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Localization of hydrogen peroxide production in *Zinnia elegans* L. stems

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Abstract

Hydrogen peroxide is required for a variety of physiological processes associated with plant cell wall biosynthesis. Several recent studies have suggested that starch-KI could be used as an effective histochemical stain for studying the spatial and temporal regulation of H_2O_2 production during plant development. In this study, we found that detection of H_2O_2 in *Zinnia elegans* stems using starch-KI staining was highly dependent upon plant age, developmental status, and the manner in which the tissue was handled prior to staining. An alternative staining technique based on cerium perhydroxide deposition clearly showed that most of the H_2O_2 detected by starch-KI resulted from wounding at the cut surfaces of stem sections. However, with cerium staining, substantial H_2O_2 production was apparent well below the cut surface in collenchyma cell walls where previous work had demonstrated localization of peroxidase activity. Co-localization of H_2O_2 and peroxidase in collenchyma tissues may suggest an involvement in crosslinking of the pectins and hemicelluloses that predominate the walls of these cells. Cerium deposition also suggested that H_2O_2 production in xylem vessel walls may lag slightly behind secondary cell wall thickening.

Keywords: *Zinnia elegans*; Asteraceae; Biosynthesis; Active oxygen; H_2O_2; Cerium; Lignification; Starch-KI; Xylem; Collenchyma

1. Introduction

Plants synthesize a multitude of peroxidases in response to a wide variety of developmental and environmental cues (Siegel, 1993). The common substrate for these enzymes, hydrogen peroxide, can be generated by a number of enzymatic systems that are more or less poorly understood, but are very often stimulated by stress (Lane, 1994; Baker & Orlandi, 1995; Smirnoff, 1993; Foyer, Lelandais & Kunert, 1994). Many peroxidase isozymes are localized in plant cell walls where they have been implicated in various physiological processes, including lignin deposition (Barcelo, 1995; Dean & Eriksson, 1992), the cross-linking of structural proteins (Cooper & Varner, 1984) and polysaccharides (Miyamoto et al., 1994), as well as auxin metabolism (Gazaryan, Lagrimini, Ashby & Thorneley, 1996). Although some of these peroxidases are known to be capable of oxidizing certain substrates in the absence of H_2O_2 (Liu, Dean, Friedman & Eriksson, 1994; Yamazaki & Piette, 1963), many absolutely require H_2O_2 for activity, and thus, an understanding of the spatial and temporal production of H_2O_2 is critical to defining the functions of peroxidases in plant development.

Olson and Varner (1993) used a histochemical stain (starch-KI) to examine the localization of H_2O_2 production in *Zinnia elegans*. This technique, which depends on soluble starch turning blue when elemental iodine (I_2) is produced by H_2O_2 oxidation of iodide (I^-), had previously been used to localize plant amine oxidases (Kaur-Sawhney, Flores & Galston, 1981; Smith, 1970). More recently, starch-KI staining was

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Table 1
The influence of tissue age and buffer washing on the detection of H_2O_2 production in Zinnia stem sections by starch-KI staining

Plant age	Time required for a visible positive reaction		Tissues stained
	no wash	water wash	
4–6 weeks (young internode)	0.6–3 h	no stain	Xylem
4–6 weeks (old internode)	0.6–3 h	> 18 h	xylem, epidermis
7–8 weeks (middle of the stem)	0.2–0.3 h	0.6–6 h	xylem, epidermis, phloem
12–13 weeks (middle of the stem)	0.03–0.09 h	0.5–0.6 h	xylem, epidermis, phloem, collenchyma

used to study localization of H_2O_2 production in various tissues from a variety of plant species (Hotter, 1997; Ogawa, Kanematsu & Asada, 1997, Schopfer, 1994). Hydrogen peroxide production can also be localized by cerium deposition (Halbhuber, Hulstaert, Feuerstein & Zimmerman, 1994; Kausch, 1987), and this technique has been used to examine H_2O_2 production in plant vascular tissues (Bestwick, Brown, Bennett & Mansfield, 1997; Czaninski, Sachot & Catesson, 1993; Liu, Eriksson & Dean, 1995). Cerium staining has recently been used to localize hydrogen peroxide accumulation during a hypersensitive response in lettuce (Bestwick et al., 1997). Whereas starch-KI stain is limited to the cut surface of sectioned tissues, cerium can penetrate to relatively undisturbed cells where its deposition is less likely to reflect H_2O_2 produced in response to wounding. In this study, we used both techniques to re-examine the production of H_2O_2 in *Z. elegans* stems, and found that starch-KI staining gave erratic results that were highly dependent on the age of the plant and the way in which the tissues were handled. In addition to detecting H_2O_2 production in developing xylem vessel walls, cerium highlighted the production of large amounts of H_2O_2 in the walls of collenchyma cells found around the periphery of *Z. elegans* stems. This is the first study to show high levels of H_2O_2 production in the collenchyma tissues from any plant, and the possible significance of this observation is discussed.

