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Cytological Investigation of Resistance to *Leptosphaeria maculans* Conferred to *Brassica napus* by Introgressions Originating from *B. juncea* or *B. nigra* B Genome

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ABSTRACT

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Introgressions into *Brassica napus* from the B genome, either the *B. nigra* chromosome B4 or the *B. juncea* fragment carrying the *Jlm1* gene, have given rise to the *B. napus-B. nigra* addition line (LA4+) and the *B. napus-B. juncea* recombinant line (MXS), respectively. The resistance of these two lines to *Leptosphaeria maculans* is characterized by a hypersensitive reaction (HR) on both the cotyledons and leaves, while the collar displays a high degree of resistance. Responses induced in cotyledons of the two lines by *L. maculans* inoculation were investigated with em-

phasis on cytological events underlying the HR and on host defense reactions. Features of host cell changes including condensation and lobing of nuclei, fragmentation of chromatin, disruption of the nuclear membranes, and plasma membrane withdrawal were reminiscent of HR cell death in MXS and LA4+ plants. Restriction of pathogen growth to the infection areas in LA4+ was correlated to reinforcement of cell wall barriers, including wall apposition, papillae, and vessel plugging. In MXS, the lower expression of resistance was associated with a delay in plant responses. These results indicate that mechanisms underlying the HR in the *B. napus* recombinant and addition lines are differently controlled according to the introgressed genes.

Additional keywords: HR-type symptoms, resistance gene.

Blackleg, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.), is one of the most threatening diseases of *Brassica*, including oilseed rape (*B. napus* L. var. *oleifera*; AACC, 2n = 38). After invading the leaf mesophyll in susceptible plants, the fungus reaches the vascular bundle and colonizes the plant via petioles and stems (16). This intercellular systemic phase of growth is biotrophic and symptomless. It is followed by invasion of cortical cells in the basal part of the stem and subsequent development of collar necrosis. This necrotrophic phase results in the formation of stem canker, leading to lodging, which is the most damaging manifestation in the field. Because control of the disease by cultural and chemical practices is currently inadequate, the use of resistant cultivars has become the most promising strategy for sustainable management of blackleg.

Two types of resistance in *B. napus* have been identified. The first type, which occurs at the seedling stage, is thought to be mono- or oligogenic (2,24,27), whereas the second type is partial and polygenic (13,26) and only expressed at the collar level, though cotyledons and leaves remain susceptible. Because the resistance to *L. maculans* expressed by most commercial cultivars of *B. napus* does not prevent significant crop losses, an increase in resistance has become a major challenge in oilseed rape breeding programs.

Brassica species containing the B genome, such as *B. juncea* (L.) Czern. (AABB, 2n = 36) and *B. nigra* (L.) W. Koch (BB, 2n = 16), generally exhibit a hypersensitive response (HR) to *L. maculans* at the cotyledon stage. This resistance is thought to be mono- or oligogenic (19,20,27,33). Recently, introgression of either the *B. nigra* chromosome B4 or the *B. juncea* *Jlm1* gene into *B. napus* has given rise to a *B. napus-B. nigra* addition line (LA4+; AACC +

chromosome B4, 2n = 38 + 1) and a *B. napus-B. juncea* recombinant line (MXS; AACC, 2n = 38), respectively (8,9). Resistance in the MXS and LA4+ lines, efficient under artificial and field conditions, is characterized by a HR on cotyledons and leaves and by a high degree of resistance at the collar level (8,9). Recent genetic investigations demonstrated that the genes borne by each introgression had two different locations on the B genome; three markers, tightly linked to the *Jlm1* gene from *B. juncea*, were located on the chromosome B8 from *B. nigra* cv. Junius, but not on the chromosome B4 (8).

Biochemical analyses revealed that the HR of *B. juncea* to *L. maculans* correlated with the production of the phytoalexin brassilexin, which accumulated in *B. juncea* earlier and more intensely than in *B. napus* following an abiotic elicitation (32). In the *B. napus-B. juncea* interspecific progenies, phytoalexin accumulation was less intense than in *B. juncea*, but greater than in *B. napus* cultivars susceptible to *L. maculans* (31). Resistance in the *B. juncea* cotyledons was correlated with the restriction of hyphal growth, production of callose, and accumulation of a brownish material in the mesophyll cells (7).

In spite of major advances in our understanding of the events involved in the resistance of *B. juncea* to *L. maculans*, the mechanisms governed by resistance genes from the B genome and expressed in *B. napus*, *B. juncea*, and *B. nigra* are still controversial. In that context, further investigation into the resistance mechanisms to *L. maculans* conferred in *B. napus* by introgressed genetic material is essential to characterize and compare the responses induced in the *B. napus* AACC genome by the *Jlm1* gene and the chromosome B4. In the current histological and cytological study, we investigated the infection process of an A-group *L. maculans* isolate, avirulent on *B. juncea* cv. Picra and MXS and LA4+ lines and virulent on *B. napus* cvs. Darmor and Samouraï, with particular emphasis on the ultrastructure of tissues undergoing HR and host defense reactions. In the MXS and LA4+ lines, evidence

is provided that shows that the HR phenotype is associated with cell death features, restriction of pathogen growth, and elaboration of various host responses.

MATERIALS AND METHODS

Plant material. Two oilseed rape lines resistant to *L. maculans* were previously obtained through interspecific crosses between *B. napus* and either *B. juncea* or *B. nigra* cultivars. The first was the *B. napus-B. juncea* recombinant line MXS (*B. napus* cv. Samouraï carrying one resistance gene [*Jlm1*] from *B. juncea*) (8). The second was the *B. napus-B. nigra* addition line LA4+ (*B. napus* cv. Darmor carrying the chromosome B4 from *B. nigra* cv. Junius, responsible for resistance at the cotyledon stage) (9). Both the MXS and LA4+ lines are winter-type brassicas.

The winter-type *B. napus* cvs. Samouraï and Darmor (from INRA-SERASEM, La Chapelle d'Armentieres, France) (susceptible at the cotyledon stage), the resistant spring-type *B. juncea* cv. Picra, and the MXS and LA4+ lines were used in this study. Because cotyledons of the resistant spring-type *B. nigra* cv. Junius exhibited yellowing and chlorosis in our experimental conditions, cytological investigations were not carried out with this cultivar.

Seeds from each cultivar were sown in a sterilized soil mixture (sand/loam/compost, 1:1:1) in $7 \times 7 \times 8$-cm plastic pots and grown, under a 12-h photoperiod, in a growth cabinet at 15°C. Before inoculation of the plants, leaves were removed as they developed in order to prevent early senescence of cotyledons. Preliminary tests showed that cotyledon reaction was not modified by leaf removal (H. Brun, *unpublished data*).

Fungal culture. The single-ascospore isolate A290 of *L. maculans*, obtained from oilseed rape stem residues, was assigned to the A group (highly aggressive) by isozyme banding pattern and pigment production (36) and belonged to pathogenicity group 4 (PG4) (H. Brun, *unpublished data*) according to virulence tests on the three differential cvs. Westar, Glacier, and Quinta (24). Pycnidiospore suspensions were produced as described by Somda et al. (36) and adjusted to a final concentration of 10^7 pycnidiospores per ml.

Inoculation test. Cotyledons of 20 plants per genotype were inoculated 10 days after sowing. Immediately before inoculation, a small puncture was made in the middle of each cotyledon lobe (four inoculation sites per plant) with a thin sterile needle. Preliminary tests showed that wounding was necessary to ensure homogeneous infection and to generate consistent results (S. Roussel, *unpublished data*). A 10-μl droplet of the pycnidiospore suspension was deposited on each wound using an Eppendorf repeater pipette. Inoculated plants were then incubated in darkness for 24 h at 20°C and 100% relative humidity. Ten untreated plants and 10 wounded plants with a drop of sterile water deposited in place of inoculum were used as controls in each experiment. Compatible interactions included the *B. napus* cv. Samouraï-PG4 and *B. napus* cv. Darmor-PG4 interactions, and incompatible interactions included the *B. juncea* cv. Picra-PG4, MXS-PG4, and LA4+-PG4 interactions.

Evaluation of disease severity. Cotyledon reactions were classified according to lesion size 12 days after inoculation (d.a.i.), using the 0 (no visible symptoms) to 9 (collapse of tissue) rating scale of Williams and Delwiche (39). Plants in classes 1 to 5 were considered resistant to *L. maculans*, and plants in classes 6 to 9, susceptible. A disease index was calculated as $DI = \Sigma(N_i \times i)/N_t$, in which N_i represents the number of inoculation sites with disease score i ($i = 0$ to 9) and N_t represents the total number of inoculation sites.

Electron microscopy. Fragments (3×2 mm) centered on the wounds were excised from untreated, water-control, and inoculated cotyledons 1, 2, 3, 5, 8, and 12 d.a.i. For each genotype and observation date, eight inoculated sites from different plants together with four fragments from each of the two controls were examined under a light microscope. Electron microscopy observations were made on three inoculated sites, two water-infiltrated sites, and two untreated sites per genotype and per observation date.

