Mode of Penetration by *Phoma macdonaldii* in Susceptible and Tolerant Sunflower Genotypes

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Abstract

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An ultrastructural investigation on the infection of susceptible and tolerant lines of sunflower (*Helianthus annuus* L) using artificial inoculation with *Phoma macdonaldii* Boerema conidiospores was carried out using scanning, transmission electron microscopy, and light microscopy to elucidate the host-parasite relationship. Results revealed the occurrence of direct penetration through the cuticle of cotyledon, petiole and roots after adhesion to the plant surface without prior formation of appressoria. Mechanical penetration of cell walls by the fungal hyphae was demonstrated, and no enzymatic degradation at the penetration point was observed. The evolution and progression of the fungus within stem and root tissues after penetration differed according to host susceptibility. The pathogen has similar morphological features in different root tissues of the susceptible genotype roots. In contrast, differences in morphological features of hypha based on location of the pathogen in root tissues was noted in the tolerant genotype. The pathogen was observed only in the roots cortex of tolerant line, and could not proceed further, with the presence of many empty hyphae. Whereas the whole root tissues including the vessels of susceptible line were colonized by the pathogen with only few empty hyphae.

Keywords: Black stem, direct penetration, *Helianthus annuus*, host-pathogen interactions, *Phoma macdonaldii*.

Introduction

Phoma black stem of sunflower (*Helianthus annuus* L.), caused by the soil-borne fungus *Phoma macdonaldii* Boerma (= *Phoma helianthi taberosi* Sacc.), teleomorph *Leptosphaeria lindquistii* Frezzi (11), is characterized by large jet-black lesions on the stem. In addition, the fungus produces lesions on leaves, back of the head, and at crown or base of the stalk. The typical stem lesions originate with leaf infections which progress down the petiole to the stalk. Under favorable conditions, the leaf wilts, the petiole turns black, and the stem lesions expand to form a large shiny black patch with definite borders. Small circular fruiting bodies of the fungus are produced on the stem surface, but they are inconspicuous to the naked eye and require a hand lens to be observed. Early plant senescence results in yield losses of 10–30% (25), and reduction in oil content of seeds (22) as well as thousand seed weight (8).

The pathogen penetration into plants may proceed through enzymatic and toxin effects, mechanical pressure, or through wounds or natural openings such as lenticels or stomata (28).

The symptoms of black stem have been described by various authors (2, 3, 15, 21), but the histological and ultrastructural aspects of the infection of sunflower roots and stems by *Phoma macdonaldii* have been reported in few studies (28, 29).

In the present study, an ultrastructural investigation of the infection of stem and root of susceptible and tolerant genotypes of sunflower by artificial inoculation with *P. macdonaldii* conidiospores was undertaken to investigate further the host–parasite interaction.

Material and methods

Seeds of susceptible (C150) and tolerant (C137) sunflower lines were disinfected for 20 min. in 6 chlorometric degree of sodium hypochlorite solution, then rinsed three times in sterile distilled water. Seeds were placed on the surface of a growth medium in Magenta boxes (three seeds/box) containing Murashige and Skoog medium solidified with Phytagel (Sigma). Experiments were performed in a growth chamber at 25±1°C, with14-h light (200 µE m^{-2} s^{-1}) and 10-h darkness.

A single-spore conidial isolates of *P. macdonaldii* was used and conserved according to Arabi *et al.* (5) for *Drechslera teres*. The pathogen inoculum was prepared from 10 days culture grown on PDA medium at 25±1ºC in continuous light (37 µE m^{-2} s^{-1}) and used for 10 days sunflower seedlings infection. The proximity of main roots of two pairs of well developed seedlings were inoculated by syringe with 20 µl of inoculum suspension (10^6 conidiospores/ml water) mixed with 0.5% orange juice and 0.25% gelatin. The stem infection was carried out by using a 20 µl of the above mentioned inoculum which was introduced into the pit formed by the cotyledon petiole and the epicotyl of sunflower seedlings. Sterile distilled water was used for the control treatments, and trials were repeated three times.

All seedlings were incubated under a polyethylene bag to create high humidity conducive to disease development.

