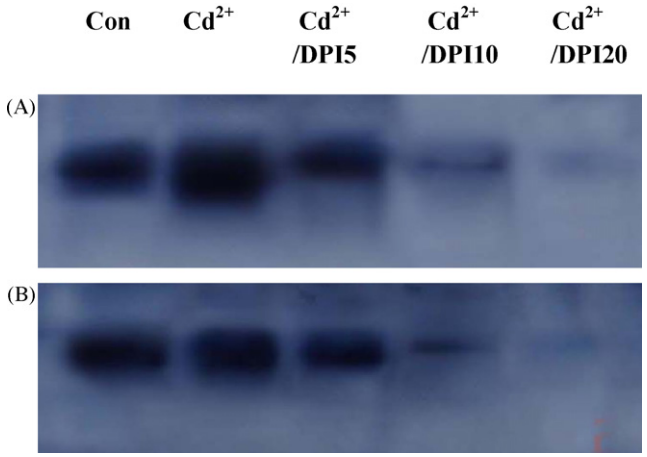

Fig. 7. NADPH oxidase activity of plasma membrane proteins from *P. aureus* and *V. sativa* leaves fractionated by native PAGE. Samples applied to the gels contained the same amount of protein. Plants were grown in full nutrient solution with 100 μM $CdCl_2$ for 6 d and then treated with 0, 5, 10, or 20 μM DPI for 24 h. Plants grown in the full nutrient solution without Cd and DPI served as the control.

Table 2
In vitro effects of DPI on plasma membrane NADPH oxidase activity of Cd-treated *P. aureus* and *V. sativa*.[a].

DPI (μM)	NADPH oxidase activity (μmol reduced XTT min^{-1} mg^{-1} protein)			
	P. aureus		*V. sativa*	
	0	100	0	100
0	0.47 ± 0.03 a (100%)	0.98 ± 0.04 a (100%)	0.46 ± 0.03 a (100%)	0.63 ± 0.04 a (100%)
5	N a	0.23 ± 0.02 b (23.0%)	N a	0.11 ± 0.01 b (17.5%)
10	N a	N b	N a	N b

Values are means ± SE (n = 3) of three different experiments. N: nondectable. Different letters at the same column indicate a significant difference at $p < 0.05$ according to the Duncan's test.

[a] The seedlings were treated with 0 and 100 μM $CdCl_2$ for 6 d.

4. Discussion

Accumulation of H_2O_2 and $O_2^{\bullet-}$ has been observed in some Cd-exposed plant species [5,19–23,39]. In this study, Cd-induced production of H_2O_2 and $O_2^{\bullet-}$ in the leaves of *P. aureus* and *V. sativa* was demonstrated using both histochemical and cytochemical methods. *P. aureus* produced more H_2O_2 and $O_2^{\bullet-}$ than *V. sativa* exposed to toxic Cd. These results are in agreement with our previous work that reported variation in the content of H_2O_2 and $O_2^{\bullet-}$, as measured using a colorimetric method, as well as variation in the level of lipid peroxidation in the leaves of *P. aureus* and *V. sativa* [25]. Cytochemical methods showed that the H_2O_2-dependent $CeCl_3$ precipitates and the $O_2^{\bullet-}$-dependent DAB precipitates were mainly localized in the cell walls of mesophyll cells of both species after Cd treatment. Similarly, the accumulation of H_2O_2 normally took place in the cell walls or the outer side of the PM during the oxidative burst induced by pathogens, ozone, and Cu toxicity [13,15,18]. In Cd-treated tobacco BY-2 cells, H_2O_2 was predominantly located in the PM and in the tonoplast [19]. However, Romero-Puertas et al. [20] reported that accumulation of H_2O_2 induced by Cd was found mainly on the inner side of the PM and tonoplast in pea leaves, and accumulation of $O_2^{\bullet-}$ was found mainly in tonoplasts of bundle sheath cells close to the vascular tissue.

In the leaves of *P. aureus* and *V. sativa* treated with 100 μM Cd, $O_2^{\bullet-}$-dependent DAB precipitates were also observed in chloroplasts. Chloroplasts, the major component of photosynthetic tissue, are highly sensitive to damage by Cd toxicity [6]. ROS are also produced by the reaction of chloroplast O_2 and electrons that escape from the photosynthetic electron transfer system under normal circumstances [4]. Cd inhibits the photoactivation of photosystem II (PSII) by inhibiting electron transfer [40]. Thus, Cd could lead to the generation of ROS indirectly by producing a disturbance in the chloroplasts.