To facilitate observations, three zones were defined around the infection site in the mesophyll: zone A, two cellular layers adjacent to the wounding zone; zone B, three cellular layers adjacent to zone A; and zone C, three cellular layers adjacent to zone B.

For histochemistry and immunocytochemistry, cotyledon fragments were fixed for 2 h in 1% (vol/vol) glutaraldehyde (Sigma Chemical Co., St. Louis) and 4% (vol/vol) paraformaldehyde (Sigma Chemical Co.) buffered with 0.1 M sodium cacodylate (Sigma Chemical Co.), pH 7.2. Samples were rinsed in the same buffer, dehydrated in a graded series of ethanol, and embedded in LR White resin (London Resin Co., TAAB, Reading, England).

For conventional electron microscopy and cytochemistry, samples were fixed for 2 h in 4% (vol/vol) glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2, postfixed for 1 h in 1% (vol/vol) osmium tetroxide (Sigma Chemical Co.) (OsO_4) in water, dehydrated in ethanol, and embedded in Epon 812 resin (TAAB, Reading, England).

Ultrathin sections (80 to 90 nm) were stained with uranyl acetate (Sigma Chemical Co.), followed by lead citrate (E. Merck AG, Damstadt, Germany), before being examined under a JEOL 100EX transmission electron microscope (JEOL, Ltd., London) operating at 80 kV.

Histochemistry. Semithin sections (1 to 2 μm) of fragments embedded in LR White resin were stained with 1% toluidine blue (Sigma Chemical Co.) in borax (Sigma Chemical Co.), pH 8.9, and observed under the Diaplan light microscope (Leitz, Lyon, France). A pink staining indicated the presence of pectic-like components. The percentage of xylem vessels occluded by pectic compounds was based upon the ratio between the number of occluded vessels and the number of observed vessels on a section. The number of observed vessels on a section was estimated to be 20. One section per block was observed, and four blocks were examined. Hyphae were also monitored in zones A, B, and C on each section.

Autofluorescence of phenolics was investigated on semithin sections embedded in LR White resin examined by epifluorescence microscopy using both a UV filter set (365-nm excitation and 400-nm barrier filter) and a blue filter set (420-nm excitation and 515- to 560-nm barrier filter) to detect the presence of phenolic compounds.

Cytochemistry. Cytolocalization of β-1-4-glucans was performed using a purified β-1-4-exoglucanase complexed to colloidal gold at pH 9.0 in 0.1 M phosphate-buffered saline (PBS) polyethylene glycol (PEG) (0.01 M) (4). Sections were incubated for 30 min at 37°C on a drop of the gold probe (dilution ¼, pH 6.5) before rinsing with PBS, washing in distilled water, and staining. Specificity of the labeling was assessed by incubating sections with the gold-complex protein previously saturated with an excess of β-1-4-glucans from barley.

Immunocytochemistry. Polyclonal antibodies raised against β-1-3-glucans (Cambridge Research Biochemicals, Cardiff, United Kingdom) were used to immunolocalize callose. Sections from cotyledons were incubated for 30 min at 37°C on a drop of primary antibodies (in 1/500 0.1 M PBS [pH 7.2], 0.5% bovine serum albumin, and 0.05% Tween) and rinsed with PBS before being incubated on gold-labeled goat anti-rabbit antibodies (1/20) (GAR-15; BioCell Research Laboratories, Cardiff, United Kingdom).

The monoclonal antibody JIM 5, raised against epitopes of unesterified pectin, was used to detect galacturonic acid-containing molecules. Immunogold localization of pectin was performed as described by Knox et al. (21). Sections were incubated on a drop of the primary antibody for 2 h at 37°C and then on a drop of a gold-labeled goat anti-rat antibodies (GAT 15) for 30 min at 37°C.

Specificity of labeling was assessed through the following control experiments performed on sections from untreated and infected cotyledons: (i) incubation with the antiserum previously adsorbed with the antigen, laminarin, and galacturonic acids, respectively; (ii) incubation with preimmune rabbit or rat serum instead of the primary antiserum; and (iii) omission of the primary antibody incubation step.

dation was observed. Wall appositions were observed by 3 d.a.i. and were usually found in zones A and B, but not in zone C, except by 12 d.a.i. (Table 3), when they showed a fibrillar-like organization with electron-dense bodies (Fig. 2A, arrows). Papillae were detected earlier in cv. Picra (5 d.a.i.) than in cotyledons of the susceptible cv. Samouraï (8 d.a.i.) The material that accumulated in the lumen of vessels also showed a fibrillar organization (Fig. 2B). The main changes detected in the host cells were characterized by a spatial reorganization of the tonoplast invaginated into the cytoplasm (Fig. 2C) by 3 to 8 d.a.i. in the three zones. In addition, enhanced vacuolation (Fig. 2D), localization of mitochondria close to the plasma membrane, fragmentation of the chromatin (Fig. 3A), and presence of highly lobed nuclei were striking features observed in the three zones of plants exhibiting HR.

In the resistant recombinant MXS line, no apparent cell wall degradation was observed. Wall appositions and papillae were similar to those observed in the cvs. Samouraï and Picra. Wall appositions were observed by 3 d.a.i. in zone A and by 12 d.a.i. in zone C (Table 3). Papillae were seen by 8 d.a.i. and extended to the three zones by 12 d.a.i. The vessel-accumulating material was similar in structure and electron density to that observed in the resistant cv. Picra and susceptible cv. Samouraï. Invagination of the tonoplast into the cytoplasm, similar to that observed in the resistant cv. Picra, was seen by 3 d.a.i. Vacuolation, localization of mitochondria close to the plasma membrane, and lobed nuclei with fragmented chromatin were also typical features observed in the cytoplasm of parenchyma cells by 3 d.a.i.

In the resistant addition LA4+ line, no apparent cell wall degradation was observed. Wall appositions were found in zone A by 5 d.a.i. and in zone B by 12 d.a.i. No papillae were detected (Table 3). Invagination of the tonoplast, vacuolation, fragmentation of chromatin, and highly lobed nuclei (Fig. 3B) were also observed in this line at 3 d.a.i. The material that accumulated in the lumen of vessels was similar to that described above.

Wall appositions, formation of papillae, and occlusion of vessels by a fibrillar material were observed in the LA4+ line at the same location and with a similar intensity as in cv. Darmor, but at a lower intensity than in the MXS line.

Our observations in mesophyll and vascular parenchyma cells were reproducible from one sample to another for the cvs. Picra

and Samouraï and the MXS line. In such cultivars, the magnitude of the host reactions was high. By contrast, a great variability between samples with respect to wall appositions, papillae, and vessels obstruction was observed in both the cv. Darmor and LA4+ line.

Compared with the untreated cotyledons, wall appositions were the only ultrastructural features found in sections from water-control cotyledons. They were observed in all genotypes by 3 days after wounding. As they appeared only in the zone A, the closest zone to the wound, they may likely result from wounding during inoculation. Nuclei did not display any morphological changes (Fig. 4A).

Cytochemical localization of β-1,4-glucans. In the susceptible cv. Samouraï, the interface between hyphae and mesophyll cells was characterized by a marked modification of the primary cell wall (Fig. 5A), with areas showing a weaker labeling with the exoglucanase-gold complex (Fig. 5A, arrows) as compared with unmodified wall portions. In the susceptible cv. Darmor, labeling was associated with detached fragments of the cell walls (Fig. 5B), but labeling was absent over wall appositions and papillae (data not shown).

In contrast, cell wall modifications were not observed in the resistant cv. Picra and the MXS and LA4+ lines (Fig. 5C). Wall appositions (Fig. 6A) were slightly labeled by the exoglucanase probe in cvs. Samouraï and Picra and the MXS and LA4+ lines. Labeling of papillae was positive in the cv. Samouraï (data not shown). Gold particles were also seen over compounds occluding vessels in the resistant cv. Picra (Fig. 6B), but not in the other genotypes (Fig 6C). In the controls, labeling over the cell walls was regularly distributed (Fig. 4B).

Immunocytochemical localization of β-1-3-glucans. Labeling of papillae occurred in the cvs. Samouraï, Picra, and Darmor (Fig. 7A) and the MXS line (Fig. 7B). Gold particles were distributed over wall appositions in cvs. Picra and Darmor and the MXS line (Fig. 7C), but not in cv. Samouraï or the LA4+ line. No labeling of the fibrillar material accumulating in vessels was observed; in contrast, the fungal cell wall showed uneven distribution of gold particles. Incubation of sections of control plants with the anti-β-1-3-glucans polyclonal antibody did not reveal any significant labeling.

