Eur J Plant Pathol (2009) 125:73–85
DOI 10.1007/s10658-009-9460-0

Colonisation and histological changes in muskmelon and autumn squash tissues infected by *Acremonium cucurbitacearum* or *Monosporascus cannonballus*

Ana Alfaro-Fernández · Amparo García-Luis

Received: 11 May 2008 / Accepted: 5 March 2009 / Published online: 20 March 2009

Abstract Muskmelon (*Cucumis melo* cv. Temprano Rochet) and autumn squash (*Cucurbita maxima*) seedlings were inoculated either with *Acremonium cucurbitacearum* or *Monosporascus cannonballus*, two of the soil-borne fungi implicated in 'melon collapse'. Inoculation was achieved in two different ways: by growing the plants in pots containing infested soil to study the histological changes produced in the infected tissues using light microscopy and by growing seedlings in Petri dishes together with fungal colonies in order to observe the colonisation of the plant tissues using scanning electron microscopy. Both muskmelon and autumn squash roots infected with *A. cucurbitacearum* showed a suberised layer in the epidermis and the outermost layers of the parenchymatic cortex, but these symptoms developed earlier in the muskmelon plants. Muskmelon plants infected by this fungus also presented hypertrophy and hyperplasia, which led to a progressive separation of the vascular bundles in the lower stems of the affected plants. This response was not observed in autumn squash during the study. On the other hand, few histological changes were observed in tissues infected with *M. cannonballus* and only a slight increase in the size of cortical intercellular spaces was noted in the lower stems of muskmelon plants, and infected autumn squash tissues remained free of these symptoms throughout the study. The scanning electron microscope observations revealed that both fungi were able to colonise the tissues of the two host plants which were studied. *A. cucurbitacearum* colonised the epidermis and cortex of both muskmelon and autumn squash. The hyphae grew both inter- and intracellularly, and the density of the colonisation decreased within the endodermis. The same colonisation of host plants was observed as a result of *M. cannonballus* infection. The xylem vessel lumina of both muskmelon and autumn squash showed hyphae and tylose formation as a result of both fungal infections. However, non-fungal structures were detected in the hypocotyl vascular tissues. The present study demonstrates that both fungi are capable of infecting the tissues of a species which is resistant (autumn squash) and a species which is susceptible (muskmelon) to melon collapse.

Keywords *Cucumis melo* · *Cucurbita maxima* · Infection process · Light microscopy · Scanning electron microscopy · Vine decline

A. Alfaro-Fernández (✉)
Instituto Agroforestal Mediterráneo (IAM),
Universidad Politécnica de Valencia (UPV),
Camino de Vera s/n. 46022 Valencia, Spain
e-mail: analfer1@doctor.upv.es

A. García-Luis
Departamento de Biología Vegetal,
Universidad Politécnica de Valencia (UPV),
Camino de Vera s/n, 46022 Valencia, Spain

Introduction

'Melon collapse', also referred to as 'sudden death', 'sudden wilt' or 'root rot and vine decline', is a syndrome which has become prominent in all the muskmelon production areas in Spain. The symptoms of this syndrome are similar to those described for other vine decline diseases (García-Jiménez et al. 2000). These symptoms include the yellowing and death of the crown leaves and a gradual decay of the vine, as the fruits approach maturity. The roots of infected plants develop brown, corky and necrotic areas. The fruits of affected plants are small in size, have a low sugar content and are more prone to sunburn (Mertely et al. 1991; Martyn and Miller 1996).

Vine decline is a generic term applied to a group of diseases with similar symptoms but different causal agents and are rarely caused by a single pathogenic organism. A disease complex is usually involved, although one pathogen may predominate (Bruton et al. 1998). The principal fungal pathogens associated with this syndrome in Spain are *Acremonium cucurbitacearum*, and *Monosporascus cannonballus* (García-Jiménez et al. 2000; Beltrán et al. 2007). *A. cucurbitacearum* is a mitosporic fungus, the vegetative hyphae of which are 1–3 μm wide and mostly thin-walled. This fungus survives as conidia or chlamydospores in the soil. Conidia are elongated, ellipsoid and hyaline, and usually aggregate to form slimy heads (García-Jiménez et al. 1994; Alfaro-García et al. 1996). *M. cannonballus* is a homothallic pyrenomycete adapted to hot semi-arid melon and watermelon-producing areas which produces fertile perithecia in host roots. It forms one large ascospore per ascus, which is dark brown when mature and 25–55 μm in diameter. The ascospores are released into the soil from the perithecia when the infected roots start to decompose (Pollack and Uecker 1974; Martyn and Miller 1996; Cohen et al. 2000) and are able to survive in the soil in the absence of muskmelon production, even under flooding conditions (Beltrán et al. 2005).

