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Localization of Hydrogen Peroxide in Pumpkin (*Cucurbita ficifolia* Bouché) Seedlings Exposed to High-Dose Gamma Ray

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Hydrogen peroxide (H_2O_2) was detected cytochemically, via transmission electron microscopy (TEM), in pumpkin tissues exposed to high-dose gamma ray. Its reaction with cerium chloride produced electron-dense precipitates of cerium perhydroxides. Their patterns of deposition in the tissues of both control plants and those irradiated with gamma ray (PIG) were typically found in the plasma membranes and cell walls. However, gamma irradiation remarkably increased the intensities of cerium perhydroxide deposits (CPDs) in the plasma membranes and cell walls for all tissue types, but especially the leaves. The only exception was for vessels in the cotyledons. After gamma irradiation, the H_2O_2 content in all tissues was higher than in the control samples, except for the cotyledons of PIG, where the H_2O_2 content was lower than for all others. The increased appearance of CPDs may have been due to the enhancement of H_2O_2 accumulation by gamma radiation. This accumulation also varied according to the cell or tissue type examined.

Keywords: cerium chloride, gamma irradiation, hydrogen peroxide, pumpkin, transmission electron microscopy

Stresses such as UV exposure, herbicides, drought, temperature stress and intensive light induce the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion ($O_2 \cdot -$), hydroxyl radicals (OH) and singlet oxygen, in plant tissues (Noctor and Foyer, 1998; Desikan et al., 2003). These oxidative stresses can directly damage cells by modifying target molecules, including proteins, lipids and DNA (Fridovich, 1986; Wolff et al., 1986; Imlay and Linn, 1988; Bolwell and Wojtaszek, 1997) and by decreasing membrane integrity (Lee et al., 1998).

H_2O_2, a key player in oxidative stress (Cho and Sohn, 2004), is required for a variety of physiological processes associated with cell wall biosynthesis (Olson and Varner, 1993; Wi et al., 2005b). It can be produced by a number of enzymatic systems, and is commonly synthesized in response to various environmental stimuli (Sutheland, 1991). In addition, although H_2O_2 is a normal metabolite and not particularly cytotoxic at optimal concentrations, when these concentrations are increased by environmental stresses and ionizing radiation, they lead to cell lethality (Halliwell, 1974). Thus, H_2O_2 is one of the most important agents in terms of cell damage.

H_2O_2 contents can be remarkably increased by water radiolysis derived from gamma rays (Croute et al., 1982). High doses also inhibit plant growth, promoting H_2O_2 production that is harmful to cell organelles, while also inducing the formation of leaf trichomes and the alteration of morphologies (Nagata et al., 1999; Wi et al., 2005a). Although reports have described this relationship between H_2O_2 and gamma rays, none has yet demonstrated the distribution of H_2O_2 after such irradiation.

Histochemical localization of H_2O_2 production has relied on starch/KI reagents (Olson and Varner, 1993; Schopfer, 1994) or staining for peroxidase activity (Angelini and Federico, 1989). However, these techniques are indirect methods and are limited to detecting H_2O_2 produced only at the cut surfaces of tissue sections (Ros Barceló, 1998). Precise histochemical detection of H_2O_2 on an ultrastructural level is based on its reaction with cerium chloride ($CeCl_3$), which forms electron-dense insoluble precipitates of cerium perhydroxide, $Ce(OH)_2OOH$: $Ce^{3+} + 2H_2O_2 \rightarrow Ce(OH)_2OOH + H^+$. Although the Ce^{3+} that originates from $CeCl_3$ only slowly penetrates into the tissues, this second method is now widely used for cytochemical detection (Bestwick et al., 1997; Wi et al., 2005b).

In this study, we applied the cerium chloride

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method to tissues of pumpkin seedlings to examine the effects of high-dose gamma rays on H_2O_2 production, and to obtain more detailed information on the pattern of H_2O_2 deposition after irradiation.