Scanning electron microscopy

After two and four days of incubation, 1-2 cm pieces of seedling roots and stems, including inoculation points (IP), were used for examination by scanning electron

microscope. Samples were fixed for 24 hours at 4 ºC in 50 mM sodium cacodylate buffer (pH 7.15) containing 2.5 % (w/v) glutaraldehyde (Oxford Agar, Oxford, UK) and then washed in the same buffer without glutaraldehyde, washed in water and dehydrated twice in series of aqueous ethanol solutions (20, 40, 60, 80 and 95 % v/v), for 30 minutes each, and twice in absolute ethanol for 1h. Ultra thin sections were performed using a CPD750 Emscope apparatus. Dry samples were placed on a block and covered by 50 nm conductive layer of or-palladium (model JFC1100 JEOL). Sections were examined by a scanning electron microscope (HITACHI S450) at 20 kV. Photographs were taken using CCD color camera (Color CoolView, Photonic Science, Milham, UK).

Transmission electron microscopy

Pieces of approximately 2 mm^2 were fixed and dehydrated as previously described. Progressive infiltration with Spurr's epoxy resin (London Resin White, Oxford, UK) was carried out by serial incubation in ethanolic solutions of increasing concentrations of Spurr's resin (1/2, 1/1, 2/1, v/v, for 12 hours each) and incubated in undiluted resin for 12h at 4 ºC. Infiltrated samples were then embedded in capsules and allowed to polymerize for 24 hours at 70 ºC. Semi-thin (1 µm) and Ultra-thin sections (80-90 nm) were prepared using an UltraCut E ultra-microtome (Reichert-Leica, Germany) and collected on gold grids. Semi-thin sections were stained with Toluidine blue (0.5% w/v) in 2.5% sodium carbonate buffer, pH 11. Ultra-thin sections were subjected to the periodic acid-thiocarbohydrazide-silver proteinate reaction (PATAg) according to Thiéry (31). For PATAg staining, sections were floated on a 1% (w/v) aqueous solution of periodic acid for 30 min., at room temperature, and rinsed in distilled water, treated for 12h. at 4°C with a 20% aqueous solution of acetic acid containing 0.2% thiocarbohydrazide, and washed in water. Sections were then floated on a 1% (w/v) aqueous solution of silver proteinate for 30 min in the dark, washed in water and air dried before examination with a transmission electron microscope.

Light microscopy

Fresh materials were coated by 5% aqueous agarose solution. Transversal and longitudinal sections were obtained using a vibratome (Microcut H1250; Energy Beam Science Inc., St Louis MO, USA). The samples were stained with Evans blue (0.1% w/v) for 2 min. The sections were mounted in distilled water and examined under a light microscope.

Results

Twenty-two plants per replication and ten sections per plant were observed. Two days after inoculation, an extensive colonization of the susceptible line roots by the fungal mycelium was developed. Pathogen grew from the inoculation point along the roots, and formed a thin layer of long ramified hyphae on the root surface (Figure 1-A). The pathogen penetration point was monitored by scanning electron microscopy, where a specific site for penetration of the fungus was not detected neither on cell wall nor on cell junction points. At the point of contact with the host cell wall, no structural alterations were visible. Pathogen penetration was characterized by a mechanical perforation of the host wall. Cuticle was slightly wrinkled, without evidence for enzymatic alteration (Figure 1-B and 1-C). Hyphae developed through the epidermal cell wall matrix were also observed (Figure 1-D). It was clear that pathogen could penetrate the host at the site of emerging secondary roots (Figure 2-A and 2-B). Cell tanning at penetration site (Figure 2-A and 2-B) as well as infection of epidermal cells along the roots were also observed. The fungus was visible within the host cell wall, inside the epidermal and cortical cells where cytoplasmic content was gradually colonized (Figure 2-C). However, hyphae within the xylem and phloem cells of the central cylinder and between two vessels of xylem have been shown in longitudinal sections (Figure 2-D).