Immunocytochemical localization of pectin. In the cv. Samouraï, incubation of sections with the JIM 5 anti-pectin mono-

TABLE 3. Ultrastructural changes of host cells in *Leptosphaeria maculans*-inoculated or control cotyledons of *Brassica* genotypes

Type of reaction	Genotype (*L. maculans*-genotype interaction)		Time (days) after inoculation											
			3			5			8			12		
			A[x]	B	C	A	B	C	A	B	C	A	B	C
Wall appositions	cv. Samouraï (compatible)	i[y]	−[z]	+	−	−	+	−	−	+	−	−	+	−
		c[y]	−	−	−	+	−	−	+	−	−	−	−	−
	cv. Darmor (compatible)	i	−	−	−	+	+	−	+	+	−	+	+	−
		c	−	−	−	−	−	−	−	−	−	−	−	−
	cv. Picra (incompatible)	i	+	+	−	+	+	−	+	+	−	+	+	+
		c	+	−	−	+	−	−	−	−	−	−	−	−
	MXS line (incompatible)	i	−	+	−	−	+	−	−	+	−	−	+	+
		c	−	−	−	+	−	−	+	−	−	+	−	−
	LA4+ line (incompatible)	i	−	−	−	+	−	−	+	+	−	+	+	−
		c	−	−	−	−	−	−	−	−	−	−	−	−
Papillae	cv. Samouraï (compatible)	i	−	−	−	−	+	−	−	+	−	−	+	−
		c	−	−	−	−	−	−	−	−	−	−	−	−
	cv. Darmor (compatible)	i	−	−	−	−	+	−	−	−	−	−	−	−
		c	−	−	−	−	−	−	−	−	−	−	−	−
	cv. Picra (incompatible)	i	−	−	−	−	+	+	−	+	+	−	+	+
		c	−	−	−	−	−	−	−	−	−	−	−	−
	MXS line (incompatible)	i	−	−	−	−	−	−	−	+	−	−	+	+
		c	−	−	−	−	−	−	−	−	−	−	−	−
	LA4+ line (incompatible)	i	−	−	−	−	−	−	−	−	−	−	−	−
		c	−	−	−	−	−	−	−	−	−	−	−	−

[x] Zones are defined from the wounding zone: A, two cellular layers adjacent to the wounding zone; B, three cellular layers adjacent to zone A; and C, three cellular layers adjacent to zone B.
[y] i = Inoculated cotyledons; and c = water-control cotyledons.
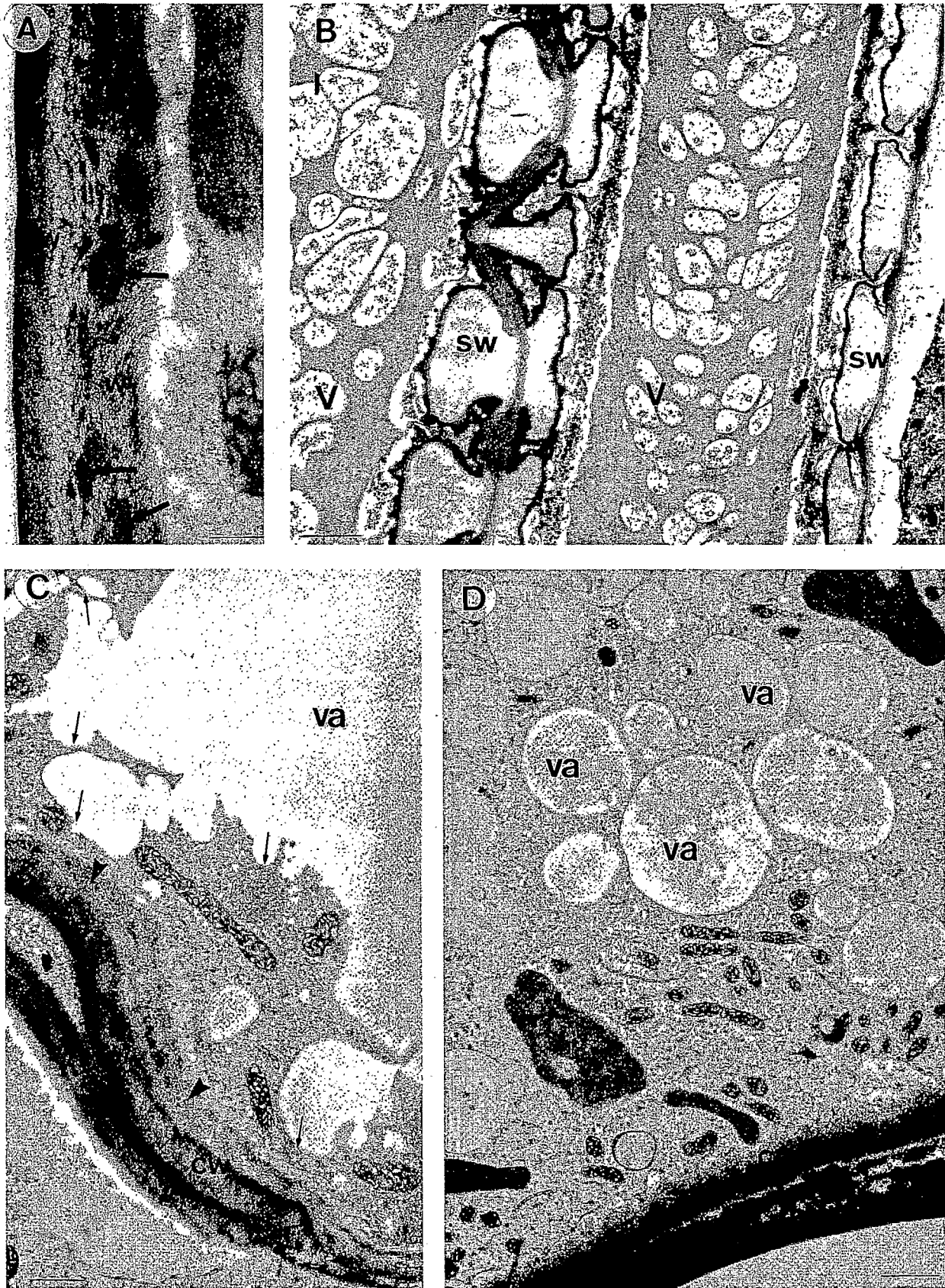[z] Rating scale: −, reaction absent; and +, reaction present.

Fig. 2. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons of the resistant *Brassica juncea* cv. Picra. Glutaraldehyde and osmium tetroxide fixation 12 days after inoculation. **A,** Electron-dense bodies (arrows) are included in the fibrillar material of a wall apposition (w) in the paramural area of a parenchyma cell. Bar = 0.3 μm. **B,** The material that accumulates in the xylem vessels (V) displays a fibrillar texture. sw = Secondary cell wall. Bar = 1.2 μm. **C,** The tonoplast shows invaginations (arrows) from the vacuole (va) into the cytoplasm of a parenchyma cell. A fibrillar wall apposition (arrowheads) is seen close to the cell wall (cw). Bar = 0.5 μm. **D,** The marked vacuolation (va) in the cytoplasm of this parenchyma cell is associated with localization of numerous mitochondria close to the plasmalemma. cw = Cell wall. Bar = 1.2 μm.

RESULTS

Symptoms. By 12 d.a.i., cotyledon lesions reached 5 to 9 mm in diameter in the compatible interactions *B. napus* cv. Samouraï-PG4 and *B. napus* cv. Darmor-PG4, with an average disease index of 7.9 ± 1.2 and 6.4 ± 1.9, respectively. In the incompatible interaction *B. juncea* cv. Picra-PG4, the diameter of HR-induced lesions never exceeded 3 mm, with an average disease index of 1.9 ± 0.8 at 12 d.a.i. In the incompatible interactions, *B. napus-B. juncea* MXS-PG4 and *B. napus-B. nigra* LA4+-PG4, the lesions reached 1 to 5 mm in diameter, with a disease index of 3.8 ± 1.2 and 2.6 ± 0.9, respectively, at 12 d.a.i. Due to variability in the diameter of lesions, microscopic investigations on the MXS and LA4+ lines were carried out on the plants that appeared to be the most resistant to *L. maculans* (plants exhibiting small brown necrotic symptoms). No visible symptoms were observed in plants treated with water.

Colonization of cotyledon tissues by *L. maculans*. Light microscopy revealed that, in all genotypes, hyphae were localized in intercellular areas of the mesophyll. In the lesions of the susceptible cv. Samouraï, hyphae were detected as soon as 3 d.a.i. in zones A and B and 5 d.a.i. in zone C. The intensity of colonization increased between 8 and 12 d.a.i., with hyphae multiplying abundantly in the three zones (Table 1). In the susceptible *B. napus* cv. Darmor, hyphae were detected 5 d.a.i. in the three zones. The intensity of colonization increased progressively up to 12 d.a.i. in the three zones (Table 1). Damaged hyphae with degraded or empty cells were never observed in either genotype at that time.

In the lesions of the resistant *B. juncea* cv. Picra, hyphae appeared only at 5 d.a.i. (Table 1). The few hyphae were consistently seen in zones A and B and seldom seen in zone C, except after 8 d.a.i. An average of 30% of the fungal cells showed an altered cytoplasm, including vacuolation of the cytoplasm and disorganization of organelles, at 5 d.a.i. (data not shown).

Within the lesions of the MXS line, hyphae were detected 3 d.a.i. and colonized the three zones at 5 d.a.i. The level of colonization was similar to that observed at up to 8 d.a.i. in the susceptible cv. Samouraï, but was lower than that observed in the susceptible cv. Samouraï at 12 d.a.i. (Table 1). Within the lesions of the LA4+ line, hyphae were not detected before 12 d.a.i. (Table 1). At that time, the level of colonization in the three zones was very low. Hyphae showing signs of damage were seen in both lines.