MATERIALS AND METHODS

Plant Materials and Gamma Irradiation

Seedlings of pumpkin (*Cucurbita ficifolia* Bouché)

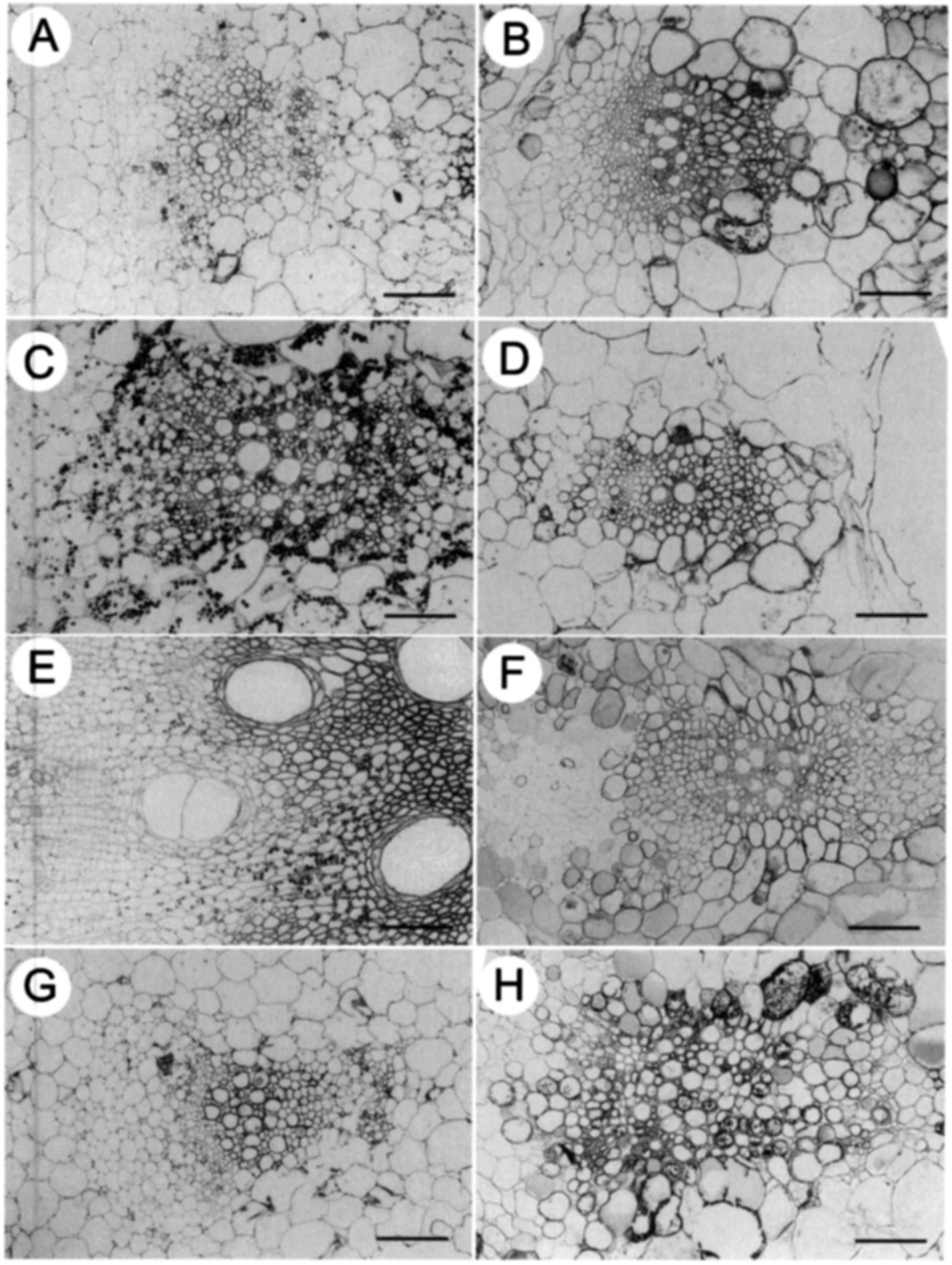

Figure 1. Microphotographs of leaf (**A** and **B**), petiole (**C** and **D**), hypocotyls (**E** and **F**), and cotyledon (**G** and **H**) of control (**A, C, E,** and **G**) and plant irradiated at 1 kGy (**B, D, F,** and **H**). Bar = 100 μm.