Establishment of root infection of the tolerant line was similar to that of the susceptible one during the first stage of seedling infection (Figure 1-E and 1-F). However, the difference was visible 5 days after inoculation, when pathogen was observed only within the root cortical tissues, compared to the susceptible line whose root center was colonized. There were no significant differences between both lines in root histological study, whereas the observations by the light microscope and transmission electronic microscope allowed describing the pathogen penetration process, as well as the host plant reaction.

Transversal sections in roots of susceptible line, showed an intensive presence of pathogen in the epidermal side of cortical tissues (Figure 3-A), or in the internal side close to the central cylinder (Figure 3-C). The hypha was intensely stained by Toluidine blue with only some empty hyphae (weak coloration) (Figure 3-D), compared to many empty hyphae in different tissues of infected tolerant plants (Figure 3-G).

Transmission electron microscopic observations allowed to precisely describe the pathogen penetration according to its location from the epidermis to the root center (Figure 4). The pathogen has the same features in different tissues of the susceptible genotype roots: hyphae were surrounded by a strongly PATAg-stained cell wall at the surface. A disseminated extracellular matrix was sometimes observed around the pathogen hyphae. Many glycogen granules and huge lipid mass were also shown in the hyphal center. In contrast, differences of hyphal morphological features based on pathogen location in tolerant line root tissues were observed. In the epidermal tissues and in the first layer of cortical parenchyma, most of hyphae were similar to those previously described for the susceptible line. However, a different type of hyphae was observed in the other cortical cells. Some hyphae were surrounded by a thick extracellular matrix strongly correlated with the host cell wall. Furthermore, many glycogen granules and some partially degraded fatty bodies were recorded. The presence of holes on walls of some hyphae was also noticed. The fatty bodies were not colored and appeared with empty vacuoles. The glycogen granules were smaller than those in susceptible line. In the internal cortical side and in the central cylinder, hyphae lacked glycogen granules, and the fatty bodies were much

degraded. Most of the hyphae were empty (40–45% in the internal cortex, and 80–85% in the central cylinder). Empty hyphae were also observed in the root tissues of the susceptible line, but with a lower rate (14-18%) compared to the tolerant line.

Discussion

The ultrastructural investigation of the infection of sunflower with *P. macdonaldii* conidia showed the occurrence of direct penetration through the cuticle after adhesion to plant surface. Issac (16) reported that the fungal adhesion to the host surface is an essential pre-penetration process that determines the success of infection and disease development. Roustaee *et al*. (28, 29) showed that germinating conidia of *P. macdonaldii* and their germ tubes appeared to be attached to the surface of sunflower cotyledon by a mucilaginous sheath covering the hyphae and conidia.

In the present study, direct penetration to cotyledon petiole and root tissues through the cuticle was observed, but without prior formation of appressoria, and these results are in agreement with Roustaee *et al*. (28, 29). Penetration without appressorium formation was also reported for other *Phoma* species, such as *P. lingam* and *P. narcissi* on Hippeastrum leaves (30). It was also reported for *Phomopsis leptostromiformis* on the stem of lupine (*Lupinus angustifolius*) (32), *Fusarium oxysporum* f. sp. *medicaginis* on the roots of *Medicago truncatula* (17), and *Septoria apiicola* (10). In contrast, appressorium formation was produced by other fungi closely related to *Phoma* spp., such as *Phomopsis helianthi* (24), *P. longicolla* (6), *Septoria tritici* (9), *Ascochyta fabae* (23) and *A. rabiei* (14). Different species of *Phoma* can infect plants with or without appressorium formation. The behavior of the fungus could vary at different parts of the plant, such as the leaf petiole or base of the stem, where pathogen penetration might be accompanied by appressorium formation. The formation of appressoria is known to depend on different factors, such as epicuticular waxes, rigidity, and surface hardness (14). The stomata on the surface of the cotyledon petiole of sunflower may facilitate the penetration of fungal pathogen. Penetration via stomata in the cotyledon petiole of sunflower was observed earlier for *P. macdonaldii* (28, 29).