Accumulation of H_2O_2 in Cd-treated plants may be the consequence of disturbing the balance between its production and scavenging. SOD, the first enzyme in the detoxifying process of ROS, converts $O_2^{\bullet-}$ radicals to H_2O_2. The accumulation of H_2O_2 is prevented by CAT, POD, and APX. The expected increase in H_2O_2 as a result of the SOD reaction was accompanied by an increased enzymatic capacity to decompose it. Thus, CAT and APX play an important role in the scavenging process of H_2O_2 when coordinated with SOD activity. Previous results showed that treatment with Cd for 6 d increased the specific activities of SOD, CAT, and APX in leaf homogenates of *P. aureus* and *V. sativa* [25]. Increased SOD, CAT, and APX activities in the leaves indicate that these two plant species have the capacity to adapt to Cd toxicity by developing an antioxidant defense system. Improvement of stress tolerance is often related to an increase in activity of antioxidant enzymes [41,42]. Hsu and Kao [43] reported that early accumulation of H_2O_2 during heat shock signaled an increase in APX and GR activities, which in turn protected rice plants from the oxidative stress caused by Cd. H_2O_2 treatment resulted in an improvement of salt tolerance by alleviation of oxidative damage [44]. Compared to those in *P. aureus*, increased antioxidant enzyme activities were more pronounced in *V. sativa*, whereas Cd-induced lipid peroxidation was lower [25], indicating that *V. sativa* was more tolerant to Cd than *P. aureus*. This was further supported by the fact that *V. sativa* had higher leaf symplastic SOD and APX activities than *P. aureus* (Table 3).

On the other hand, increased SOD, CAT, and APX activities also indicated that production of H_2O_2 induced by Cd in the leaves did not result from the inhibition of major H_2O_2-scavenging enzymes. The increase in the activity of antioxidant enzymes, such as SOD, CAT, APX, and GR, after Cd exposure has also been observed in other plants [5,10,11,39]. In contrast, Cd-induced H_2O_2 accumulation in pea leaves may be a result of the inhibition of the antioxidant enzymes CAT and POD [6]. In leaves of *Tagetes erecta*, Cd depressed the activities of SOD, CAT, APX, and GR [9]. In the less Cd-sensitive pea genotypes, APX activity decreased while the activities of CAT and POD significantly increased [8].

ROS are produced by plant cells via the enhanced enzymatic activity of PM-bound NADPH oxidases, cell-wall-bound peroxidases, and amine oxidases in the apoplast under biotic and abiotic stress conditions [4]. DPI is a relatively specific inhibitor for NADPH oxidase [45]. DPI prevents the production of H_2O_2 induced by Cd in cultured tobacco BY-2 cells [19], pea leaves [20] and roots [21], and

Table 3
Effect of Cd on the activities of antioxidant enzymes in the apoplast and symplast of *P. aureus* and *V. sativa* leaves.[a].

Plant	Antioxidant enzymes	Symplast		Apoplast	
		0 μM Cd^{2+}	100 μM Cd^{2+}	0 μM Cd^{2+}	100 μM Cd^{2+}
P. aureus	SOD (U g^{-1} FW)	1.69 ± 0.11 a (94.41%)	3.46 ± 0.02 b (86.07%)	0.10 ± 0.01 a (5.59%)	0.56 ± 0.03 b (13.93%)
	APX (μmol min^{-1} g^{-1} FW)	33.60 ± 2.31 a (96.34%)	44.45 ± 4.55 b (93.86%)	1.27 ± 0.01 a (3.66%)	2.91 ± 0.02 b (6.14%)
	POD (μmol min^{-1} g^{-1} FW)	5.84 ± 0.03 a (40.87%)	6.09 ± 0.02 a (10.73%)	8.45 ± 0.04 a (59.13%)	50.67 ± 3.33 b (89.27%)
V. sativa	SOD (U g^{-1} FW)	2.43 ± 0.01 a (96.05%)	5.02 ± 0.03 b (94.54%)	0.10 ± 0.01 a (3.95%)	0.29 ± 0.02 b (5.46%)
	APX (μmol min^{-1} g^{-1} FW)	42.93 ± 3.26 a (82.29%)	69.90 ± 6.00 b (58.52%)	9.24 ± 0.50 a (17.71%)	49.52 ± 2.74 b (41.48%)
	POD (μmol min^{-1} g^{-1} FW)	6.54 ± 0.29 a (40.20%)	7.10 ± 0.01 a (35.25%)	9.73 ± 0.73 a (59.80%)	13.04 ± 0.15 b (69.75%)

The percentage of activity in the symplast and apoplast (with respect to the total present in the whole leaf) is given between parentheses. Different letters indicate a significant difference between 0 and 100 μM Cd treatments at $p < 0.05$ according to the Duncan's test.