were cultivated in a greenhouse at the Advanced Radiation Technology Institute at Jeongeup, Korea. Uniform seedlings were planted in polyethylene bags containing a bed-soil mixture. At nine days old, the seedlings were exposed to 1 kGy, generated by a gamma irradiator (^{60}Co, ca. 164,000 Ci capacity; AECL, Canada) at the Korea Atomic Energy Research Institute, Korea. This dose was confirmed with a thermoluminescence dosimeter (Alaine, Germany). Samples of leaves, petioles, cotyledons and hypocotyls were collected 19 d after the radiation treatment.

Cytochemical Localization of Hydrogen Peroxide

H_2O_2 was detected by the cerium chloride ($CeCl_3$) method, as described by Bestwick et al. (1997). Briefly, small pieces (1 mm^3) of the middle zone from every fragment of the irradiated leaves, petioles, cotyledons and hypocotyls were excised and incubated for 1 h in freshly prepared 50 mM Mops buffer (pH 7.2) containing 5 mM $CeCl_3$. The samples were then fixed for 1 h in a mixture of 1.25% (v/v) glutaraldehyde and paraformaldehyde in 50 mM sodium cacodylate buffer (pH 7.2). They were then washed

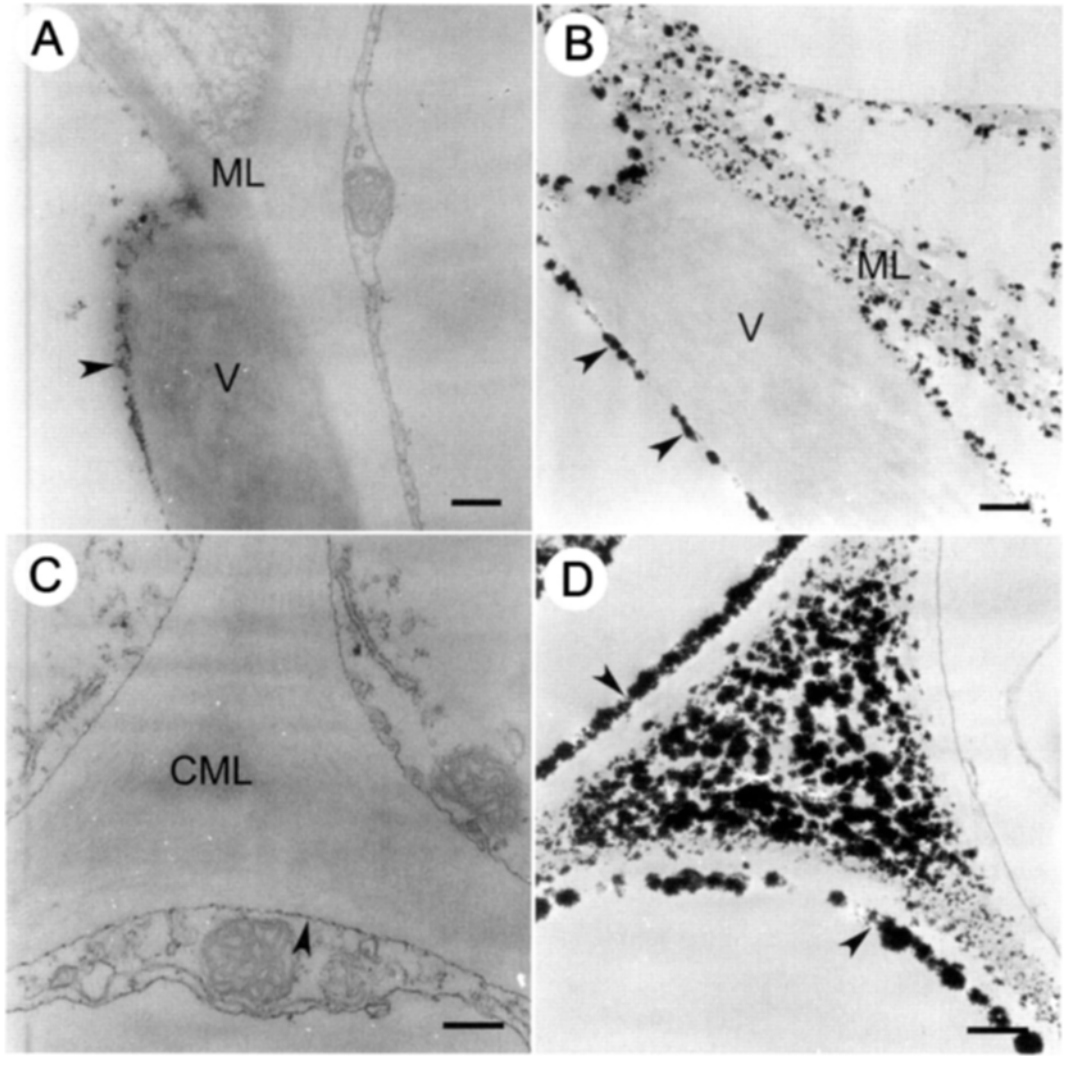

Figure 2. Localization of hydrogen peroxide in vessel (**A** and **B**) and parenchyma cell (**C** and **D**) of leaf in control (**A** and **C**) and plant irradiated at 1 kGy (**B** and **D**). Note that cerium perhydroxide deposits are significantly increased in plasma membrane (arrow heads) and cell corner middle lamella after gamma irradiation. CML, cell corner middle lamellae; ML, middle lamella; V, vessel wall. Bar = 0.5 μm.

