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Ultrastructural Studies of the Mode of Penetration by *Phoma macdonaldii* in Sunflower Seedlings

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ABSTRACT

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An ultrastructural investigation of the artificial inoculation of sunflower with *Phoma macdonaldii* conidia was undertaken using light, scanning, and transmission electron microscopy to elucidate the host-parasite relationship. The behavior of the conidia deposited on the cotyledon petiole was investigated at various time intervals after inoculation. Conidia adhesion and germination were observed first. The cotyledon petiole was invaded by the fungus directly through the cuticle and via stomata. Externally, the spore and germ tube were covered with a mucilaginous polysaccharide sheath of a cotton-like appearance and of variable thickness. At the time of penetration, the host cuticle was perforated mechanically. The cuticle was slightly depressed and no enzymatic alteration could be observed. The fungus did not form appressoria on the surface of the host tissues but developed an infection peg. As soon as the cuticle barrier was crossed, the fungus rapidly colonized the host parietal layer. In a first step, the plasmalemma of the host cell appeared to be stuck against the cell wall. As soon as the fungus passed through the epidermal cell wall to reach the host cytoplasm, the plasmalemma was disrupted, and the subsequent rapid breakdown of cell integrity favored the colonization of the tissues by the pathogen.

Additional keywords: black stem, *Helianthus annuus,* host–pathogen interactions, subcuticular hyphae.

The black stem disease of sunflower (*Helianthus annuus*) is caused by *Phoma macdonaldii* (8) whose teleomorph is *Leptosphaeria lindquistii* (18). It has been reported in many European countries (Yugoslavia, Italy, Bulgaria, Romania) (2), Iran (33), Pakistan (44), China (24), Australia and the United States (2). Since 1990, the disease has been expanding steadily and is now recognized as one of the most serious diseases of sunflower in France (40). The disease is mainly characterized by the appearance of black spots on the stem around the petiole insertion point. Coalescing spots at the base of the stem develop into a wide black sleeve (40), and black maculae surround the back of the flower head. Premature ripening results in yield losses of 10 to 30% (39), decreased oil content (34), and decreased thousand seed weight (11). The disease can also cause early plant death (45,16). The symptoms of black stem disease of sunflower have been described by various authors, but the histological and ultrastructural aspects of the infection of sunflower by *P. macdonaldii* have not been documented.

Fungal pathogen entry into plants may proceed through direct penetration via enzyme and toxin effects or mechanical pressure or indirectly through wounds or natural openings such as lenticels or stomata (25). The different tissues of the host (cotyledon petiole, leaf petiole, leaf lamina, stem, etc.) may be penetrated differently by the same fungus. Each species of fungal pathogen may display different modes of penetration under different conditions (25). In the present study, an ultrastructural investigation of the artificial inoculation of the cotyledon petiole of sunflower by *P. macdonaldii* conidia was undertaken to elucidate the host–parasite relationship.

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MATERIALS AND METHODS

Plant material. Seeds of a susceptible sunflower hybrid (Santiago) were disinfected for 5 min in a 0.5% sodium hypochlorite solution, then rinsed three times in sterile distilled water. They were sown regularly at a depth of 2 cm in 40×30×25-cm plastic containers filled with vermiculite. Plants were raised in a growth chamber regulated at 24 ± 1°C (14-h illuminated period) and 17 ± 1°C (10-h dark period), and 75 to 85% relative humidity. The light intensity during the illuminated period was 200 µE m^{-2} s^{-1}, provided by SON–T AGRO 400 lamps (Philips, Eindhoven, Netherlands). Each container was watered every 3 days with 500 ml of water that contained 1 ml of a nutrient solution (N-P-k, 6-3-6, and micronutrients [Substral, Boulogne-Billancourt, France].

Fungal isolate. A single conidial isolate of *P. macdonaldii* isolated from naturally infected plants collected in the southwest of France was used. The pathological and physiological characteristics of this isolate, which is one of the most aggressive isolates of the pathogen (42), remained constant with time, using the method of conservation described by Arabi et al. (4) for *Drechslera teres.* This method has proven to be efficient for the conservation of *P. macdonaldii* isolates since 1996.