A. cucurbitacearum and *M. cannonballus* have a wide host range within the *Cucurbitaceae*, but their hosts display different degrees of susceptibility (Mertely et al. 1993; Armengol et al. 1998). Muskmelon (*Cucumis melo*) and watermelon have been classed as susceptible or highly susceptible to *A. cucurbitacearum*. *Cucurbita maxima* (autumn squash) was included in a group which is highly resistant to this fungus (Armengol et al. 1998). All the cucurbits tested were susceptible to *M. cannonballus*. The pathogen was readily isolated from both infected watermelon and muskmelon plants, whereas it was difficult to recover from *Cucurbita* species. Therefore, while the growth of watermelon and muskmelon plants was severely affected, *Cucurbita* species were less stunted and there was a smaller reduction in their maximum weight (Mertely et al. 1993).

The grafting of muskmelons and watermelons onto *Cucurbita* sp. rootstocks is a technique commonly used to control these diseases in the Mediterranean region. The success that grafting melons onto *Cucurbita* rootstocks has in reducing the incidence of *M. cannonballus* 'vine decline' and increasing yield has been reported a number of times (Cohen et al. 2000; 2005). In Spain muskmelon has been routinely grafted onto *C. maxima* x *C. moschata* to control Fusarium wilt (Miguel et al. 2004). This practice has also been used in Spain to control Acremonium collapse in watermelon crops (Armengol et al. 1998).

Histopathological studies of the penetration and colonisation of melon roots by *M. cannonballus* have been conducted by Waugh et al. (2001, 2005). These studies demonstrated that ascospore germination occurred in the rhizosphere of melon plants and one to three germ tubes emerged from the ascospores. Subsequently, these adhered to the surface of the root without forming appressoria (Waugh et al. 2001). Once the root had been penetrated, the hyphae of the fungus grew mainly intracellularly until the hyphae reached the vascular cylinder and colonised both the protoxylem and the metaxylem vessels (Waugh et al. 2005). However, no information is available about the penetration and colonisation of muskmelon roots by *A. cucurbitacearum*.

The aim of this study was to compare the response of *C. melo* and *C. maxima* to infection by *A. cucurbitacearum* or *M. cannonballus*. The main objectives were: (i) to compare the colonisation, by the hyphae of these fungi, of the infected tissues of muskmelon and autumn squash plants and (ii) to determine the early histological changes produced by the infection.

Materials and methods

Plant material

Muskmelon (*C. melo* cv. Temprano Rochet) and autumn squash (*C. maxima*) seeds were surface-sterilised in a 1.5% sodium hypochlorite solution for 2 min and washed twice by soaking them in sterile distilled water. The seeds were placed in Petri dishes containing moistened sterile filter paper and incubated in the dark at a temperature of 25°C and 86% relative humidity for 7–10 days until they germinated.

Fungal cultures

Isolates of *A. cucurbitacearum* and *M. cannonballus* were obtained from the culture collection of Grupo de Investigación de Hongos Fitopatógenos at the Instituto Agroforestal Mediterráneo (IAM) of the Universidad Politécnica de Valencia, Spain, where hyphae are maintained in sterile peat at room temperature. In the present study the fungal isolates were cultivated for 15 days in a general fungal growth medium (potato dextrose agar, PDA) in Petri dishes at 26°C with a 12 h photoperiod provided by Sylvania S40 BLB lamps.

Histological changes in affected tissues

Production of inocula Fungal mycelium grown in PDA, as described above, was placed in an autoclaved sand-oat hull medium (0.5 l of sand, 46 g of ground oat (*Avena sativa*) hulls and 37.5 ml of distilled water) and incubated in the dark at 25–30°C for 21–28 days. Colony-forming units (CFUs) were quantified using the method of serial dilution in 1% hydroxyethyl cellulose, as described by Dhingra and Sinclair (1985). Briefly, 1 g of the sand-oat hull medium colonised by the fungus was added to a 1% hydroxyethyl cellulose dilution. Ten-fold serial dilutions were prepared using 1 ml of the previous solution until dilutions of 10^{-5} or 10^{-6} were obtained. One ml of each dilution was poured into a Petri dish containing PDA amended with streptomycin sulphate (500 ppm). Two replications were performed for each dilution. After 4–6 days at 25–27°C with a 12 h photoperiod, the number of colonies grown in the Petri dishes for each dilution was counted and the total amount of CFU g^{-1} of inocula was calculated.