2. Results and discussion

2.1. H_2O_2 production detected with starch-KI staining

Previous work with *Z. elegans* (Liu et al., 1994) demonstrated that in stems of different ages there were temporal and spatial variations in the activities of laccases and peroxidases associated with lignifying tissues. To determine whether H_2O_2 production in these tissues followed the pattern observed for peroxidase activity, tissues of various ages were stained using the starch-KI method (Olson & Varner, 1993). However, the staining pattern generated by this technique was found to vary drastically depending on the age of the plant, as well as the way in which the tissue sections were handled prior to stain application (Table 1). In general, staining was much faster in tissue sections taken from older plants, while washing or floating the tissue sections in buffer prior to stain application slowed the staining process considerably. Whereas Olson and Varner (1993) reported no staining of pith cells in young *Z. elegans* stems, we found that the central pith cells in older plant stems always stained more rapidly and more intensely than any of the surrounding cells (Fig. 1(a)). Vascular bundles and some of the nearby cortical cells were the next most strongly stained regions in older stem sections, but the rate of staining was more gradual in these tissues. This differential rate of staining was a problem because the blue reaction product was water-soluble and tended to diffuse readily into the incubation buffer. As a consequence, the length of incubation prior to observation had a critical influence on how the results were interpreted. Specifically, large pith cells, while staining most strongly overall, rapidly lost that stain by diffusion, while small diameter vascular cells tended to retain the stain longer. Also in contrast to the results of Olson and Varner (1993), when this staining pattern was examined at higher magnifications (Fig. 1(b)), it appeared that most of the stain was localized to the cell lumen and very little was actually associated with the cell wall. This phenomenon was particularly striking in the highly lignified phloem fiber cells. Some stain was also apparent in the lumen of epidermal and collenchyma cells.

When viewed at low magnifications similar to those used by Olson and Varner (1993), tissue sections from 4–5 week old plants showed no staining in the pith cells and relatively little staining in the region containing phloem primordia, while the xylem vessel walls appeared to be intensely stained (Fig 1(c)). However, examination of unstained sections showed that xylem vessel walls were already relatively dark and opaque prior to the application of any staining reagent (Fig. 1(d)), and examination of stained tissues at higher magnification showed that after staining, most of the stain was in the lumen of xylem and phloem cells (Fig.