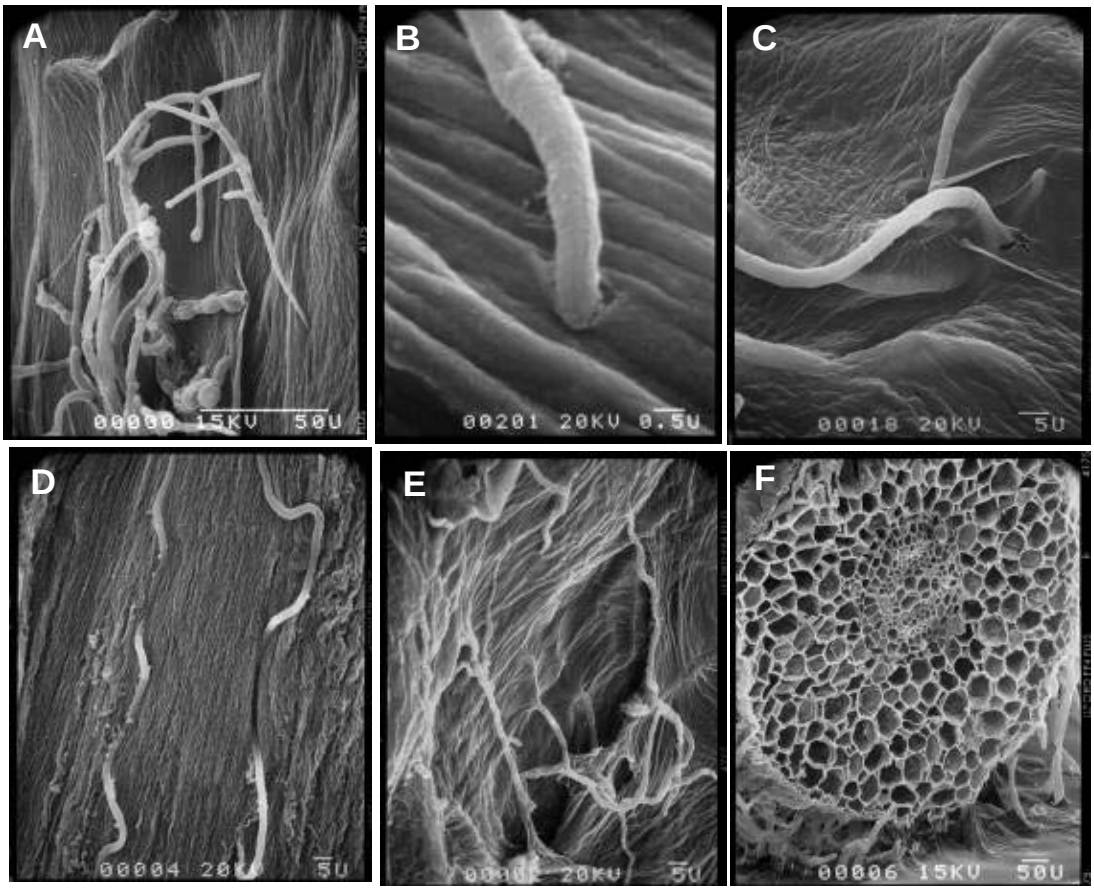

Figure 1. Scanning electron micrographs of *Phoma macdonaldii* on sunflower roots. Development of hyphae at the root surface of C150 (a). Direct penetration by a mechanical perforation of the host wall (C150) (b). Direct penetration (solid arrow), hyphae in the cell matrix of the plant (dotted arrows) (c). Hyphae developed through the cell wall matrix (d). Development of hyphae at the root surface of C137 (e). Transversal section of C137 roots showing the hyphae at the surface (f). (Scale bar: a, b, c, d = 10 µm; e = 5 µm; f = 15 µm).