Obstruction of xylem vessels. In the compatible interaction *B. napus* cv. Samouraï-PG4, a material that stained pink with toluidine blue accumulated in the lumen of 8% of the xylem vessels at 5 d.a.i. This percentage increased to 97% by 12 d.a.i. (Table 2). In the compatible interaction *B. napus* cv. Darmor-PG4, 56% of the vessels were obstructed at 8 d.a.i., increasing to 87% by 12 d.a.i. In the incompatible interaction *B. juncea* cv. Picra-PG4, 49% of the vessels were occluded at 5 d.a.i. and 98%, at 12 d.a.i. (Table 2).

In the incompatible interaction MXS-PG4, no vessels were seen occluded at 5 d.a.i., but 75 and 91% contained an occluding material by 8 and 12 d.a.i., respectively. In the incompatible interaction LA4+-PG4, 57 and 97% of the vessels showed a pink material at 8 and 12 d.a.i., respectively (Table 2). In the controls, no material was seen in the vessels.

Autofluorescence. Except for lignin fluorescence in secondary vessel cell walls, no autofluorescence was observed on sections from any of the genotypes when infected with *L. maculans*.

Ultrastructural changes of host cells in the compatible interaction. Infection of cotyledons from the susceptible cv. Samouraï was accompanied by severe alterations of middle lamellae (Fig. 1A, arrows). Swelling of primary cell walls and lysis were noticed in areas adjacent to the pathogen. Eight days after inoculation, the cytoplasm of infected tissues was disorganized, including retraction of the plasmalemma from the cell wall. Wall appositions (deposition of wall material along the cell wall in the paramural area, with

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TABLE 1. Intensity of cotyledon colonization by the *Leptosphaeria maculans* pathogenicity group 4 isolate in *Brassica* genotypes

Plant genotype (*L. maculans*-genotype interaction)	3			5			8			12		
	A[x]	B	C	A	B	C	A	B	C	A	B	C
B. napus cv. Samouraï (compatible)	3 ± 0.7[y]	1 ± 0.2	...[z]	3 ± 0.9	1 ± 0.8	2 ± 1	4 ± 1.9	4 ± 2.1	2 ± 1.4	29 ± 3	34 ± 2.8	31 ± 2.4
B. napus cv. Darmor (compatible)	...	...	...	2 ± 0.6	1 ± 0.9	1 ± 1.2	11 ± 3	6 ± 1.6	2 ± 1.4	27 ± 2.2	22 ± 1.8	21 ±1.8
B. juncea cv. Picra (incompatible)	...	...	...	1	1 ± 0.6	...	2 ± 0.4	1 ± 0.3	1 ± 0.5	3 ± 0.5	1 ± 0.6	2 ± 0.2
B. napus-B. juncea recombinant MXS line (incompatible)	2 ± 0.4	1 ± 0.2	...	4 ± 0.8	1	1 ± 0.1	5 ± 1.1	3 ± 1.6	1	16 ± 2.1	12 ± 2.3	11 ± 2
B. napus-B. nigra addition LA4+ line (incompatible)	...	...	...	...	...	...	...	...	...	3 ± 1.1	2 ±1.3	1 ± 1.5

[x] Zones are defined from the wounding zone: A, two cellular layers adjacent to the wounding zone; B, three cellular layers adjacent to zone A; and C, three cellular layers adjacent to zone B.

[y] The intensity of mycelial colonization of host tissues was evaluated as follows. Hyphae were counted in zones A, B, and C. Two sections per sample were observed and two samples per plant were examined. Five plants were observed. Each value represents the mean of 20 measurements ± standard deviation.

[z] ... = Absence of mycelium.

TABLE 2. Intensity of staining with toluidine blue of vessel-accumulated material and percentage of occluded vessels in cotyledons of three *Brassica* genotypes following *Leptosphaeria maculans* inoculation

Plant genotype (*L. maculans*-genotype interaction)		3	5	8	12
B. napus cv. Samouraï (compatible)	i[x]	–[y]	+ (8 ± 0.9[z])	++ (77 ± 5.2)	+++ (97 ± 6.3)
	c[x]	–	–	–	–
B. napus cv. Darmor (compatible)	i	–	–	++ (56 ± 3.5)	+++ (87 ± 9)
	c	–	–	–	–
B. juncea cv. Picra (incompatible)	i	–	+ (49 ± 0.8)	++ (81 ± 5.1)	+++ (98 ± 5.6)
	c	–	–	–	–
B. napus-B. juncea recombinant MXS line (incompatible)	i	–	–	++ (75 ± 5.9)	+++ (91 ± 6)
	c	–	–	–	–
B. napus-B. nigra addition LA4+ line (incompatible)	i	–	–	++ (57 ± 6.2)	+++ (97 ± 5.8)
	c	–	–	–	–

[x] i = Inoculated cotyledons; and c = controls (water-treated or nontreated cotyledons).

[y] Intensity of staining: –, none; +, weak; ++, medium; and +++, intense.

[z] Percentage of occluded vessels. The percentage is based on the ratio between the number of occluded vessels and the number of observed vessels. Two sections per block and two blocks per plant were observed. Four plants were examined. Each value is presented ± standard deviation.

no particular location) and papillae (localized deposition of wall material in the paramural area, close to the pathogen) were often seen in the vicinity of hyphae and were always located in zone B (Table 3). The occluding material accumulating in xylem vessels displayed a fibrillar structure (Fig. 1B). In *L. maculans*-inoculated cotyledons of the susceptible cv. Darmor, the pattern of fungal infection and host modifications was similar to that described in the susceptible cv. Samouraï. Wall appositions were detected 5 d.a.i. in zones A and B (Table 3). The only visible papillae (Fig. 1C) were detected 5 d.a.i. in zone A. In the cv. Darmor, wall appositions and papillae were observed, but with a lower intensity than in the cv. Samouraï.

Ultrastructural changes of host cells in the incompatible interaction. In the resistant cv. Picra, no apparent cell wall degra-
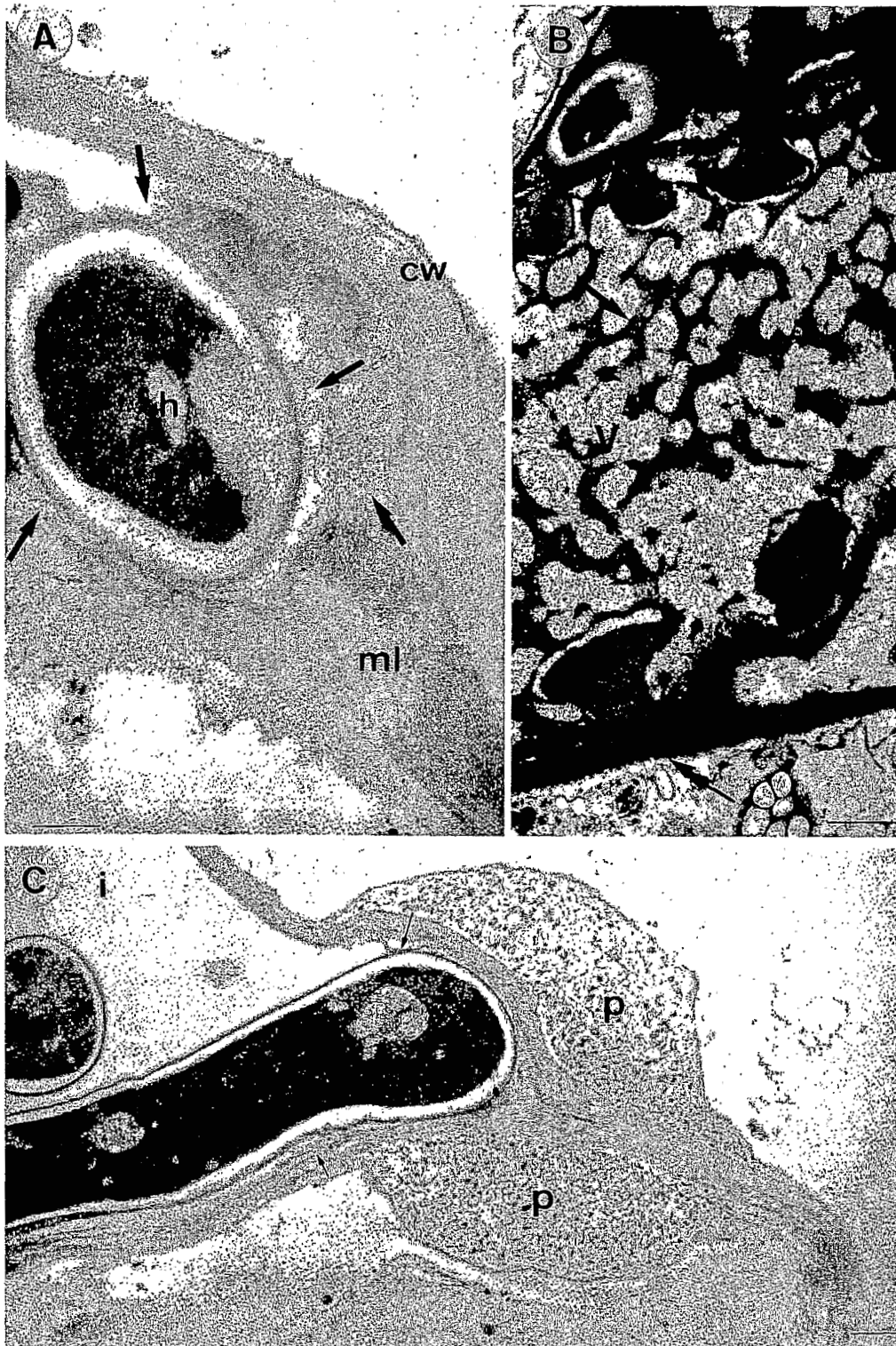
Fig. 1. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons of the susceptible *Brassica napus* cvs. Samouraï and Darmor. Glutaraldehyde and osmium tetroxide fixation 12 days after inoculation. **A,** Degradation of middle lamella (ml; arrows) is seen close to the fungal hypha (h) in the cv. Samouraï. Bar = 0.3 μm. **B,** The lumen of a xylem vessel (V) is occluded by a fibrillar material (arrow) that is closely associated with the secondary cell wall (sw). The middle lamella is highly electron dense (double arrow). Bar = 0.1 μm. **C,** Two papillae (p) are localized in the periplasmic areas of cells adjacent to the intercellular hypha (h) in cv. Darmor. The middle lamella and the cell wall (cw) show degradation patterns (arrows). i = Intercellular space. Bar = 0.8 μm.