Artificial inoculation. The pathogen was incubated on a potato dextrose agar (PDA) medium at 25 ± 1°C in the light (37 µE m^{-2} s^{-1}). After 10 days of incubation, the addition of sterile distilled water to the surface of the culture followed by mechanical stirring allowed a conidial suspension to be obtained. The suspension concentration was adjusted to 10^6 conidia per ml. Using a micropipette, 20 µl of spore suspension were introduced into the pit

formed by the cotyledon petiole and the epicotyl of 12-day-old sunflower seedlings (first pair of developed leaves, stages 1 to 2 [31]). A microdrop of sterile distilled water was used on the control plants. After inoculation, the containers in which the plants were grown were covered with a polyethylene bag to promote pathogen development. Observations of five samples were carried out at different time intervals after inoculation: 12, 16, 20, and 36 h.

Light Microscopy. The petiole of the cotyledon was cut into pieces of approximately 5 mm^2. The upper epidermis of the cotyledon petioles (seat of inoculation) was then peeled. The samples were stained with lactophenol-cotton blue and rinsed in lactophenol for 10 min. The sections were mounted in distilled water and examined under a light microscope (Leica, Rueil-Malmaison, France).

Scanning electron microscopy. Fresh samples of sunflower seedlings cotyledon petioles were prepared by the cryopreservation technique. Each sample was mounted with kaolin on the stub of a Hexland CT1500 CryoTrans system (Oxford Instruments Ltd., Eynsham, England) and plunged into nitrogen slush at –210°C. After eventual fracturing, specimens were transferred from an evacuated pre-chamber to the microscope stage, which was cooled by nitrogen gas at –160°C. Surface frost was sublimed by raising the stage temperature and keeping it at –80°C for 5 min. The specimen was withdrawn and put back in the prechamber, where it was sputter-coated with gold then transferred back to the microscope stage at –160°C. Micrographs were obtained at 10 kV using a 525 M scanning electron microscope (Philips).

Transmission electron microscopy. The cotyledon petiole was cut into pieces of approximately 3 mm^2 for examination by transmission electron microscopy. Samples were fixed with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, for 2 h at room temperature.

Material was postfixed in 2% osmium tetroxide in the same buffer for 2 h at 20°C before being dehydrated in a graded ethanol series. Preparations were embedded in Spurr's epoxy resin (46).

Ultrathin sections (100 nm thick) were cut on a Reichert OMU3 microtome (Leica) and collected on gold grids. Sections were stained for 10 min with uranyl acetate and for 5 min with lead citrate for examination at room temperature. Sections were examined on a 301 electron microscope at 80 kV (Philips).

Cytochemical techniques. Cytochemical detection of polysaccharides was performed by PATAg labeling (periodic acid, thiocarbohydrazide, silver proteinate) according to Dargent and Touzé-Soulet (13). The specificity of the reactions was assessed on control sections without thiocarbohydrazide (TCH) treatment.

RESULTS

Light Microscopy. Light microscopy (LM) observations of the fungus in sunflower cotyledon petioles are presented in Figure 1. Sixteen hours after inoculation, most spores with a 4 ± 0.5 µm diameter had germinated (Fig. 1A). Germination was first accompanied by the formation of a filament constituted of 6 ± 2 µm-long