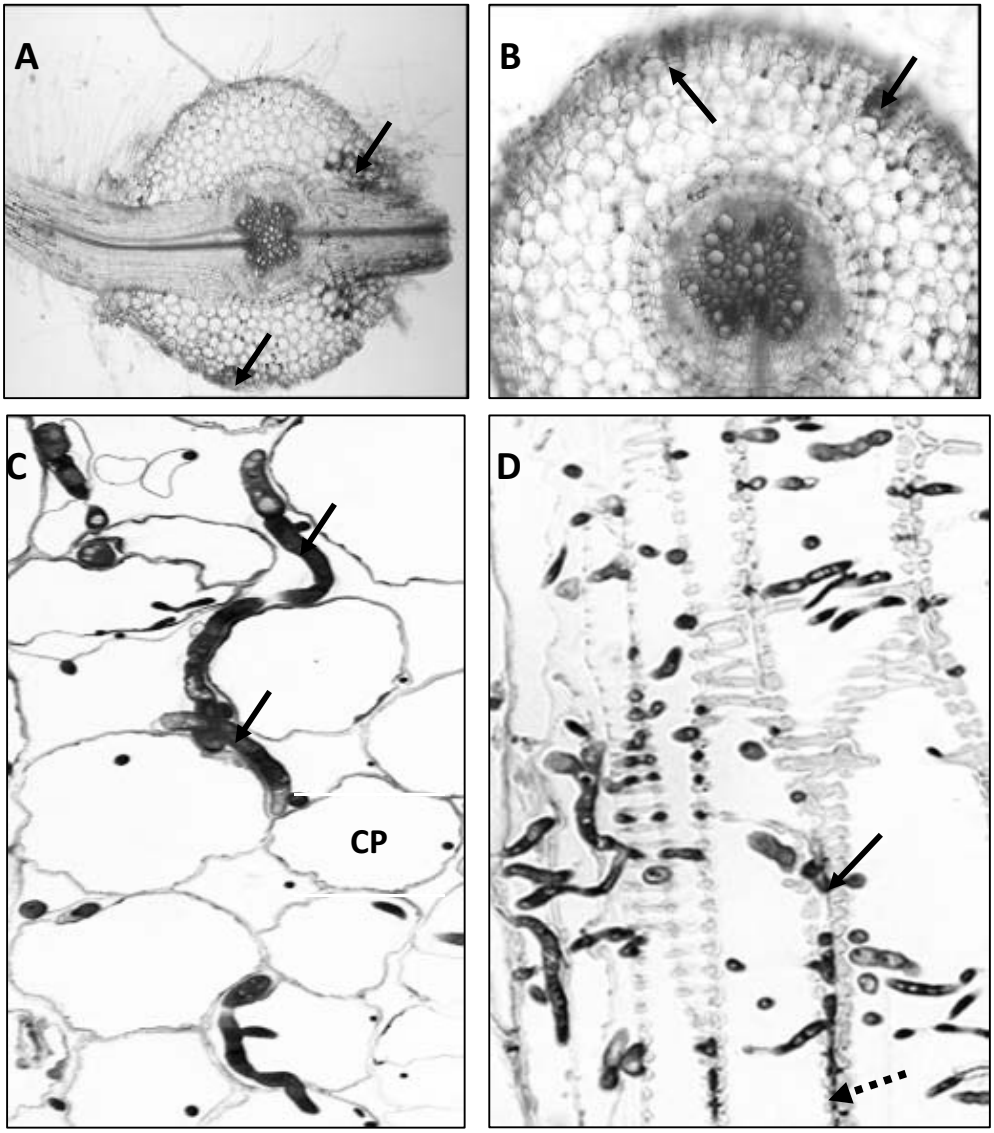

Figure 2. Light micrographs of *Phoma macdonaldii* on sunflower roots. Pathogen penetrates at the site of emerging secondary roots (a). Brown coloration of the penetration zone (arrows) (a and b). Hyphae were present in cell walls and colonized the cytoplasm, in the cortical parenchyma (CP), and in the intercellular space (arrows) (c). Longitudinal section showing the presence of hyphae in the xylem and phloem cells, inter-vessels (dotted arrow), and the passage of pathogen between two vessels of xylem (solid arrow) (d). (Scale bar = a: 300 µm, b, c, d: 150 µm).

Results of the present study showed a mechanical penetration of cell walls by the fungal hyphae without any enzymatic degradation at the penetration point. 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 (16) or through the effects of degrading enzymes, such as cutinase in the case of *Fusarium solani* f. sp. *pisi* (20). 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 (18). Direct penetration has also been reported for other fungi such as *Phomopsis scabra* on sycamore leaves (4), *Fusarium* spp. (19) and *Phoma* sp. (anamorph of *Didymella ligulicola*) the causal agent of ray (flower) blight of Chrysanthemum (27).