clonal antibody resulted in a general labeling of primary cell walls and middle lamellae of infected cells (Fig. 8A). Altered areas of middle lamellae were associated with an uneven labeling (Fig. 8A). Papillae were labeled in the cv. Samouraï, but not in the cvs. Picra and Darmor and the MXS and LA4+ lines. Wall appositions were labeled in cvs. Samouraï and Picra (Fig. 8B), but not in cv. Darmor or the MXS and LA4+ lines. In all genotypes, the material that occluded vessels was evenly but slightly labeled (Fig. 8C and D). In control plants, gold particles were distributed regularly over middle lamellae (Fig. 4C).

DISCUSSION

The current microscopic study investigated plant cytological responses induced by resistance genes to *L. maculans* of the B ge-nome expressed in *B. napus*. We demonstrated that restriction of pathogen growth and expression of host defense reactions in the *B. napus-B. juncea* recombinant line (MXS) are different from those observed in the *B. napus-B. nigra* addition line (LA4+).

This analysis clearly emphasizes variations in location and timing of the cytological responses in both the compatible and incompatible interactions, including the infected introgressed *B. napus* lines. In the susceptible cvs. Samouraï and Darmor, the strong and extended fungal colonization of cotyledon tissues was associated with an important cell wall + degradation pattern. Cytochemical evidence for pectin and cellulose alteration close to *L. maculans* suggests the production of fungal hydrolases such as polygalacturonases and cellulases, the activity of which could be responsible for degradation of middle lamellae and cell walls (1,11,17). The local weakening or loosening of the host cell walls likely facili-
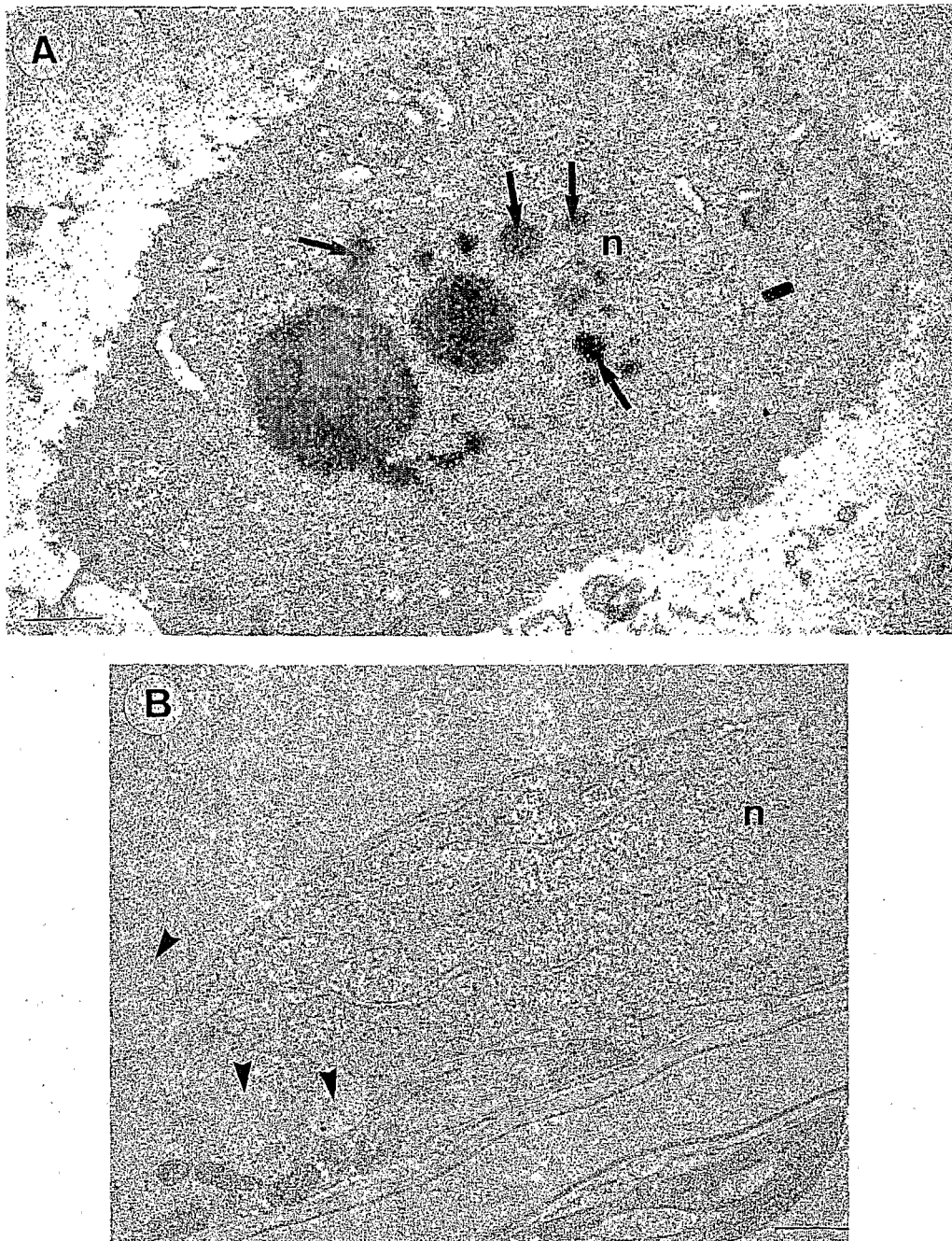

Fig. 3. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons. Glutaraldehyde and osmium tetroxide fixation 8 days after inoculation. A, In the resistant *Brassica juncea* cv. Picra, chromatin in the nucleus (n) of a parenchyma vascular cell shows a highly fragmented pattern (arrows). Bar = 0.6 µm. B, In the resistant *B. nigra-B. napus* addition line (LA4+), the highly lobed nucleus (n) of a parenchyma cell is associated with vacuolation (arrowheads) of the cytoplasm. Bar = 1.2 µm.

tated fungal ingress inside leaf tissues. By contrast, during the incompatible interactions *B. juncea* cv. Picra-*L. maculans*, MXS-*L. maculans*, and LA4+-*L. maculans*, the low rate of host invasion by the pathogen was associated with an apparent integrity of the host cell walls that did not display any striking modification of labeling. This suggests that inhibition of either the activity or the production of fungal lytic enzymes occurred in

infected tissues of plants challenged by the PG4 avirulent *L. maculans* strain.