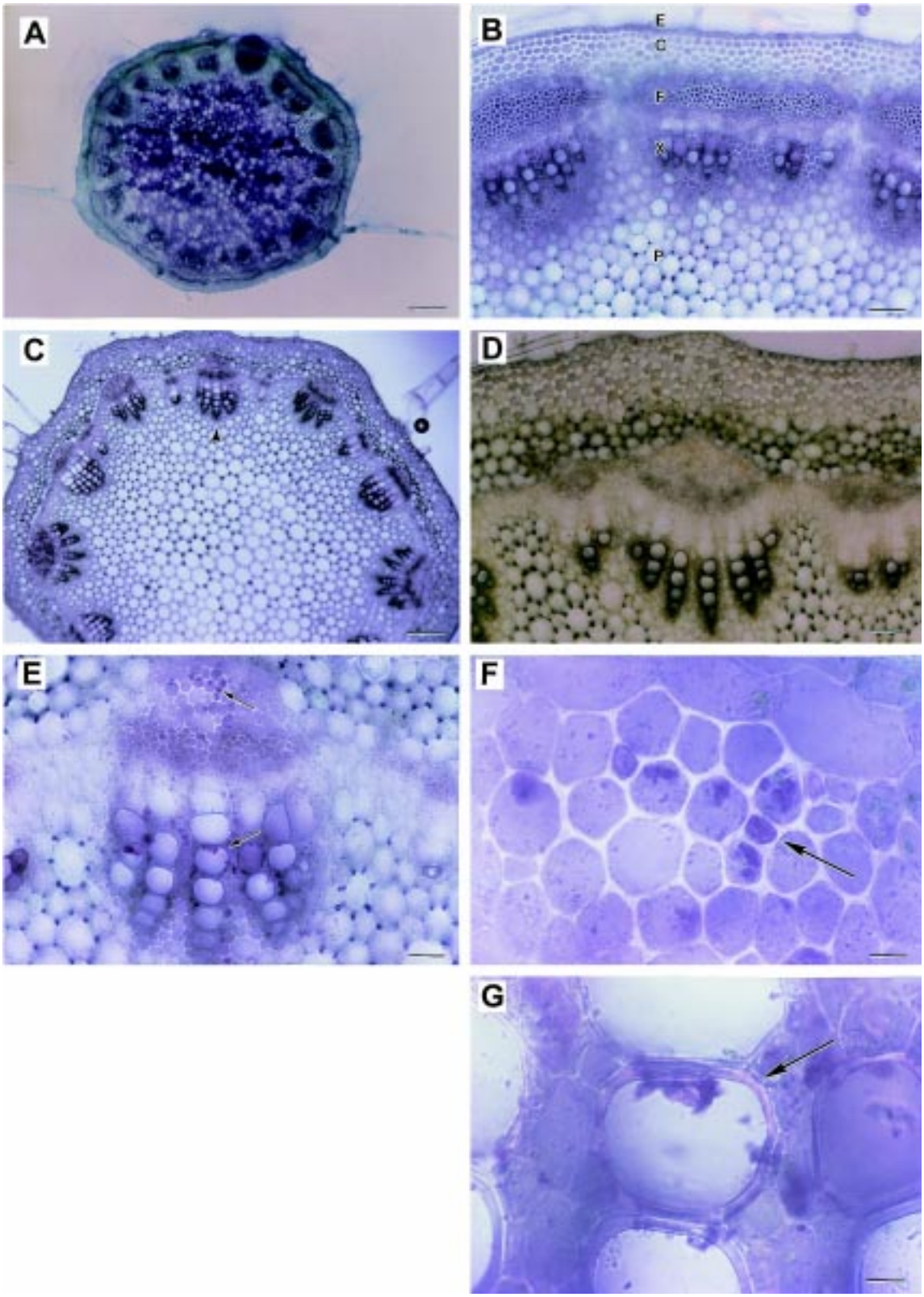

Fig. 1. Detection of H_2O_2 production by starch-KI staining of zinnia stem sections. Sections from a 12 week old zinnia plant (panels A, B) showed strong staining in the pith (P), as well as in the lumen of xylem (X), phloem (F), collenchyma (C), and epidermal (E) cells. Sections from the same internode of a 6 week old plant were either stained with starch-KI (panel C) or left unstained (panel D) for comparison. Higher magnifications of the vascular bundle marked with an arrowhead in panel C are shown in panels E–G. Arrows in panel E point to specific cells depicted in panels F and G. Scale bars = 2500, 500, 1250, 250, 125, 50 and 50 μm for panels A–G, respectively.

Table 2
The influence of various reagents on the detection of H_2O_2 production in Zinnia stem sections by starch-KI staining

Reagent	Concentration	Effect
Putrescine	10 mM	no staining
Spermidine	10 mM	no staining
Catalase	100 U ml^{-1}	no staining
Aminotriazole	50 mM	very reduced staining, limited to pith cells

1(e)), just as was seen after staining older tissues. At very high magnifications it could be seen that the most intense staining was associated with blebs of material that appeared to be fragments of cytoplasm, and that in many cases, stain appeared to be excluded from the cell walls (Fig. 1(f)). On the other hand, some xylem vessel walls did appear to contain stain (Fig. 1(g)), but because of the diffuse nature of the stain, the thickness (100 μm) of the tissue sections, and the discontinuous nature of the helical secondary walls in these cells, it was difficult to determine with any certainty whether the stain was localized within the secondary cell walls or between the helical bands. It was very apparent that the heaviest staining associated with xylem tissues actually occurred in the nonlignified parenchyma cells surrounding the vessels.

A number of enzyme systems, including those based on polyamine and diamine oxidases, have been proposed to act as the source of H_2O_2 in plants (Auh & Murphy, 1995; Bolwell, Buti, Davies & Zimmerlin, 1995; Lane, 1994; Medda, Padiglia & Floris, 1995). Although polyamine and diamine oxidases have mainly been associated with H_2O_2 production in grasses and legumes, respectively, putrescine and spermidine were each applied to *Z. elegans* stem sections in the presence of starch-KI stain to test whether either of these enzymes was also produced in *Z. elegans*. In contrast to what was seen in oat leaves using this technique (Kaur-Sawhney et al., 1981), the addition of either polyamine completely inhibited the staining of *Z. elegans* stem sections by starch-KI (Table 2).

Heavy staining of putative cytoplasm fragments was not unexpected in light of work demonstrating rapid release of H_2O_2 by mechanically stressed cells (Yahraus, Chandra, Legendre & Low, 1995); however, it did suggest that wound responses in the cut tissue might be more of a problem than was suggested by Olson and Varner (1993). Senescent tissues are often more sensitive to wounding than younger tissues, and evidence suggests that this may arise from a decline in levels of anti-oxidative enzymes, such as catalase and superoxide dismutase (Hurng & Kao, 1994; Longa, Delrio & Palma, 1994). Thus, the strong staining noted in pith cells of older *Z. elegans* stem sections may reflect a decline in antioxidant levels in this tissue. Older, senescent tissues also contain elevated levels of lipoxygenases that can produce active oxygen species when cells are damaged (Thompson, Paliyath, Brown & Duxbury, 1987). Polyamines have long been known to act as antagonists of senescence and wounding (Galston & Kaur-Sawhney, 1987), at least in part by stabilizing membranes and preventing the lipid peroxidation catalyzed by lipoxygenase (Tiburcio et al., 1994). Thus, it may be that addition of putrescine or spermidine to the starch-KI staining reagent eliminated H_2O_2 production by stabilizing membranes and prevented lipid oxidation at the wounded surface.