in the same buffer, post-fixed for 1 h in 1% (w/v) osmium tetroxide (OsO_4), dehydrated in a graded acetone series, and embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections (70 to 90 nm thick) were mounted on uncoated nickel grids and examined with a transmission electron microscope (TEM) (J-1010; Jeol, Japan) at 80 kV without post-staining (uranyl acetate and/or lead citrate). To confirm the specificity of $CeCl_3$ staining for H_2O_2, catalase was used for its decomposition. Semi-thick sections were cut with a glass knife on an ultramicrotome (MT-7000; RMC, USA), stained with 0.1% aqueous toluidine blue, and examined with a light microscope (TE300; Nikon, Japan).

Determination of H_2O_2 Content

The content of hydrogen peroxide was measured colorimetrically, as described by Jena and Choudhuri (1981). H_2O_2 was extracted by homogenizing the leaves, petioles, cotyledons and hypocotyls with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6000*g* for 25 min. Afterward, the extracted solution was mixed with 0.1% (v/v) titanium chloride in 20% (v/v) H_2SO_4, then centrifuged at 6000*g* for 25 min. Absorbance was measured at 410 nm, and the H_2O_2 content was calculated using an extinction coefficient of 0.28 μmol^{-1} cm^{-1} per fresh weight (Jena and Choudhuri, 1981).

RESULTS AND DISCUSSION

Pumpkin seedlings treated with a high dose of gamma irradiation (PIG) showed inhibited growth and premature senescence of their leaves, petioles and