Ultrastructural changes of host cells conferred by the introgressed *Jlm1* gene or the chromosome B4 in the *B. napus* genetic background (AACC genome) resembled those shown by cells undergoing the HR in the incompatible interaction *B. juncea* cv. Picra-*L. maculans* PG4. Comparison of cellular changes in water-infiltrated
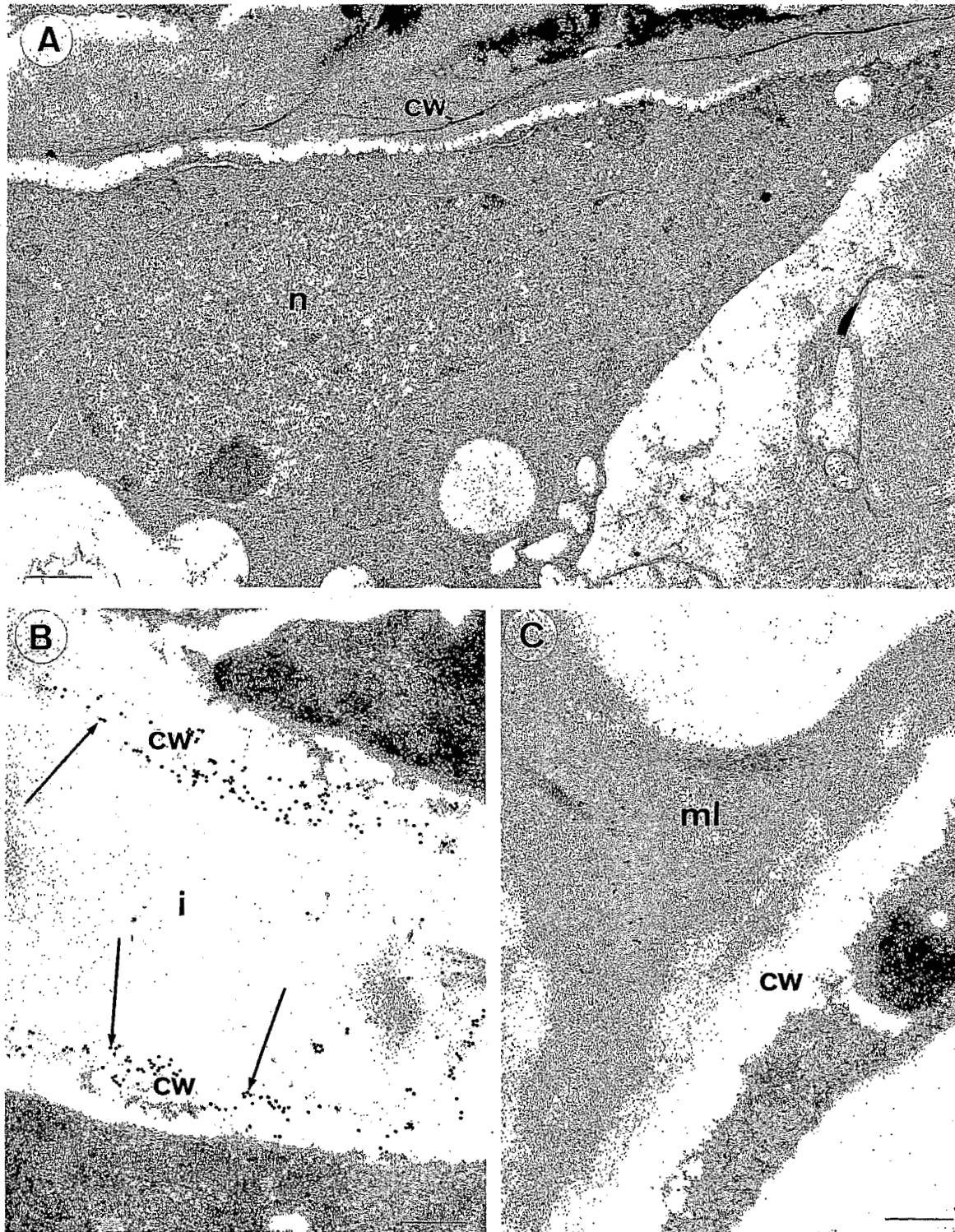
Fig. 4. Transmission electron micrographs of transverse sections in cotyledons of control plants. Glutaraldehyde and osmium tetroxide fixation. A, In the water-treated cotyledons of the resistant *Brassica juncea* cv. Picra, the nucleus (n) of this parenchyma cell exhibits a regular shape. c = Cell wall. Bar = 0.5 μm. B, Localization of β-1-4-glucans using an exoglucanase-gold probe. Cell walls (c) of a parenchyma cell in the water-treated cotyledons of the resistant *B. napus-B. juncea* recombinant MXS line are regularly labeled (arrows). Very few particles are seen over the adjacent cytoplasm and the intercellular area (i). Bar = 0.3 μm. C, Immunocytolocalization of pectin using the JIM 5 monoclonal antibody followed by GAT-10 secondary antibodies conjugated to gold. Labeling mainly occurs over the middle lamella (ml) in a cell of a healthy cotyledon in the susceptible *B. napus* cv. Samouraï. Few gold particles are associated with the cell wall (cw). Bar = 0.3 μm.

cotyledons with those observed in *L. maculans*-inoculated ones allowed the distinction between modifications triggered by the wounding process and those induced by the pathogen. Condensation and lobing of nuclei, fragmentation of chromatin, and disruption of nuclear membranes are cytological features that have been reported for plant cell death triggered by avirulent fungi (5,18,34). Invagination of the tonoplast, withdrawal of host plasmalemma, and cytoplasm vacuolation have also been reported during HR cell collapse (6,14). According to our observations, resistance controlled by the *Jlm1* gene or the chromosome B4 in the AACC genome of *B. napus* is characterized by a HR associated with restriction of the fungal development. The positive correlation between the HR and the ultrastructural features of cell death observed in the modified *B. napus* genotypes indicates that the chromosome B4 and the *Jlm1* gene were able to activate pathways leading to resistance in the *B. napus* genetic background.
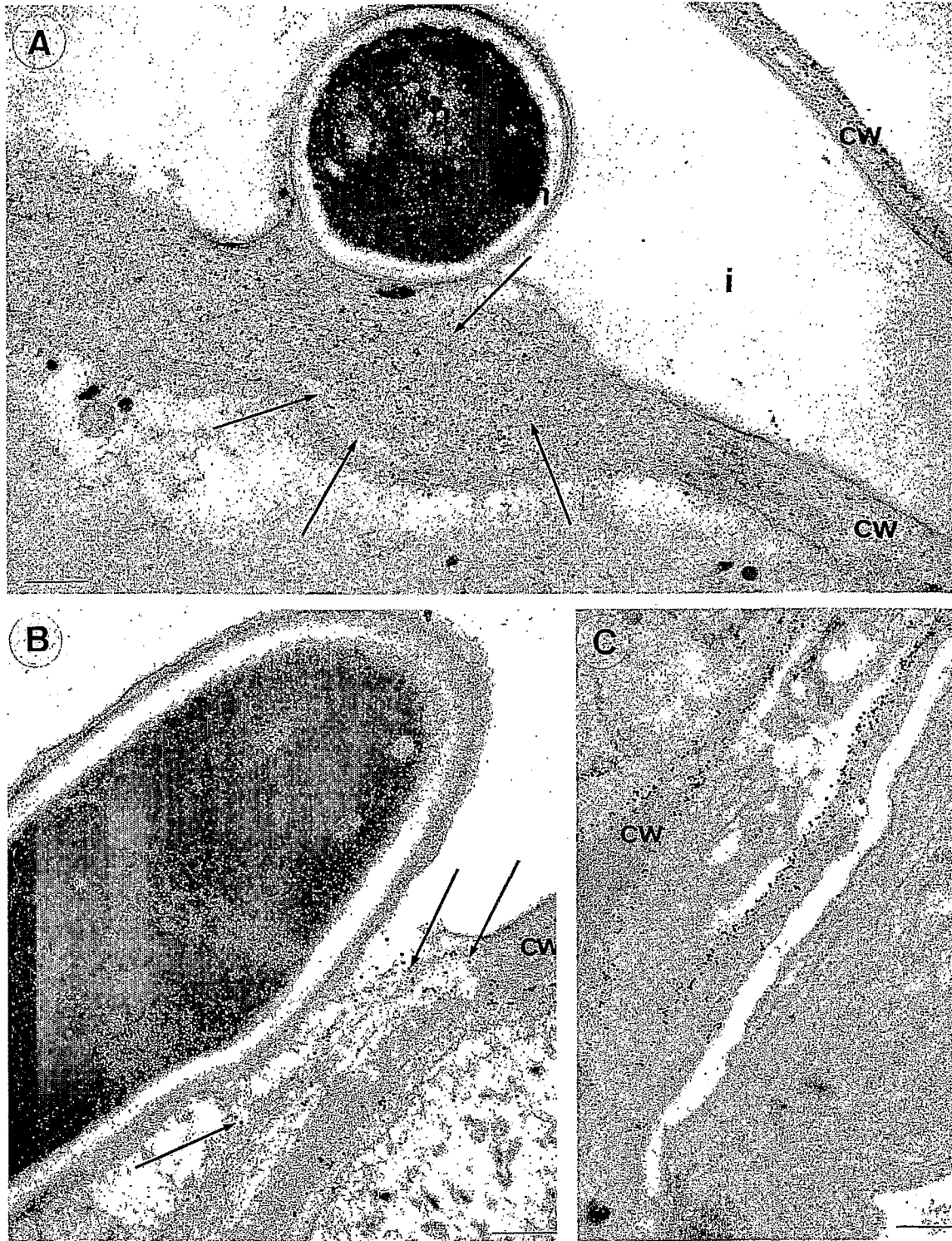

Fig. 5. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons. Glutaraldehyde and osmium tetroxide fixation 12 days after inoculation. Localization of β-1-4-glucans using an exoglucanase-gold probe. A, In the susceptible *Brassica napus* cv. Samouraï, swelling of the primary cell wall (cw), in contact with the hypha (h), is associated with areas that show weak labeling (arrows). Very few particles are associated with the fungal cytoplasm. i = intercellular space. Bar = 0.7 μm. B, In the susceptible *B. napus* cv. Darmor, disruption of the cell wall (cw) close to the hypha (h) displays labeled fragments (arrows). Bar = 0.3 μm. C, Walls of cells (cw) in the resistant *B. napus-B. nigra* addition LA4+ line show an even distribution of gold particles. Bar = 0.3 μm.