As expected, addition of catalase to the staining reagent also prevented staining of *Z. elegans* stem sections, but surprisingly, nearly all staining in the pith cells of older tissues was also inhibited by addition of the catalase inhibitor, aminotriazole. The observation that aminotriazole reduced staining in stem segments would seem to contravene its common use as a specific catalase inhibitor (Margoliash, Novogrodsky & Schejter, 1960), since catalase inhibition would be expected to increase the amount of H_2O_2 available for staining. However, Bestwick et al. (1997) noted that incubation in aminotriazole did not lead to increased levels of detectable hydrogen peroxide in lettuce leaf tissues. As aminotriazole has been shown to inhibit certain peroxidases (Castelfranco, 1960; Tuisel, Grover, Bumpus & Aust, 1992), these results could be interpreted to suggest the staining in *Z. elegans* pith tissues derived primarily from a peroxidase-based H_2O_2 production system similar to that described by Elstner and Heupel (1976). Alternatively, aminotriazole has also been shown to inhibit other oxidative enzymes, such as NO synthase (Buchmüller-Pouiller, Schneider, Betz-Corradin, Smith & Mauël, 1992), and it can also inhibit the oxidation of certain fatty acids (Casteels, Croes, Vanveldhoven & Mannaerts, 1994; Castelfranco, 1960; Hashimoto & Hayashi, 1994). This latter possibility fits with the suggestion that polyamine addition might limit H_2O_2 production by stabilizing membranes so as to prevent fatty acid oxidation by lipoxygenases.