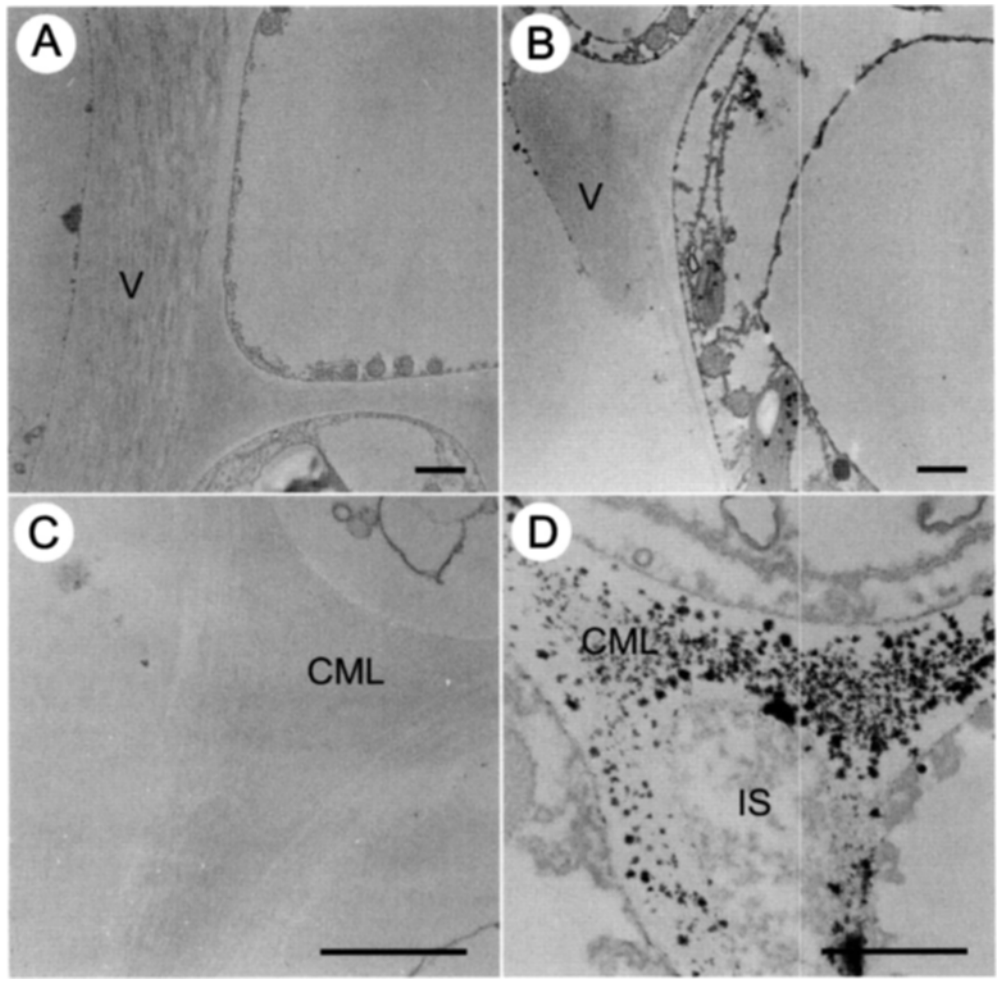

Figure 3. Localization of hydrogen peroxide in vessel (**A** and **B**) and parenchyma cell (**C** and **D**) of petiole in control (**A** and **C**) and plant irradiated at 1 kGy (**B** and **D**). CML, cell corner middle lamellae; IS, intercellular space; V, vessel wall. Bar = 1 μm.

hypocotyls. This phenomenon was especially remarkable in the leaves. In contrast, senescence proceeded more slowly in the PIG cotyledons than in the controls.

Cross-sections of histochemically stained tissues were examined by light microscopy (Fig. 1). Vessel sizes did not differ significantly between leaves from control and PIG (Fig. 1A and B). However, compared with the irradiated tissues, sections of petioles and hypocotyls from the controls were well developed as large and open vessel elements (Fig. 1C-F). In contrast, the vascular bundle regions of PIG cotyledons were much larger in both their dimensions and cell numbers than those from the controls (Fig. 1G and H).

We used cerium perhydroxide deposits (CPDs) as markers to determine the distribution of H_2O_2 on the plasma membranes in both vessels and parenchyma cells in the leaves (Fig. 2A and C). Although the distribution patterns were similar in the vessels of both control and PIG (Fig. 2A and B), the CPD intensity in treated plants was significantly higher. Moreover, the deposition pattern and intensity of CPDs in the parenchyma cells of PIG (Fig. 2D) differed significantly from the controls (Fig. 2C); CPDs were heavily deposited in the PIG cell corner middle lamellae and plasma membranes (Fig. 2D).