Three defense reactions were identified microscopically in cotyledons challenged by *L. maculans* in compatible and incompatible interactions: wall appositions, papillae, and vessel plugging. These are nonspecific responses that are not based on gene-for-gene interactions (23), since they were observed during the infection process in the susceptible *B. napus* cvs. Samouraï and Darmor. Only detected in zone A in the water controls, we can assume that those responses were triggered by the infection process in the tested genotypes. Wall appositions represent a nonspecific response to cell injury, possibly functioning as part of a mechanism restricting the effects of injury to host cells (29). Similarly, formation of papillae is a common event described in multiple compatible and incompatible interactions (10,12,37), since this process is likely involved in the reinforcement of barriers to lateral fungal spread. Occlusion of vessels has also been reported to usually be associated with colonization of xylem elements by vascular pathogens in many plants species (3,25). Vessel plugging is believed to slow down the active movement of microbes or spore transportation, including *L. maculans* (30), within the xylem (22,28,35), which is the way *L. maculans* reaches the stem collar (15).
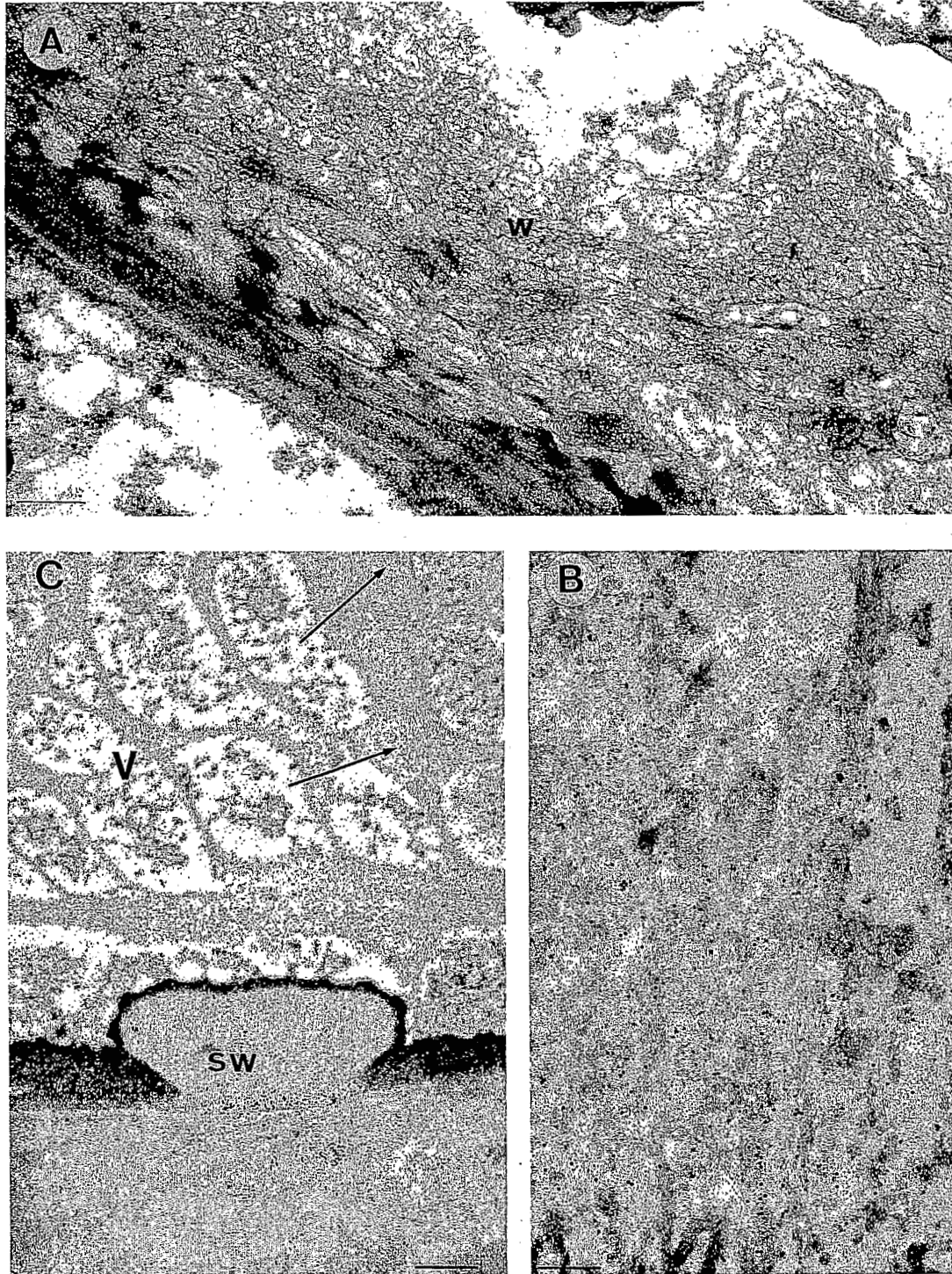

Fig. 6. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons. Glutaraldehyde and osmium tetroxide fixation. Localization of β-1-4-glucans using an exoglucanase-gold probe 12 days after inoculation. **A,** Few gold particles are associated with the primary cell wall (cw) and wall appositions (w) in a cotyledon cell of the susceptible *Brassica napus* cv. Samouraï. Bar = 0.3 μm. **B,** In the resistant *B. juncea* cv. Picra, labeling is intense over the vessel-occluding material. Bar = 0.3 μm. **C,** In the MXS line, the labeling (arrows) is no significant over the vessel-accumulating material (arrows) in contrast to the secondary walls (sw). Bar = 0.7 μm.

The results of the current cytochemical study provided evidence that wall appositions and papillae contained polysaccharides including cellulose, callose, and pectin, and that material obstructing xylem vessels contains pectic-like molecules and cellulose. The presence of electron-dense bodies within these newly formed structures indicates that compounds other than polysaccharides were deposited sequentially at these sites. The opacity of this material probably is caused by an interaction with osmium tetroxide, suggesting the presence of phenolics, although no autofluorescence under UV illumination was detected within the inoculated host cell structures.

While no difference was seen in frequency and timing of vessel obstruction between the MXS resistant line and the susceptible cv. Samouraï, more extensive wall appositions and papillae were observed in the resistant line. Introgression of the *B. juncea Jlm1* gene in the *B. napus* AACC genome induced a phenotype that was associated with enhanced formation of structural barriers. As compared with the resistant *B. juncea* cv. Picra, from which the *Jlm1*
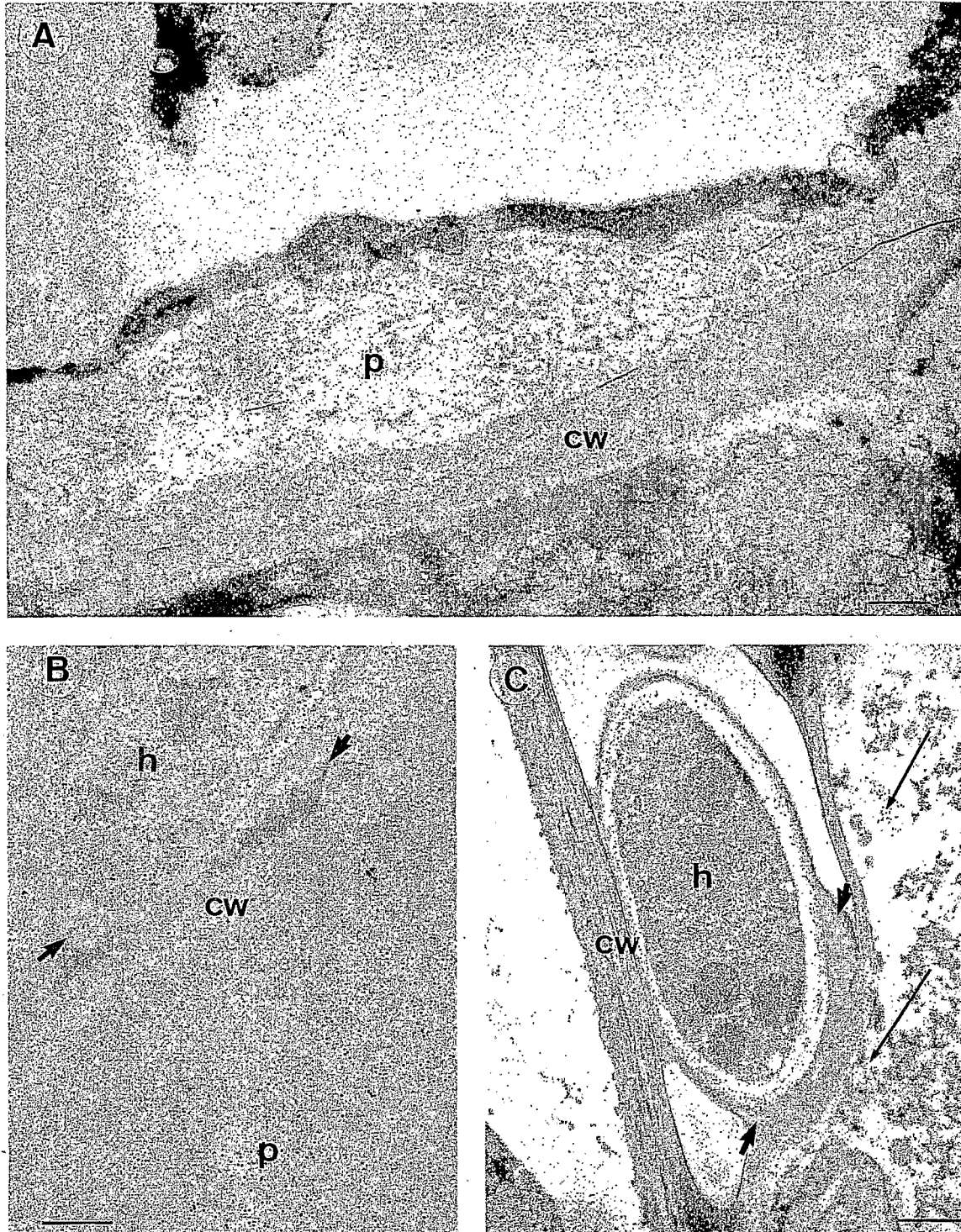