2.2. H_2O_2 production detected by cerium deposition and epi-polarization microscopy

In light of the ambiguous results obtained using

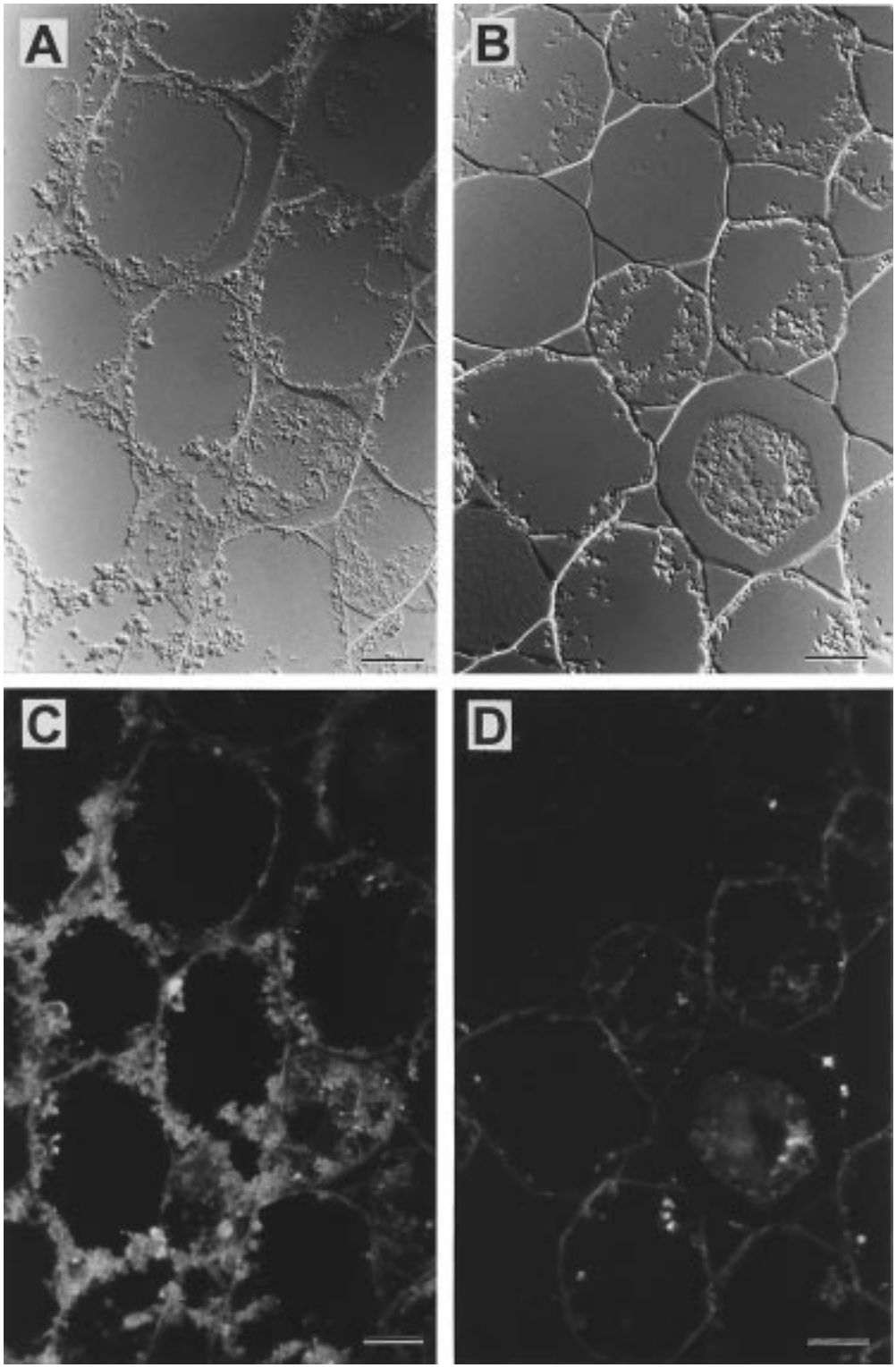

Fig. 2. The effect of washing on detection of H_2O_2 production at cut surfaces. Pith cells in sections from a 12 week old stem were left unwashed (A, C) or were washed in buffer (B, D) prior to staining with cerium. Unwashed cells retained significantly more cytoplasmic fragments, as detected by DIC (A, B), and most cerium deposition, as seen by epi-polarized illumination, was associated with these cytoplasmic fragments (C, D). Scale bars = 50 μm.