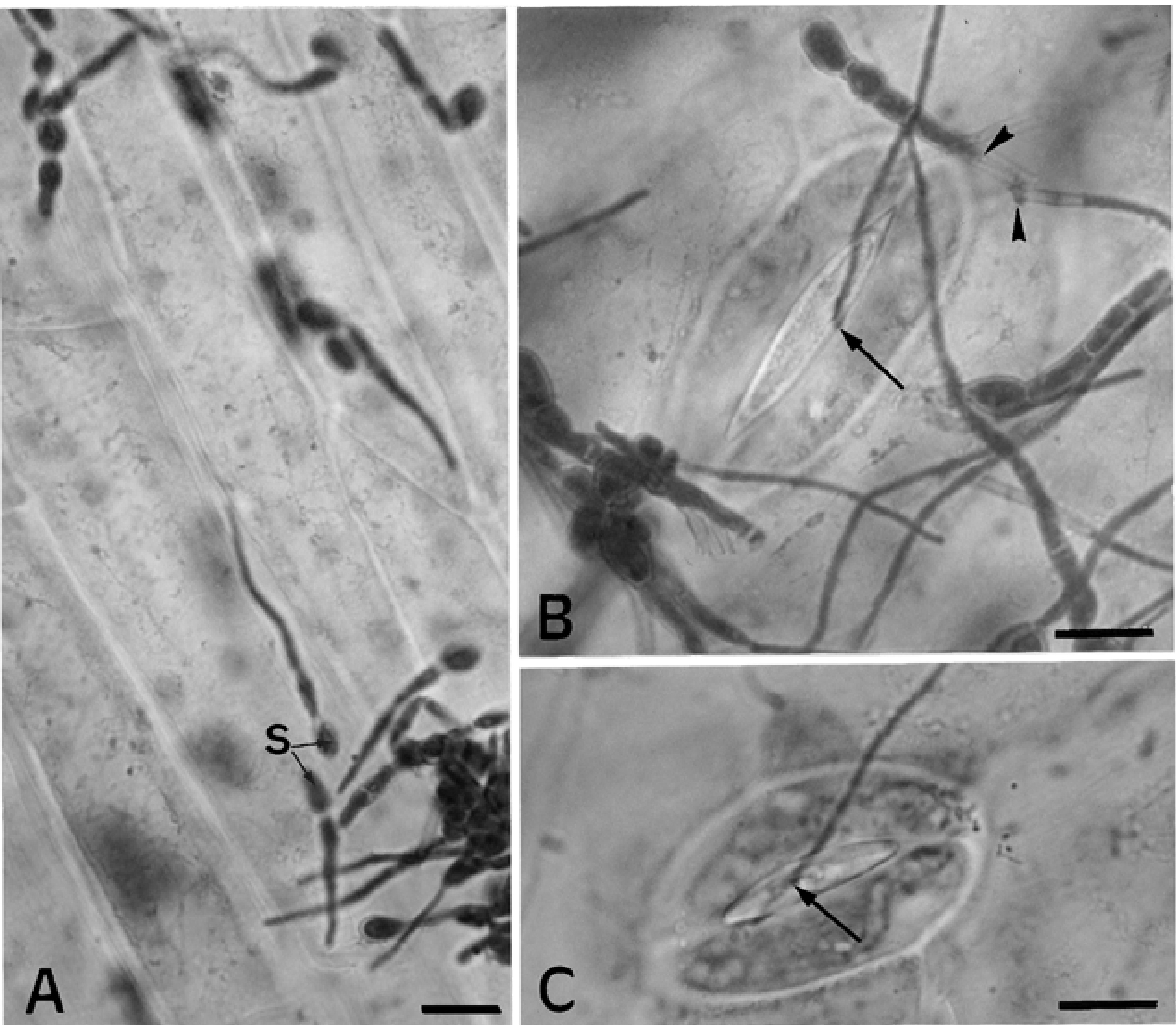

Fig. 1. Light micrographs of *Phoma macdonaldii* on sunflower cotyledon petioles. **A,** Germinated pycniospores (S) 16 h after inoculation (scale bar = 10 µm). **B,** Penetration via stomata (arrow) and directly through cuticle (arrow head). The hyphae beneath the cuticle were less intensely stained than those at the surface (arrow head) (scale bar = 10 µm). **C,** Penetration via stomata (arrow): a hypha passing through the stomatal opening (scale bar = 10 µm).

cells with the same diameter as the spores. Germ tubes eventually reached a 1 ± 0.3 µm diameter and did not form any appressoria at the surface of the tissues. Penetration proceeded either directly through the cuticle or through the stomata (Fig. 1B and C). The hypha at the surface of the host tissues was intensely stained by cotton blue; in contrast, after penetration, the mycelium color was lighter (Fig. 1B).

Scanning electron microscopy. Twelve hours after inoculation, some of the spores with a 4 ± 0.5 mm diameter gave rise to a variable-length germ tube (not exceeding 7 µm; Fig. 2A and B). Twenty hours after inoculation, most of the spores had germinated to give rise to short, unbranched, and creeping tubes developing into a mycelial tapetum (Fig. 2C). The surface of the cuticle and epidermal cells in contact with the germ tube did not appear to be altered, and no adhesive structure was observed. However, in the presence of a high inoculum density, spore germination resulted in the development of aerial hyphae (Fig. 2D, arrows).

After 24 h, the external hyphae developed by the fungus on the surface of the host tissues were 1 µm in diameter, a measurement comparable to those observed in LM. From 36 h after inoculation, the fungus was observed within the host cell wall as well as inside the epidermal cells whose cytoplasmic volume was gradually colonized (Fig. 2E).