The invasion and latent period of fungus within stem and root tissues after penetration differed according to host susceptibility. In this study, pathogen had a relatively short latent period, and invaded different tissues including the vessels in the case of susceptible line (C150). Roustaee *et al*. (28) reported that the short latent period of *P. macdonaldii* (approximately 24-36 h on susceptible variety of sunflower), might be accounted for, in terms of formation of subcuticular hyphae. Our observations suggest a partial and local systemic invasion of the disease when

symptoms on collar and roots were observed. The invasion of plant vascular system and systemic invasion of the disease in the plant have been reported for other fungi such *Leptosphaeria maculans* (12), and *Diaporthe helianthi* (13, 24).

Results also showed that the pathogen was observed only in the cortex of the tolerant line (C137) roots, but unable to proceed further, and was not found in the center of the roots. Whereas, the pathogen colonized the whole root including the vessels of the susceptible line (C150). Furthermore, the colonization which found only in the cortex of tolerant plants, with the presence of many empty hyphae, is in good agreement with the partial tolerant interaction, where the pathogen hardly progressed in the plant tissues, and the hyphae died. Genetic variability of partial resistance to Phoma black stem in sunflower has been reported in both field (26) and under controlled conditions (1, 7, 28, 29). Similar observations have been reported for other fungi such as *Fusarium oxysporum* f. sp. *medicaginis* on *Medicago truncatula* (17).

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 still needed to elucidate the *Phoma*–sunflower interactions, using both tolerant and susceptible genotypes.