Fig. 7. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons. Glutaraldehyde and osmium tetroxide fixation. Localization of β-1,3-glucans using polyclonal antibody anti-β-1,3-glucans followed by incubation with GAR-15 secondary antibodies conjugated to gold 12 days after inoculation. A, A regular and dense labeling is seen over a papilla (p) in the susceptible *Brassica napus* cv. Darmor. Few gold particles are associated with the cell wall (cw). Bar = 0.5 µm. B and C, In the MXS line, B, papilla (p) and C, wall appositions (arrows) are evenly labeled. Gold particles are associated with the fungal cell wall. The adhering fungal material in contact with the plant cell wall (cw) (arrowhead) is not labeled. h = Hypha. B and C, Bars = 0.3 and 0.7 µm, respectively.

gene originated, reinforcement of these cell wall barriers occurred later in the MXS line and was correlated with a less effective restriction of pathogen spread. The weaker expression of resistance in the MXS line in comparison to that observed in *B. juncea* may result from the absence of another resistance gene that was demonstrated to be involved in the control of *L. maculans* in *B. juncea* (27,38). According to Keri et al. (20), the two resistance genes may be epistatic in *B. juncea*. This additional resistance gene could

be carried by the chromosome B4, which was introgressed in the *B. napus-B. nigra* LA4+ line.

During the incompatible interaction LA4+-*L. maculans*, wall appositions, papillae, and vessel obstruction were similar to that observed in the susceptible *B. napus* cv. Darmor; however, fungal growth was much weaker and restricted to the infection areas. Resistance conferred by the chromosome B4 in the *B. napus* genetic background was very efficient, although the current microscopic
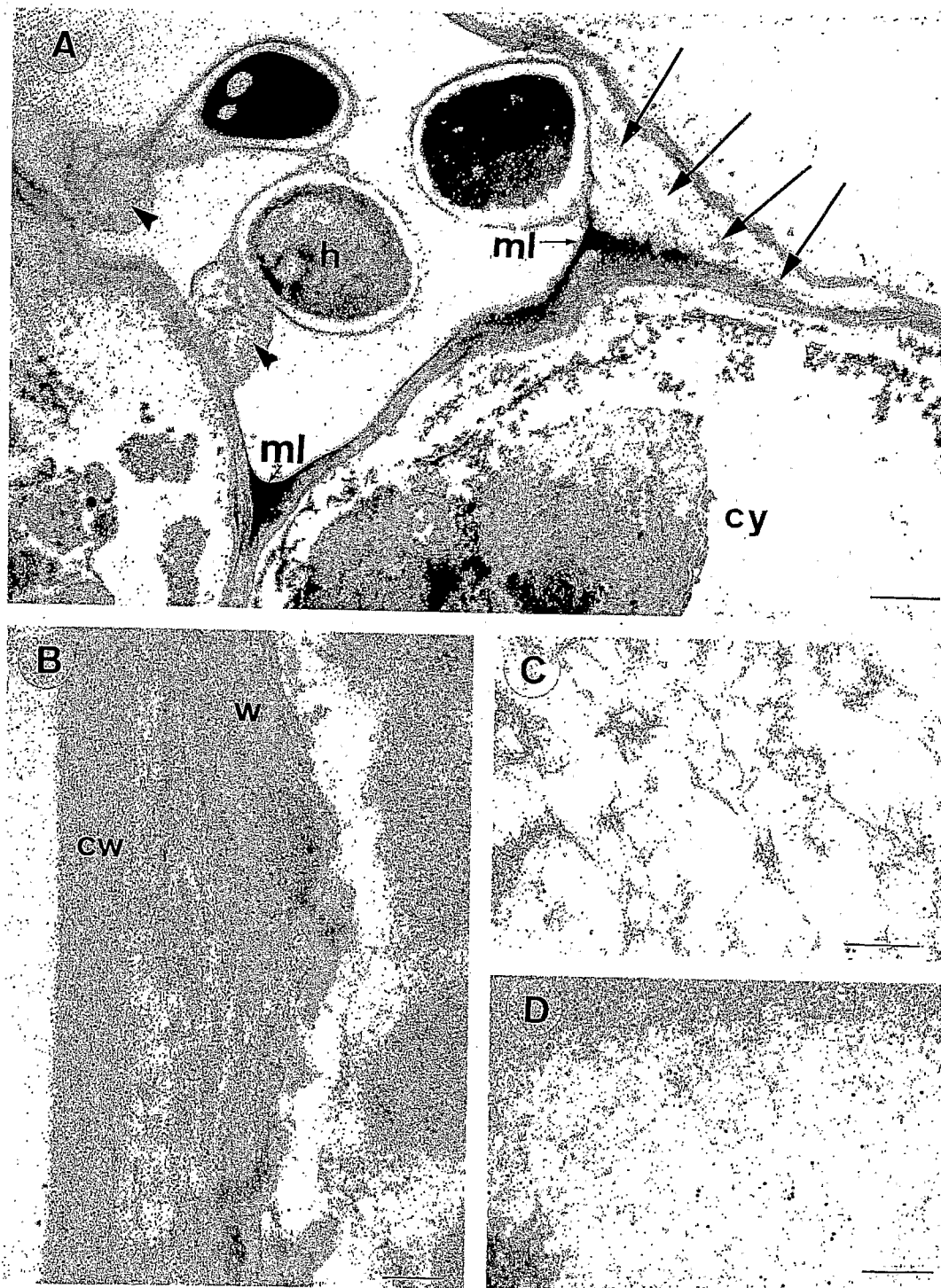

Fig. 8. Transmission electron micrographs of transverse sections of *Leptosphaeria maculans*-inoculated cotyledons. Glutaraldehyde and osmium tetroxide fixation. Localization of pectin using the JIM 5 monoclonal antibody followed by incubation with GAT-10 secondary antibodies conjugated to gold 12 days after inoculation. A, In the susceptible *Brassica napus* cv. Samouraï, a regular labeling is observed over the host cell wall and the middle lamella (ml). The fungal adhering material close to the host cell wall does not show any significant labeling (arrowheads). The degraded area of the middle lamella shows a weak labeling (arrows). Notice that the adjacent cells (cy) show pronounced collapse and plasmalemma retraction from the cell walls. h = Hypha. Bar = 0.8 μm. B, In the resistant *B. juncea* cv. Picra, the cell wall (cw) and the wall apposition (w) are labeled. Bar = 0.2 μm. C and D, Labeling is also present over the material occluding vessels in C, cv. Picra and D, the MXS line. Bars = 0.2 μm.

investigation only studied responses that were cytologically detectable. It is likely that the resistance gene carried by the chromosome B4 triggered signaling pathways in *B. napus* involved in the activation of host defense genes, the expression of which was not assessed here. It is known that the brassilexin phytoalexin accumulates in cotyledons and leaves of *B. napus-B. juncea* interspecific progenies in response to infection by *L. maculans* (31). Accordingly, investigations on phytoalexin accumulation during infection of the LA4+ line by *L. maculans* would be of interest to assess whether chromosome B4 is able to activate brassilexin or other phytoalexin biosynthesis in the AACC genome background.

In conclusion, the current study demonstrates that the *B. nigra* chromosome B4 and the *B. juncea Jlm1* gene from the B genome both induced a HR in *B. napus* with variable effects on limitation of the pathogen growth. It shows that both origins of B genome resistance trigger mechanisms that lead to ultrastructural features of HR cell death and suggests a possible differential expression of defense genes involved in wall apposition, papillae formation, and vessel plugging, but perhaps not in wall phenolic production. Investigations on the recently created *B. napus* lines that contain both the resistance gene from chromosome B4 and the *Jlm1* gene (A. M. Chèvre, *unpublished data*) would be of great interest in understanding the key role the B genome may play in mechanisms underlying resistance to *L. maculans* in the *B. napus* AACC genome.

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