Transmission Electron Microscopy. Between 36 and 48 h after inoculation, transverse sections of sunflower cotyledon petioles corresponded to the relatively important development of the fungus externally, as well as inside the host tissues (Fig. 3A). Externally, the spores observed were characterized, after PATAg staining, by a uniform wall that was weakly contrasted to electrons. The 0.3-µm-thick wall was limited, on the outside, by a cotton-like mucilaginous sheath of variable thickness. The often-retracted cytoplasmic content in every case was highly vacuolated (Fig. 3A). Spore germination gave rise to constricted, septate filaments, whose length did not exceed 5.6 µm; the wall was uniform and limited by an external mucilaginous layer identical to the spore wall. After double staining with uranyl acetate and lead citrate, these cells displayed an electron-dense, little-vacuolated cytoplasm, with various endomembrane systems. However, at the interface with the host epidermis, the mucilaginous sheath was thinner or invisible. At the point of contact with the host cell wall, no structural alteration was visible (Fig. 3B). Pathogen penetration was characterized by the mechanical perforation of the host wall. The cuticle was slightly collapsed, and no enzymatic alteration was observed. The fungus developed an infection peg and, as soon as the cuticular barrier was crossed, rapidly colonized the host parietal layer. Moreover, at the level of the constriction, a septum was formed in every case (Fig. 3C).

The fungal pathogen thus was composed of a septate hypha with cells with an average length of 7 µm. After PATAg staining, the nearly electron-transparent wall was devoid of any mucilaginous sheath. The cytoplasm of each pathogen cell was electron-dense, contained various endomembrane systems, and, at the