[a] The seedlings were treated with 0 and 100 μM $CdCl_2$ for 6 d. Values are means ± SE (n = 3) of three different experiments.

rice leaves [22]. In our study, Cd activated NADPH oxidase activity in the leaves of *P. aureus* and *V. sativa*. The activity of NADPH oxidase and the production of H_2O_2 are sensitive to DPI in the leaves of Cd-treated plants. This result strongly suggested that Cd-induced H_2O_2 production originated, at least in part, from PM-bound NADPH oxidases. The involvement of NADPH oxidase in H_2O_2 production was recently shown to be induced in some plants by Cd [19–23], Ni [16], Pb [17], and Cu [18,46]. Garnier et al. [47] identified different phases of Cd^{2+} induced ROS production in BY-2 tobacco cells. Within minutes, Cd^{2+} induced a transient increased $[Ca^{2+}]_{cyt}$ that appears to regulate the extracellular NADPH oxidase-dependent generation of H_2O_2. The mitochondrial electron transport chain disturbance by Cd induced a second wave of ROS production. Recently, Heyno et al. [48] reported that the Cd^{2+} induced production of H_2O_2 originates from inside the root cells, mainly from mitochondrial electron transport.

Besides NADPH oxidase, there are several other potential enzymatic sources of ROS [4]. PODs are widely distributed in plant tissues with different physiological roles [49]. Apoplastic PODs contribute to the production of $O_2^{\bullet-}$ and H_2O_2 during the oxidative burst through the oxidation of a reductant [4]. In our study, Cd-induced production of $O_2^{\bullet-}$ and H_2O_2 in the leaves of *P. aureus* and *V. sativa* was sensitive to 1 mM NaN_3, a well-known POD and cytochrome oxidase inhibitor. This result demonstrated that POD activation is also associated with Cd-induced ROS production in the leaves of both species, particularly in *P. aureus*. In this plant, leaf apoplastic POD activity was strongly stimulated by Cd. A POD might be implicated in the production of H_2O_2 induced by Al during seed germination of barley [50] and rice [51]. The POD inhibitor salicylhydroxamic acid strongly inhibits production of H_2O_2 and POD activity in barley roots [52]. Fecht-Christoffers et al. [53] observed that an excess of Mn stimulates H_2O_2-producing PODs and H_2O_2 formation in the leaf apoplast of cowpea. In contrast, some authors have reported that PODs are not implicated in ROS production by Cd-treated tobacco BY-2 cells [19] or Pb-treated *Vicia faba* roots [17]. In the plant apoplast, it has been postulated that POD acts by stiffening the cell walls and catalyzing lignification [54]. POD decreases the cell wall plasticity and therefore reduces cell elongation, which may represent a mechanical adaptation to stress conditions.

Results obtained in *P. aureus* and *V. sativa* with different inhibitors showed that the accumulation of Cd-induced $O_2^{\bullet-}$ and H_2O_2 was prevented by $LaCl_3$, CANT, and ODQ. These pharmacological results are in good agreement with the observations of Romero-Puertas et al. [20] for pea leaves. It has also been reported that $LaCl_3$, a Ca^{2+} channel inhibitor, inhibited production of $O_2^{\bullet-}$ and H_2O_2 induced by Cu, Ni, and Pb [16–18]. However, several other Ca^{2+} channel inhibitors were not able to inhibit the Cd-induced H_2O_2 production [19,55]. La^{3+} may act by displacing Cd^{2+} from the cell wall and competing with Cd^{2+} for a PM transporter. It was assumed that Cd^{2+} enters cells via La^{3+}-sensitive transporters or channels to activate the PM NADPH oxidase [55]. Preceding the oxidative burst, Cd^{2+} induced a rapid and transient $[Ca^{2+}]_{cyt}$ increase requiring protein phosphorylation and an inositol triphosphate (IP_3)-mediated release of calcium from internal stores. Indeed, CANT, a protein-phosphatase A2 and -1 type inhibitor, obviously decreased the Cd-induced production of $O_2^{\bullet-}$ and H_2O_2 (Figs. 1 and 2). Moreover, our result also showed that cyclic GMP may be involved in Cd-induced production of $O_2^{\bullet-}$ and H_2O_2. Cyclic GMP could induce ROS production by causing a transient elevation in concentration of Ca^{2+} [20]. Further investigation will be required to elucidate the signal transduction pathways involved in plant cell ROS production in response to heavy metal stress.

In conclusion, our study provides new evidence of H_2O_2 accumulation in the leaf apoplast of *P. aureus* and *V. sativa*, and also clarifies the roles of different antioxidant enzymes in the defense against harmful effects of Cd stress. It is suggested that the majority of Cd-induced ROS production in the leaves of both species may be via PM NADPH oxidase. This enzyme can use cytosolic NADPH to produce $O_2^{\bullet-}$, which is quickly dismutated to H_2O_2 by SOD. In addition to NADPH oxidase, apoplastic POD also may contribute to the production of ROS.

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