In the petiole (Fig. 3), the reaction was mainly associated with the cell corner middle lamellae in PIG parenchyma cells (Fig. 3D), with the difference being negligible between PIG and control vessels (Fig. 3A and B). H_2O_2 was not deposited on the plasma mem-

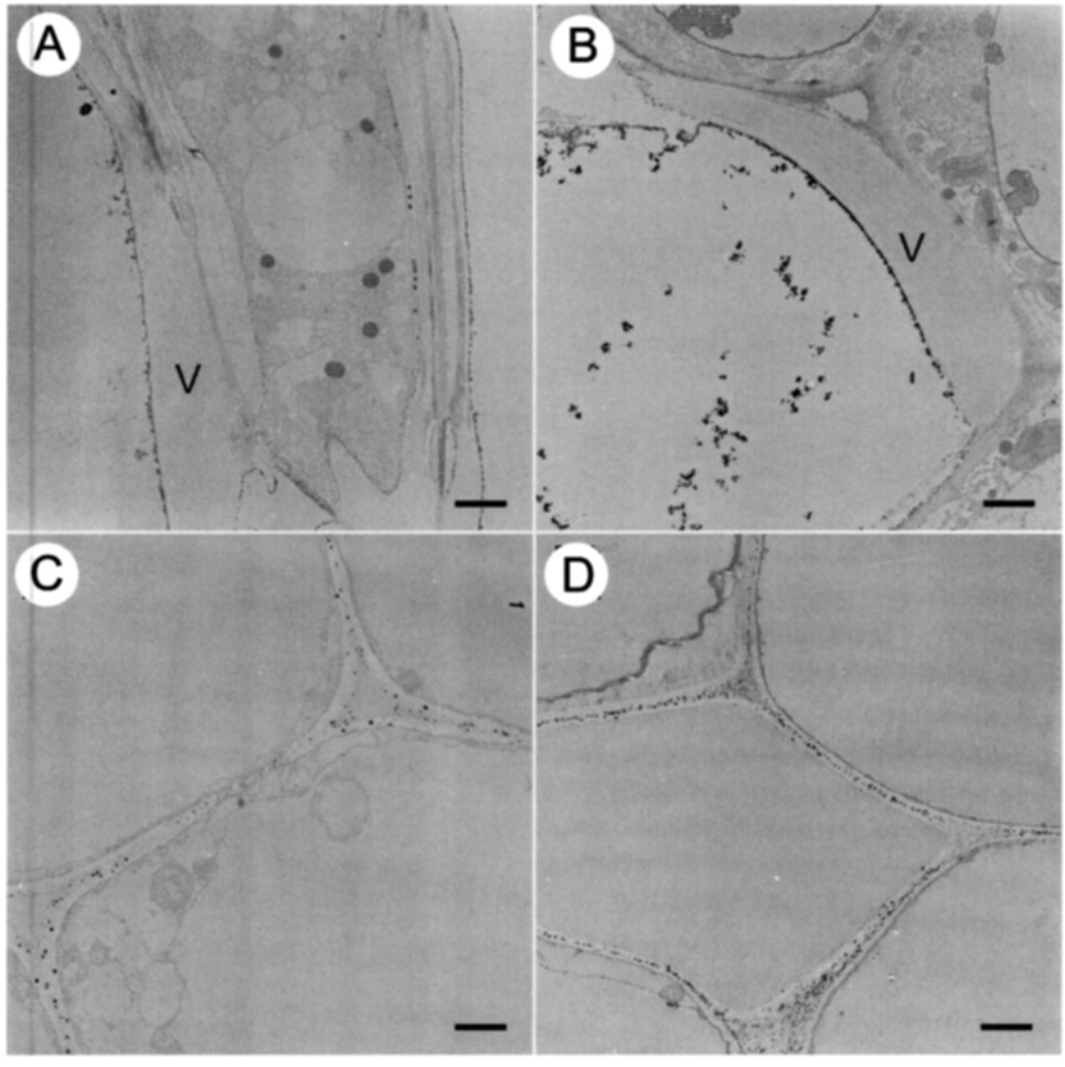

Figure 4. Localization of hydrogen peroxide in vessel (**A** and **B**) and parenchyma cell (**C** and **D**) of hypocotyl in control (**A** and **C**) and plant irradiated at 1 kGy (**B** and **D**). V, vessel wall. Bar = 2 μm.