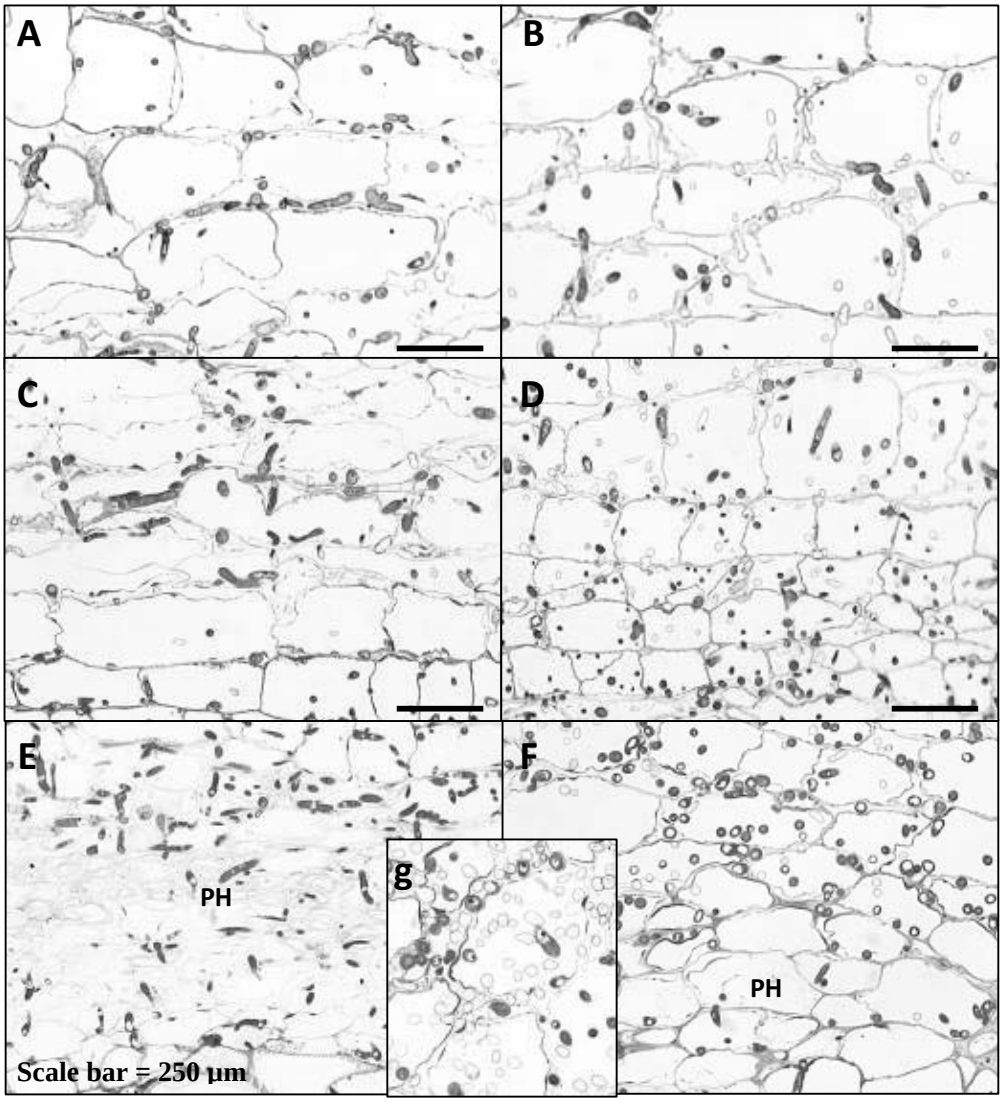

Figure 3. Semi-thin longitudinal sections of sunflower seedlings of susceptible genotype C150 (a, c and e) and of tolerant genotype C137 (b, d, f and g), stained with Toluidine blue and observed 7 days after inoculation by light microscope. (a) and (b): external side of cortical parenchyma. (c) and (d): internal side of cortical parenchyma. (e) and (f): internal side of cortical parenchyma and a part of the central cylinder. (g): phloem cells of C137 infected seedlings: it shows the presence of many empty hyphae in the plant tissues.