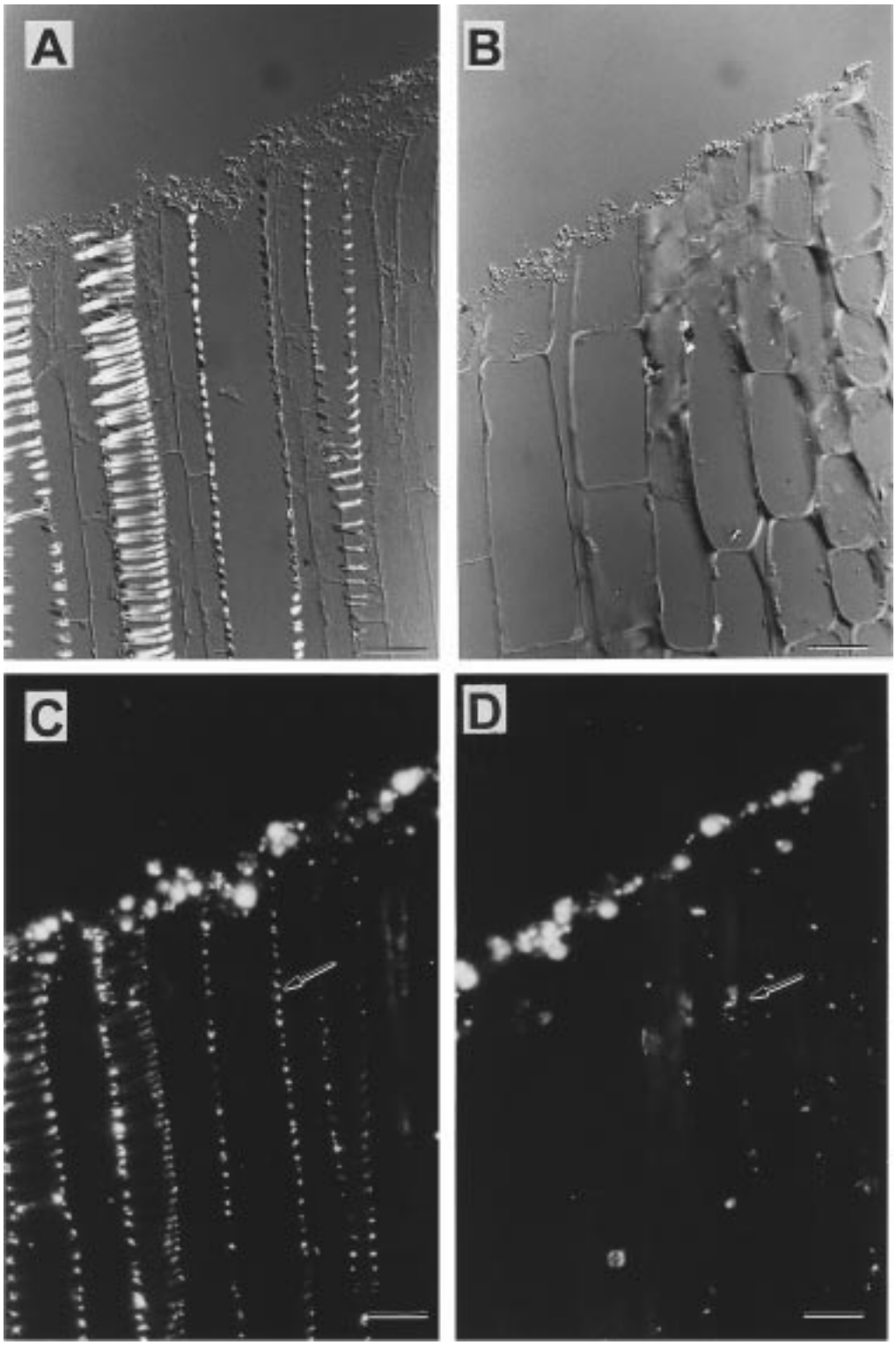

Fig. 3. Radial section of an unwashed zinnia stem segment showing cerium deposition at the cut surface. Using DIC, a layer of cytoplasmic material was observed on the original cut surface around the vascular tissues (A), as well as the collenchyma and epidermal tissues (B). Epi-polarized illumination (C, D) showed that most of the deposited cerium was associated with this cytoplasmic layer. Some cerium deposition was apparent in the deeper collenchyma cells (D, arrowhead), but cellulose in xylem vessel walls (C, arrowhead) did not allow such a conclusion to be drawn for these cells. Scale bars = 50 μm.