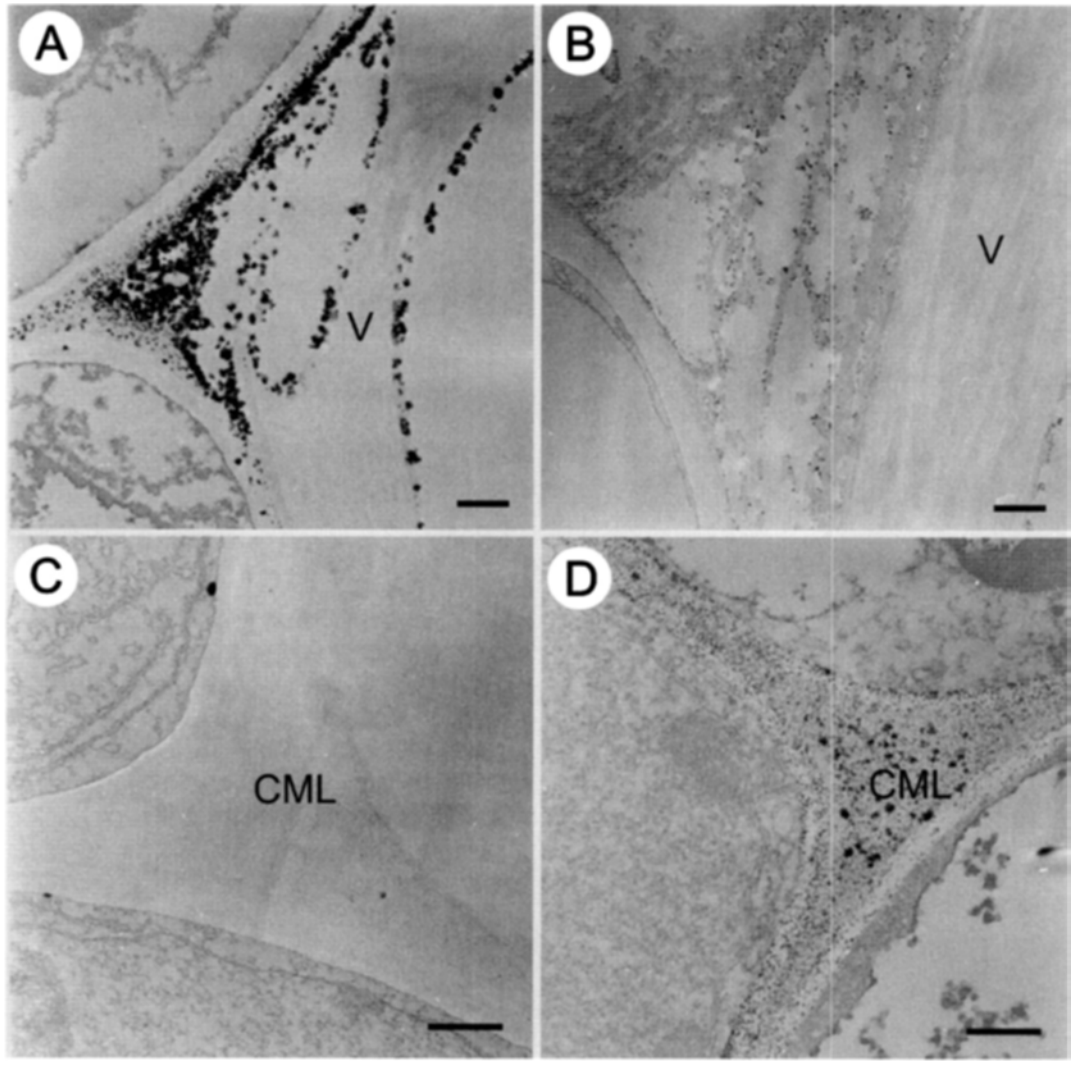

Figure 5. Localization of hydrogen peroxide in vessel (**A** and **B**) and parenchyma cell (**C** and **D**) of cotyledon in control (**A** and **C**) and plant irradiated at 1 kGy (**B** and **D**). CML, cell corner middle lamellae; V, vessel wall. Bar = 0.5 μm.

brane in either cell type.