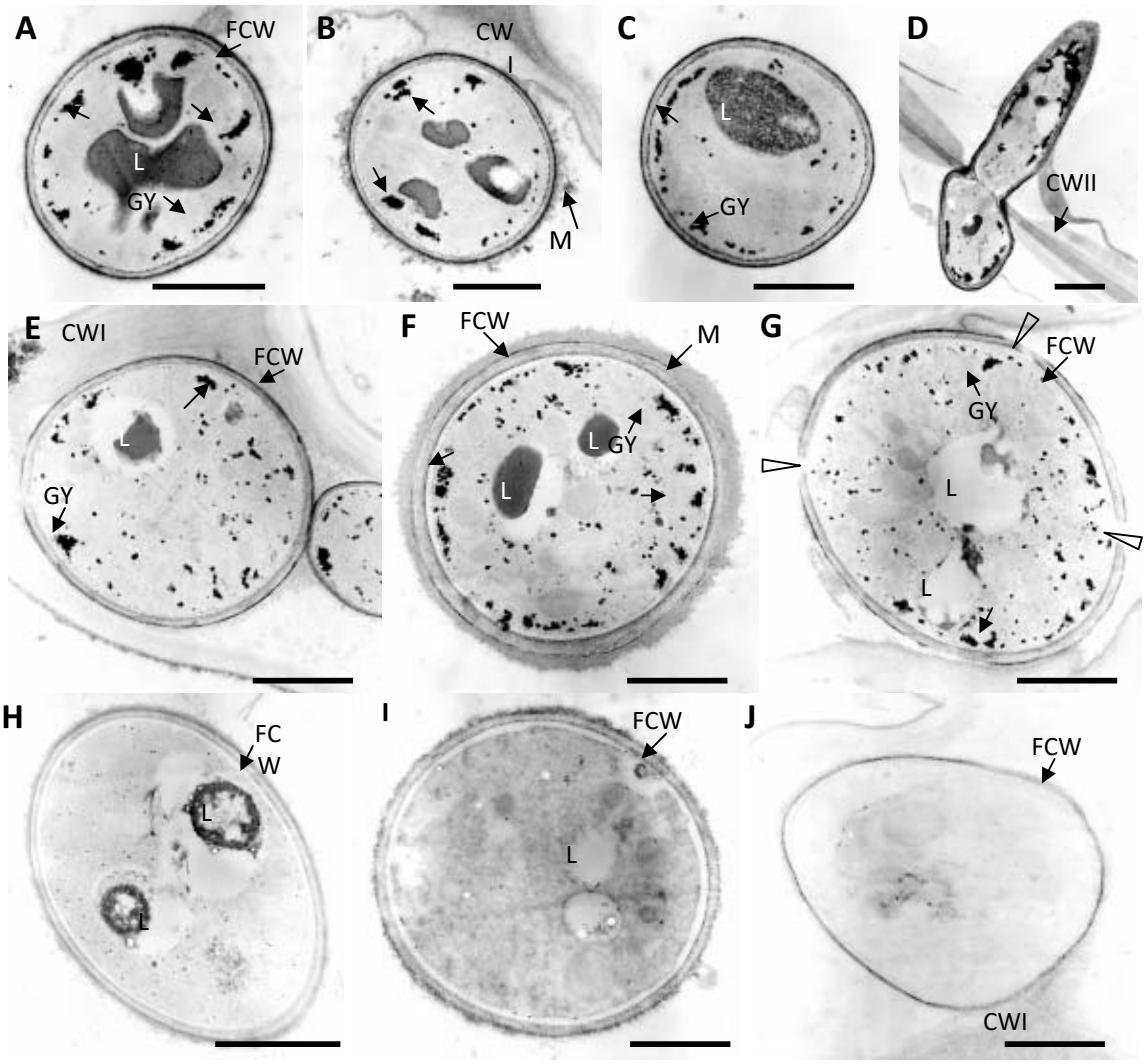

Figure 4. Transmission electron micrographs of *Phoma macdonaldii* on sunflower roots of C150 (a to d) and C137 (e to j). Differences of morphological aspect of hyphae in the epidermis (a and e), the external side of the cortical parenchyma (b, f and g), the internal side of the cortical parenchyma (c, h and i), the phloem (j) and the xylem (d). FCW: fungal cell wall, CWI: primary wall of plant, CWII: secondary wall of plant.

الملخص

أبو الفضل، تيسير، وليد نفاع، ايفز مارتيناز وغريغوري ديشام – غيوم. 2011. طريقة اختراق الفطر Boerema *Phoma macdonaldii* في الطرز الوراثي الحساسة والمتحملة من دوار/عباد الشمس. مجلة وقاية النبات العربية، 29: 131–138.

أجريت دراسة تشريحية بعد العدوى الاصطناعية بأبواغ الفطر Boerema *Phoma macdonaldii* على سلالتين من دوار/عباد الشمس (*Helianthus annuus* L.) إحداهما حساسة والأخرى متحملة للإصابة باستخدام المجهر الماسح، والمجهر الإلكتروني، والمجهر الضوئي، وذلك لدراسة العلاقة ما بين العائل والفطر الممرض. بيّنت النتائج أن الاختراق يحدث بصورة مباشرة عن طريق كيوتيكل السويقة الفلقية والجذور بعد التصاق الممرض على سطح النبات، وبدون تشكيل أعضاء التصاق. كما تبيّن أن اختراق هيفات الفطر للجدر الخلوية يتم بآلية ميكانيكية، ولم يلاحظ وجود أي هدم أنزيمي في نقطة الاختراق. وقد تباينت قدرة الفطر على الانتشار داخل أنسجة الساق والجذر بعد الاختراق تبعاً لقابلية النبات العائل للإصابة. فقد أخذ الفطر المظهر ذاته في كل أنسجة جذور الطراز الوراثي القابل للإصابة، بينما لوحظت اختلافات مورفولوجية في هيفات الفطر وذلك تبعاً لمكان توضعه في أنسجة جذور الطراز الوراثي المتحمل. واقتصر انتشار الفطر على لحاء الجذور فقط في السلالة المتحملة، إضافة إلى العديد من الخيوط الفطرية الفارغة. بينما كان الفطر قادراً على غزو كل أنسجة الجذور بما فيها الأوعية الناقلة في السلالة القابلة للإصابة مع وجود بعض الخيوط الفارغة.

كلمات مفتاحية: الساق الأسود، اختراق مباشر، *Helianthus annuus*، التفاعل بين العائل والممرض، *Phoma macdonaldii*.

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