Root inoculation Thirty plastic pots (16 cm diam) were filled with a sterilised mixture of sand and peat (1:1). Ten of the pots were inoculated with *A. cucurbitacearum* and another ten with *M. cannonballus* at a concentration of 50,000 CFU g^{-1} (as conidia) and 20 CFU g^{-1} (as mycelium), respectively. These densities are those recommended for inocula used in pathogenicity tests on these two fungal species by Bruton et al. (2000). Five germinating seeds of either muskmelon or autumn squash were planted in each pot. Ten non-inoculated pots were kept as controls.

The plants were grown in a greenhouse at 25°C and in conditions of high relative humidity. For each fungus/plant combination plants were removed from two pots and samples were taken for observation 10, 15, 20, 30 and 42 days after sowing. The roots and hypocotyls were carefully washed, superficially disinfected with a 1% sodium hypochlorite solution and washed twice in distilled water to remove any excess sodium hypochlorite solution. Some fragments of the root and hypocotyl of the plants were used for reisolation of the fungi (see below). The remaining part of the root and hypocotyl was cut into 1 cm-long fragments and fixed in FPA (formalin: propionic acid: ethanol; 1:1:18).

Fungal reisolation From every plant 14 × 1 cm-long fragments (reisolation points) from the lower stem, the root-hypocotyl transition zone and the hairy zone of the tap root were sampled at random, and placed on two Petri plates (7 fragments each) containing PDA amended with streptomycin sulphate (500 ppm). The reisolation plates were incubated at 25–27°C for 3–4 days with 12 h photoperiod exposure provided by Sylvania S40 BLB lamps. The fungal colonies which developed were transferred to PDA plates and incubated at room temperature with a 12 h photoperiod (Sylvania S40 BLB). The isolates were maintained until they sporulated and were then identified. An inoculation was considered successful when the fungus was recovered from at least two plant segments from each plate.

Light microscope observations For each fungus/plant combination 1 cm-long fragments from the root-hypocotyl transition zone and the lower stem were sampled from two plants selected at random on the dates indicated above (10, 15, 20, 30 and 42 days after sowing). The segments, which were fixed in

FPA, were dehydrated in a graded series of t-butanol and embedded in paraffin wax (Johansen 1940). Sections (35–40 μm thick) were stained with light green-saffranin and examined using a Nikon EFD-3 microscope. Photographs were taken with a Nikon Coolpix 990 digital camera.

Colonisation of plant tissues by the fungi

Due to the low concentration of hyphae within the infected roots of the plants grown in the pots, it was not possible to determine hyphal penetration accurately. Therefore, a method which ensured a higher hyphal density in the colonised tissue was designed in order to study the distribution of the fungi, as described below.

Culture of seedlings in Petri dishes A 5–7 mm thick layer of PDA medium was poured into 9 cm Petri dishes. The surface of the agar was carefully covered with a 0.3 mm thick sheet of polyethylene plastic film. One third of the film cover was removed together with the portion of the culture medium underneath it. The culture medium was then inoculated at two points with one of the fungi which were being studied by raising up the film. The sources of the inocula were the fungal isolates grown in PDA Petri dishes described above. The plates were incubated at room temperature under Sylvania S40 BLB lamps, employing a 12 h photoperiod, for 10–15 days.

Two germinated muskmelon or autumn squash seeds were planted in each of the Petri dishes close to the points where fungal colonies had developed. The seeds were placed in the section of the dish in which the agar had been removed, while the emerging root was inserted into the culture medium (Fig. 1). A small amount of liquid paraffin was placed around the germinating seeds in order to force the roots to grow inside the medium, thus preventing the film from being pushed up. Uninoculated control plants were grown in a similar way. The dishes were incubated in a vertical position in a growth chamber at 25°C, and with 86% relative humidity and a 12 h photoperiod, until the roots came into contact with the fungal mycelium (5–8 days). Then the seedlings were removed from the plate and fixed in FPA, as described above. For each fungus/plant combination up to ten seedlings grown in infected agar were obtained.