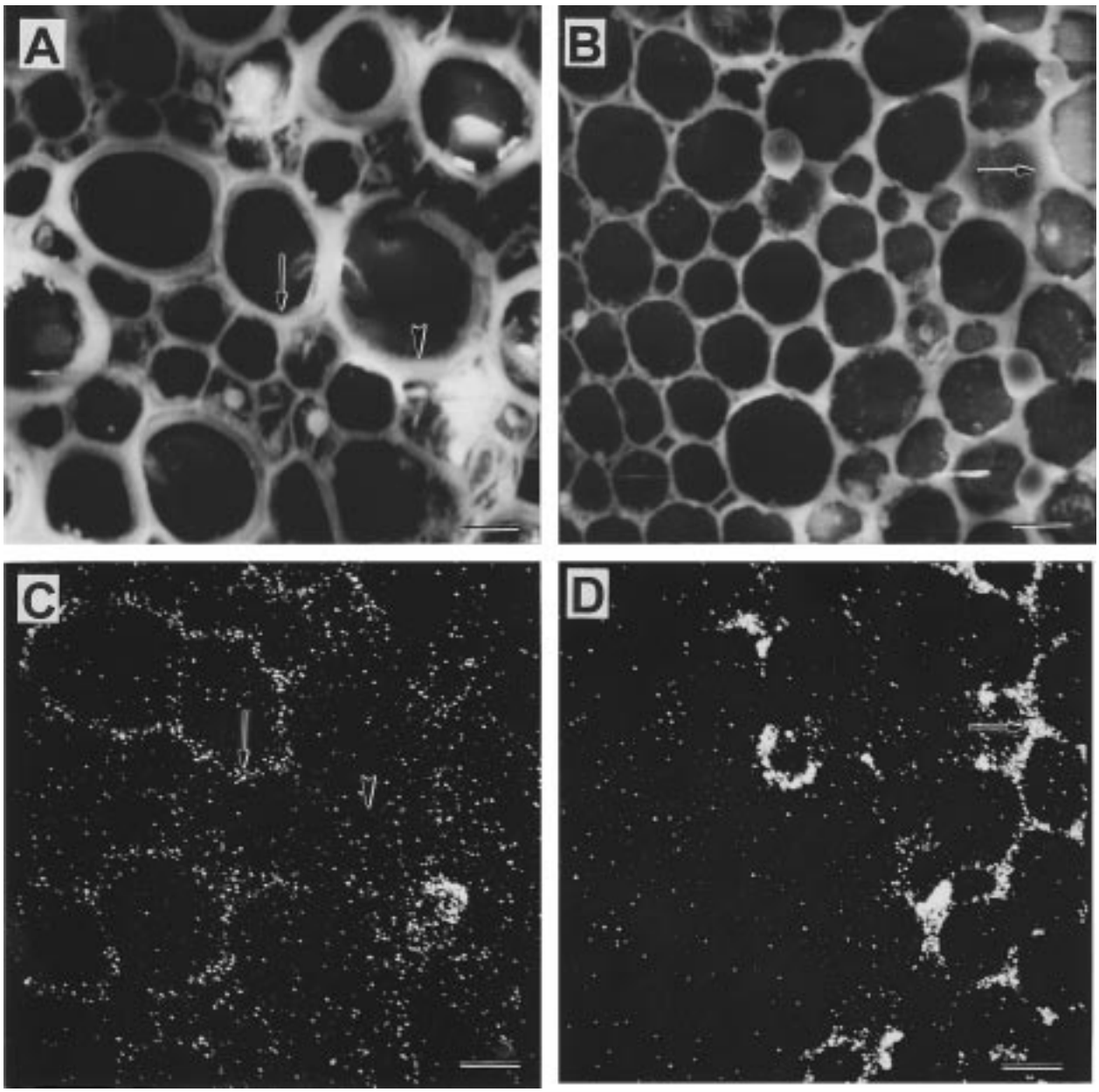

Fig. 4. SEM/XRMA of a cerium-stained zinnia stem section. Developing xylem vessels (A, C) and collenchyma tissues (B, D) in the same tissue section were examined by SEM (A, B). XRMA maps of cerium localization (C, D) clearly showed that H_2O_2 was produced in xylem vessel walls (C, arrow), and that even more H_2O_2 seemed to be produced in collenchyma cell walls (D, arrow). Note the young xylem vessel having a thickened cell wall, but showing no significant H_2O_2 production (A, C, arrowhead). Scale bars (A, C) 28 μm and (B, D) 14 μm.

starch-KI staining to detect H_2O_2 production in *Z. elegans* stem sections, a cerium deposition method (Liu et al., 1995) was tested. Whereas the chromophore in starch-KI staining is associated with macromolecular starch granules that cannot penetrate the cut surfaces of tissues, cerium can penetrate several cell layers (ca. 150–200 μm) into the tissue from the cut surface. Thus, by examining serial sections below the cut surface of cerium-treated stem segments, it is possible to gain a more accurate understanding of where H_2O_2 is produced in less disturbed tissues. However, drawbacks to the cerium technique include lengthy staining times and the potential for uneven diffusion of Ce^{3+} through different tissue types. Interpretations of cerium staining patterns should also be tempered with the knowledge that the tissues are nonetheless perturbed by removal from the intact plant, and in some situations in vivo techniques for detecting H_2O_2 production may be more appropriate (Frahry & Schopfer, 1998).

Examination of transverse sections using DIC and epi-polarization microscopy showed that large

amounts of cerium perhydroxide were deposited on the freshly cut surface of stem segments immersed in the cerium staining reagent (Fig. 2(a,c)). However, if the stem segments were rinsed in buffer prior to treatment, very little cerium deposition occurred on the cut surface (Fig. 2(b,d)). In either case, most of the cerium deposits appeared to be associated with remnants of cytoplasm. Examination of tangential sections of cerium-treated *Z. elegans* stems clearly showed that most of the H_2O_2 produced in these tissues was produced at the cut surface (Fig. 3), and thus represented a response to wounding. This highlights the fact that although the starch-KI staining technique may be useful in certain situations, the technique is limited to cut surfaces which, by definition, are wound sites. Thus, the intensity of starch-KI staining actually reflects the degree to which tissues are predisposed to produce H_2O_2 upon wounding.

Also, clearly evident under epi-polarized light was the crystalline cellulose contained in the secondary walls of xylem vessels (Fig. 3(c)). As noted in our previous study (Liu et al., 1995), epi-polarization microscopy is not effective for analyzing cerium deposition in or around the optically anisotropic cellulose in secondary cell walls. This was unfortunate since most lignin is found in secondary cell walls, and it is presumed that significant amounts of H_2O_2 should be produced in these walls for lignin biosynthesis.

2.3. X-ray mapping of cerium deposits using SEM/XRMA

Fortunately, crystalline cellulose and cerium deposits can be distinguished on the basis of their opacity to electron beams, and X-ray spectroscopy performed using a scanning electron microscope (SEM/XRMA) can be used to map the location of specific elements, such as cerium, in the presence of anisotropic biopolymers. Fig. 4 shows the SEM images of xylem vessels near the cambium (panel a) and collenchyma cells near the epidermis (panel b) in a transverse section of cerium-stained stem tissue taken from about 100 μm below the original cut surface. X-ray maps showed that cerium deposits were clearly associated with xylem vessels (panel c), but that substantially more cerium was deposited in the collenchyma tissues (panel d). In both cases, most of the cerium appeared to lie within the cell walls, although particularly intense deposits were associated with cytoplasmic fragments in some collenchyma cells. Significantly, little or no cerium was deposited in the thickening walls of young xylem vessels that were most likely undergoing active lignification (arrowheads, Fig. 4(a,c)). Given the conductive function of xylem tissues, it seems unlikely that the differences in staining intensity between collenchyma and xylem cell walls was significantly affected by diffusion of Ce^{3+} through the tissues. In light of our previous studies showing laccase activity associated with xylem cell walls from the first moment that they begin to thicken (Liu et al., 1994), it was interesting to note that relatively little H_2O_2 appeared to be produced in newly formed vessels that had obvious thickened walls and which, therefore, had likely initiated lignin deposition. This observation would seem to lend credence to our suggestion that laccases and peroxidases may work sequentially in the deposition of lignin (Sterjiades, Dean, Gamble, Himmelsbach & Eriksson, 1993). On the other hand, Bestwick et al. (1997) noted that in tissues containing high levels of peroxidase the competition for H_2O_2 could limit accumulation of cerium precipitates. Thus, the low levels of cerium deposition we noted in developing vessels may just reflect the rapid consumption of H_2O_2 during lignification.