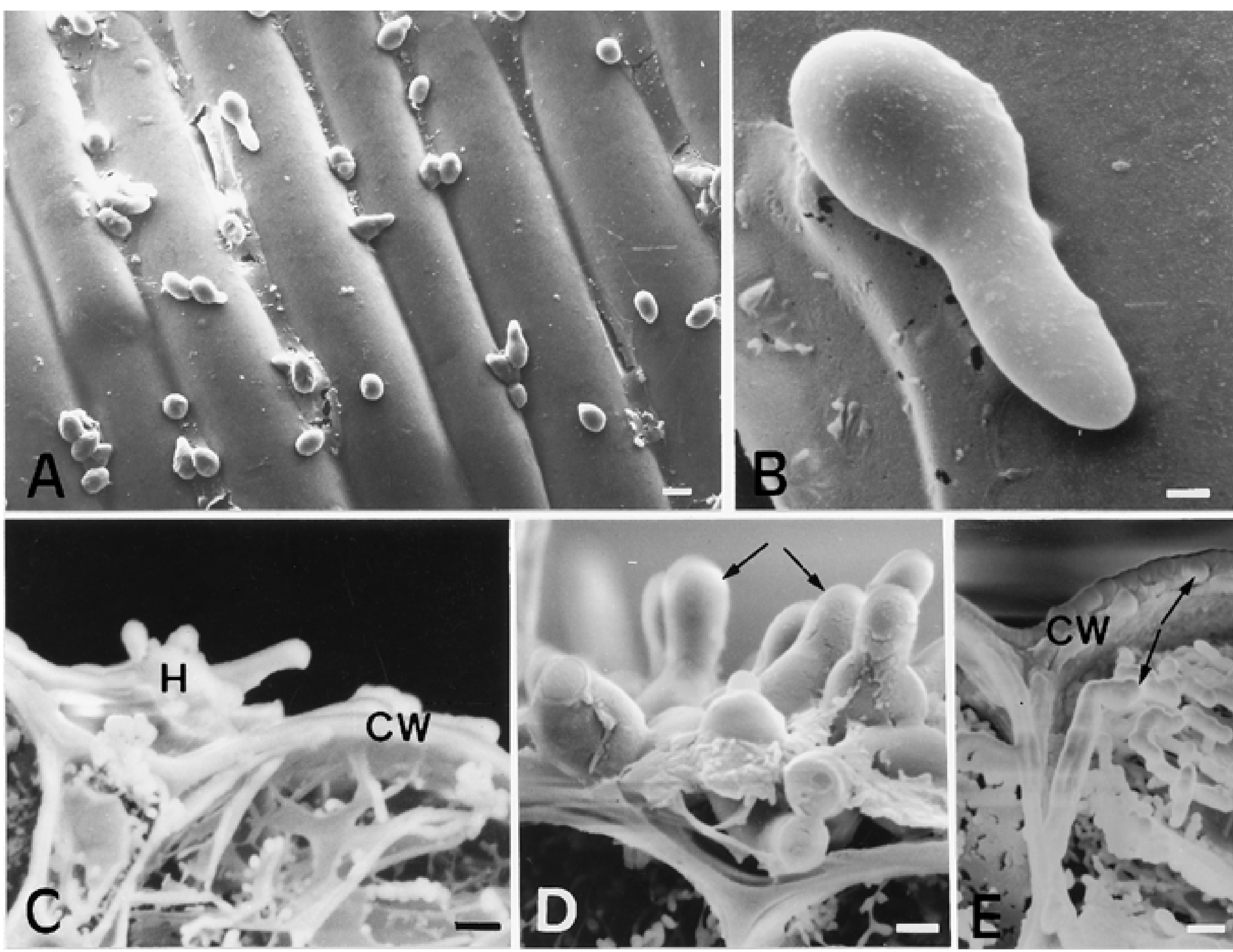

Fig. 2. Scanning electron micrographs of *Phoma macdonaldii* on sunflower cotyledon petioles. **A,** Overall view of the germinated pycniospores 12 h after inoculation (scale bar = 5 µm). **B,** Magnification of a germinated pycniospore (scale bar = 1 µm). **C,** Development of hyphae (H) at the surface of the cuticle 20 h after inoculation. Cell walls (CW) appear intact (scale bar = 2 µm). **D,** High density of pycniospores at the surface of the epidermal cells. Some of the spores gave rise to aerial hyphae (arrows) (scale bar = 2 µm). **E,** Hyphae were present in cell walls (CW) 36 h after inoculation and colonized the cytoplasm (arrows) (scale bar = 2 µm).