In the hypocotyls (Fig. 4), the distribution patterns were similar to those in the vessels and parenchyma cells of the leaf and petiole. The reactions, found mostly on the middle lamellae of both, varied in their intensities, but differences between the control and PIG plants were less than those measured in the leaves and petioles. Interestingly, a reverse pattern of CPD was observed between the vessels and parenchyma cells of the cotyledons. Although parenchyma cells were similar in pattern and intensity to those of other tissues (Fig. 5C and D), the intensity in the vessels was high in the controls but low in the PIG (Fig. 5A and B). These results were in good agreement with those recorded for H_2O_2 content (Fig. 6).

The concentration of gamma ray-induced H_2O_2 increased in all tissue types (Fig. 6), with the highest level measured in the PIG leaves. In contrast, content was higher in control cotyledons than in those of the PIG plants. In addition, a reverse pattern of CPD was observed via TEM in the cotyledon vessels (Fig. 5A and B).

In most cases, H_2O_2 accumulation, as defined by electron-dense cerium deposits, was typically associated with the plasma membrane and middle lamellae, and deposits were not detected inside the vessel walls. H_2O_2 contents were relatively high in the leaf and cotyledon but relatively low in the petiole and hypocotyl. CPD density was greatest in the PIG parenchyma cells. Although H_2O_2 contents increased

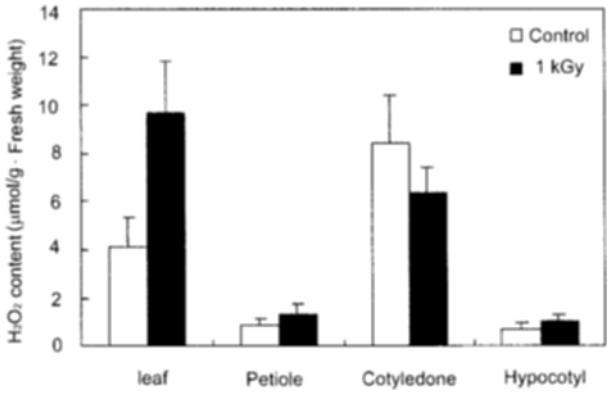

Figure 6. H_2O_2 content in total homogenates from tissues at 19 d after gamma irradiation. Data are means with standard errors (n = 4).

in all irradiated tissue types, an exception was seen with the cotyledons of PIG. Moreover, those irradiated cotyledons showed slight changes in their morphology, although the reason for such a response was not studied here.

High doses of ionizing radiation induce physiological changes (Akamine and Goo, 1971; Romani, 1984) and can alter cellular macromolecular components, including cell walls, membranes and DNA (Casarett, 1968; Wi et al., 2005a). Increases in endogenous H_2O_2 levels are also associated with the promotion and induction of leaf senescence (Mondal and Choudhuri, 1981). These responses are due to both direct interactions between the ionizing radiation and the macromolecular structure, as well as the indirect action of ROS generated by water radiolysis. Therefore, the increased appearance of CPDs in PIG may be a result of either inhibited H_2O_2 detoxification by antioxidant enzymes or the enhancement of H_2O_2 production through radiolysis, extracellular oxalate oxidase and amine oxidase in the apoplasts, or NADPH oxidase in the plasma membrane (Reggiani and Bertani, 1989; Dwyer et al., 1996; Hurkman and Tanaka, 1996).

Based on this study, we can conclude that 1) treatment with gamma rays increases H_2O_2 accumulation mainly in the plasma membrane and middle lamellae, and especially in the leaf; 2) parenchyma cells are more sensitive than vessel elements to irradiation in terms of H_2O_2 production; and 3) the accumulation of H_2O_2 in pumpkin seedlings varies according to cell and tissue type. Further studies are needed to examine why senescence in cotyledons is delayed after gamma irradiation.

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