The extensive deposits of cerium distributed throughout the collenchyma tissue (Fig. 4(d)) were somewhat unexpected, even though we had detected substantial peroxidase activity in these same tissues in a previous study (Liu et al., 1994). Interestingly, the walls of cortical cells that held a similar spatial localization in pea epicotyls (just centripetal to the epidermis) were found to contain the highest levels of H_2O_2 after treatment with putrescine, the substrate for diamine oxidase (Liu et al., 1995). Collenchyma cell walls are characterized by large amounts of pectin and hemicellulose, but no lignin, yet they have high tensile strength and are generally considered to act as a supporting tissue in many plants (Esau, 1977; Patterson, 1992). In addition to playing the role of supportive tissue, collenchyma has also been implicated in the resistance of oaks to mistletoe colonization (Hariri, Jeune, Baudino, Urech & Salle, 1992), and stem feeding by insects (Oghiakhe, Kackai, Hodgson & Ng, 1993). The observed co-localization of H_2O_2 and peroxidase activity in collenchyma cell walls may point to the active formation of covalent crosslinks between the hydroxycinnamate ester moieties commonly associated with pectins and hemicelluloses (Van Huystee & Zheng, 1993; Ralph, Quideau, Grabber & Hatfield, 1994), as well as between these polysaccharides and other cell wall components (Qi, Behrens, West & Mort, 1995; Ralph, Grabber & Hatfield, 1995). Such crosslinks would certainly serve to rigidify and strengthen the walls in this tissue (Ford & Hartley, 1989; Parker & Waldron, 1995). Evidence suggests that such crosslinkages could also play a major role in controlling cell wall elongation in such diverse plants as maize and pine (Hohl, Greiner & Schopfer, 1995; Sánchez, Peña, Revilla & Zarra, 1996; Schopfer, 1996). Further work will be required to determine whether the high levels of H_2O_2 and peroxidase activity found in *Z. elegans* collenchyma are functioning to produce hydroxycinna-

mate ester crosslinks which serve to rigidify these walls.

3. Experimental

3.1. Plants and reagents

Zinnia elegans cv. Envy seeds (Bodger Seed Co., S. El Monte, CA) were germinated on moistened filter paper for two days, and planted in 12 in. pots containing Fafard peat mix #3. Plants were grown in the greenhouse under ambient light conditions and watered daily. The reagents used in this study were obtained at the highest purity commercially available.

3.2. Sample preparation and staining

For histochemical studies, *Z. elegans* stems were cut into 90–100 μm thick sections using double-edged razor blades mounted on a Vibratome (Series 2000, Technical Products International, Inc., St. Louis, MO). Sections were either cut and placed directly into the staining solution, or were cut in a water bath and washed with buffer for 2–5 min prior to placement in the stain. Starch-KI staining for hydrogen peroxide was performed according to the method of Olson and Varner (1993).

Sample preparation for staining with cerium was as described by Liu et al. (1995). Segments (1.5 mm) were incubated for 12 h at room temperature in reaction buffer containing 100 mM Hepes, pH 7.5, and 5 mM $CeCl_3$. The reaction buffer was replaced after 2 h and 6 h of incubation. For control reactions, sections were incubated in Hepes buffer only. All solutions were made with preboiled, double glass-distilled water, and samples were agitated on a platform shaker (Kausch, 1987; Slocum & Furey, 1991). After incubation in the cerium reaction buffer, tissues were washed twice in 100 mM sodium cacodylate buffer (pH 7.0) for at least 15 min each, and then fixed using 2% glutaraldehyde in cacodylate buffer. After the fixation, tissues were rinsed thoroughly in cacodylate buffer. Sections to be embedded in paraffin were dehydrated using an ethanol/*t*-butanol series, and then embedded as described in Berlyn and Miksche (1976). Sections slated for plastic embedding were dehydrated and embedded as described previously (Liu et al., 1995). Tissues embedded in paraffin were cut into 10 μm thick sections, while tissues embedded in plastic were cut as 5 μm thick sections.

3.3. Light microscopy

Thick (90–100 μm) sections stained with starch-KI were photographed with Kodak Ektachrome 64T film using bright-field optics on a Zeiss Axioskop microscope. Thin sections (5 μm) from tissues stained with cerium were examined first using differential interference contrast (DIC) optics, and subsequently using epi-polarized light as described previously (Liu et al., 1995). Images were all recorded using Kodak T-Max 400 film.

3.4. SEM and X-ray microanalysis (SEM/XRMA)

Glass slides carrying paraffin embedded samples (10 μm) were trimmed, mounted on aluminum stubs covered with carbon tape, and coated with a heavy layer of evaporated carbon. Coated samples were analyzed as described previously (Liu et al., 1995), except that the accelerating voltage was 25 kV instead of 20 kV.

Acknowledgements

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