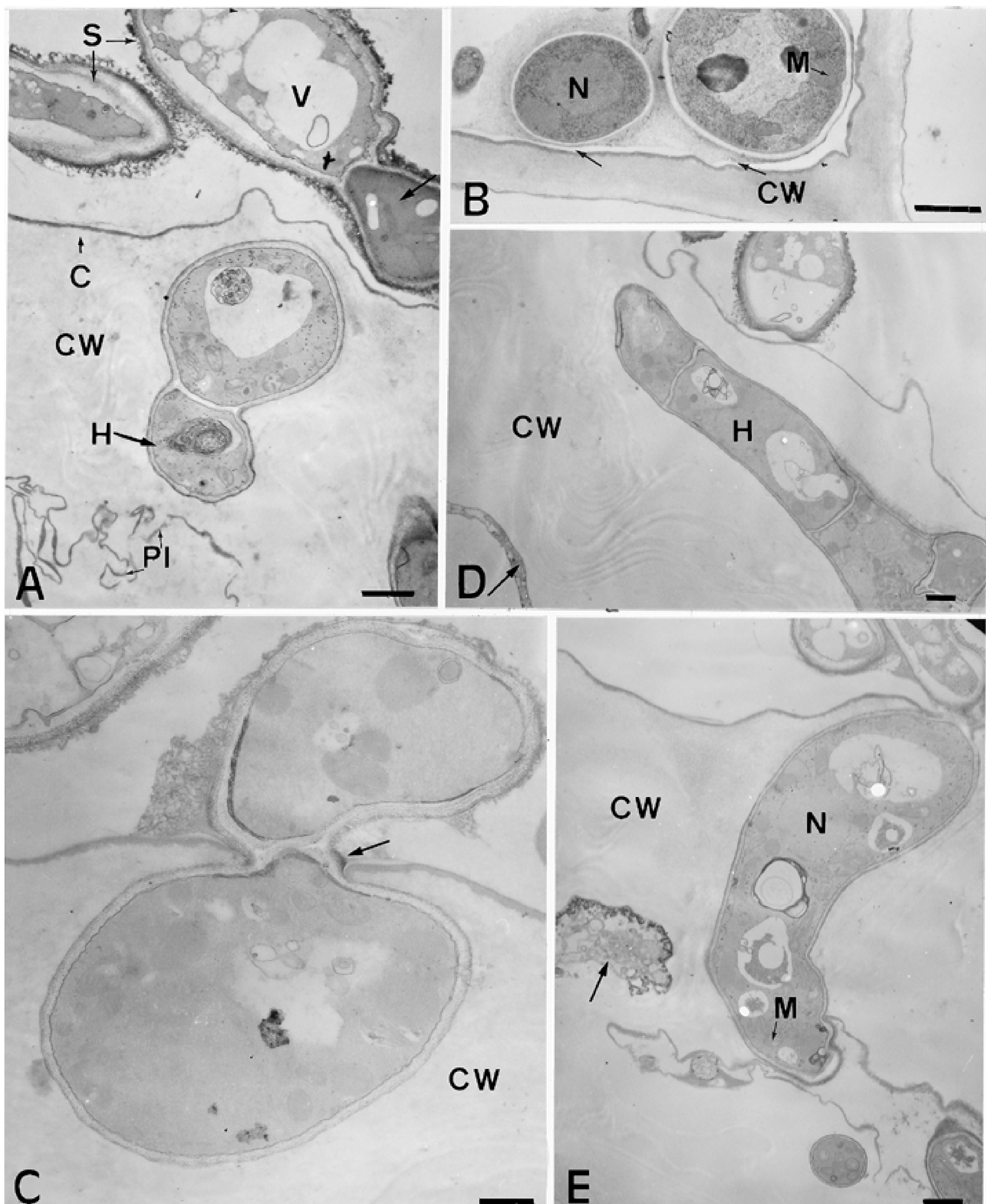

Fig. 3. Transmission electron micrographs of *Phoma macdonaldii* on sunflower cotyledon petioles. **A,** Germinating and highly vacuolated (V) *Phoma* spore (S) at the surface of the cuticle (C). The germ tube was richer (darker) in cytoplasm (arrow). The external electron-dense mucilaginous layer present on the germinating spores was absent on the hyphae (H) observed within the host cell wall (CW). The host plasmalemma was altered (scale bar = 1 µm). **B,** Transverse section of hyphae at the cuticle surface after double staining with uranyl acetate and lead citrate. Endomembrane systems shown: nucleus (N), and mitochondria (M). The mucilaginous substance at the points of contact with the cuticle (arrows) was thinner or even disappeared (scale bar = 1 µm). **C,** Direct penetration through the cuticle without appressoria formation. Hyphae penetrated the cell wall by mechanical pressure with formation of an infection peg (arrow). After penetration into the cell wall (CW) the hyphae were devoid of mucilage (scale bar = 0.5 µm). **D,** Expanding septate hyphae (H) within the cell wall (CW) beneath the cuticle. Host cytoplasm and plasmalemma (arrow) appeared to be intact (scale bar = 1 µm). **E,** Endomembrane systems shown in hyphae: nucleus (N) and mitochondria (M). The hyphae present within the cell wall (CW) eventually reached the host cytoplasm, causing structural disorganization of the host cell (arrow) (scale bar = 1 µm).

young stage, was little vacuolated (Fig. 3D). In a first step, the host plasmalemma was stuck against the host cell wall (Fig. 3E). As soon as the fungus passed through the epidermal cell wall to reach the host cytoplasm, the plasmalemma was disrupted, and the subsequent rapid breakdown of cell integrity favored the colonization of the tissues by the pathogen (Fig. 3E).

DISCUSSION

The symptoms of black stem have been described by various authors, but the histological and ultrastructural aspects of the infection of sunflower cotyledon petiole by *P. macdonaldii* have not been documented.

The ultrastructural investigation of the artificial inoculation of sunflower with *P. macdonaldii* conidia showed the occurrence of spore adhesion, germination, mucilage production, and direct penetration through the cuticle or stomata without appressorium formation. The present study also reports the presence of subcuticular hyphae in cotyledon petioles of sunflower infected with *P. macdonaldii* conidia.

Fungal adhesion to the host surface is an essential prepenetration process that determines the success of infection and disease development (25). Germinating conidia of *P. macdonaldii* and their germ tubes appeared to be attached to the sunflower cotyledon surface by the mucilaginous sheath covering the hyphae and conidia. Some fungal species produce mucilage (polysaccharides, glycoproteins, hexosamine polymers and xylans) to ensure close contact with the host (25,30,32). According to Gold and Mendgen (19), the role of fungal exudates appears to be multiple. These roles include attachment to the plant surface, sealing up of the penetration site, protection of appressoria against desiccation and other limiting environmental factors, and a reservoir for "penetrating enzymes." Staining by PATAg led to the characterization of this mucilaginous substance in *P. macdonaldii* as a polysaccharide. Such mucilaginous exudates had been reported for *P. linguam* by Abadie and Boudart (1). Other fungi closely related to *P. macdonaldii* also produce such exudates: *Ascochyta rabiei* (23), *A. fabae* (35), *Phyllosticta amplicida* (27), and *Phomopsis phaseoli* (29). For some fungal pathogens, such as *Colletotrichum graminicola* (38), *Verticillium alboatrum* (tomato roots), and *Fusarium oxysporum* f. sp. *lycopersici*, it was suggested that this mucilaginous substance also contain wall-degrading enzymes (7). Pascholati et al. (38) reported that *Erysiphe graminis* conidia release a liquid with esterase activity that partially eroded the surface of the cuticle of barley leaves. In addition to providing adhesion, these mucilages may contain substances used for recognition of the host surface.

In our study, direct penetration to cotyledon petiole tissue through the cuticle and stomata was observed without the formation of an appressorium. Penetration without appressorium was reported for other *Phoma* species, such as *P. linguam* (21) and *P. narcissi* on Hippeastrum leaves (43). Penetration without appressorium formation was also reported for *Phomopsis longicola* (*Diaporthe phaseolorum*) (6), *Phomopsis leptostromiformis* on the stem of lupins (*Lupinus angustifolius*) (50), and *Septoria apiicola* (17). In contrast, appressorium formation was observed in other fungi closely related to *Phoma* sp., such as *Phomopsis helianthi* (36), *Phoma exigua* (14), *Ascochyta pisi* (22), *A. fabae* (35), and *A. rabiei* (23,37). Different species of Phoma can infect plants with or without appressorium formation. The behavior of the fungus could be different on other parts of the plant, such as the leaf petiole or base of the stem, where pathogen penetration might be accompanied by appressoria formation. The formation of appressorium is known to depend on different factors, such as epicuticular waxes, rigidity, and surface hardness (23).

There are several types of natural openings in plants that are relatively unprotected and, therefore, vulnerable to penetration by fungal hyphae. Stomata are probably the most common route for pathogen entry (25). The stomata on the surface of the cotyledon petiole of sunflower may facilitate fungal pathogen penetration. Penetration via stomata in the cotyledon petiole of sunflower was observed for *P. macdonaldii*. This route of penetration has been reported for other pathogenic fungi, such as *Leptosphaeria maculans* (*P. linguam*) (1,20), *P. exigua* var. *linicola* (14), *Ascochyta pisi* (22), *Phomopsis phaseoli* (29), *Septoria tritici* (12), and *S. apicola* (17).

The cuticle is multilayered and separated from the plant cell wall by pectic compounds. Fungal pathogens encounter this outer layer first and usually penetrate by mechanical force (25) or through the effects of degrading enzymes, such as cutinase in the case of *F. solani* f. sp. *pisi* (51). Apparently, in *P. macdonaldii,* the infection peg breaks the cuticle by mechanical pressure. Such a mode of penetration has been reported for *Rhizoctonia solani* in bean hypocotyls (26). Direct penetration has also been reported for other fungi such as *Phomopsis scabra* on sycamore leaves (3), *Colletotrichum gloeosporoides* (15), *C. lagenarium* (9), and *Fusarium* spp. (28). The involvement of enzymes has been reported for *P. linguam* (pectinolyases and hemicellulases) (10).

Formation of subcuticular hyphae is not a phenomenon currently observed in fungal plant pathogens and, thus, might be considered an interesting feature of *P. macdonaldii* behavior on the cotyledon petiole of sunflower. As far as we know, the occurrence of subcuticular hyphae has not been reported in other species of *Phoma*. However, their presence after penetration has been reported for a number of plant pathogens. *Rhynchosporium secalis* (5), *Venturia inaequalis* (41), *Botryosphaeria vaccini* (49), *Alternaria brassicae* (48), and *Phomopsis leptostromiformis* (50) form such hyphae before further colonization of tissues. All of these pathogens have a relatively short latent period, except for *Phomopsis leptostromiformis*. The short latent period of *P. macdonaldii* (approximately 24 to 36 h on a susceptible variety of sunflower), thus, might be accounted for in terms of the formation of subcuticular hyphae.

Further histopathological studies of the mode of penetration and tissue colonization at different parts of sunflower, such as the base of the stem, leaf petiole, leaf lamina, or back of the flower head, are necessary to elucidate the *Phoma*–sunflower interactions, using both tolerant and susceptible genotypes. The role of the *P. macdonaldii* toxin (zinniol) (47) in the pathogenesis and infection process should also be investigated.

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