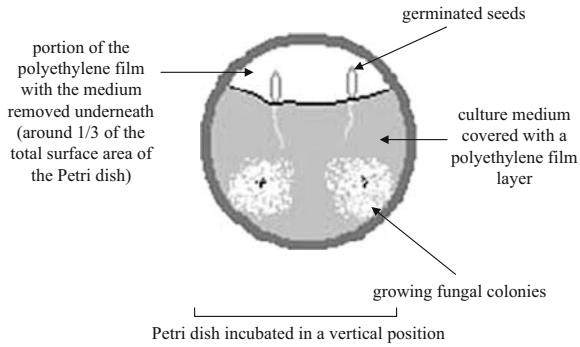


Fig. 1 Diagram illustrating the culture of seedlings in infected Petri dishes

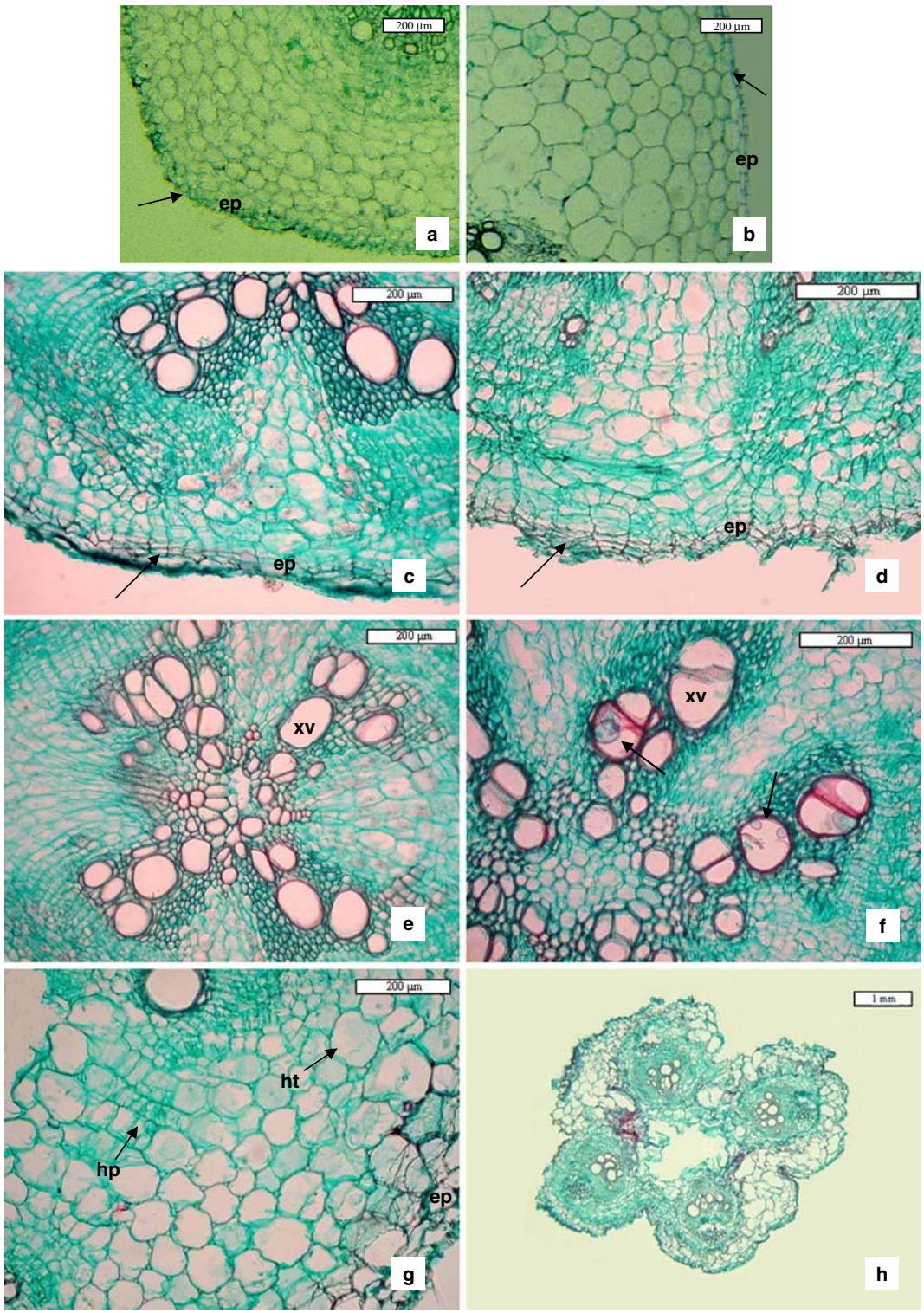
Scanning electron microscope examination One cm-long sections of roots and hypocotyls were fixed in FPA and dehydrated in ethanol series. Sections were cryofixed with CT 1500C Cryotransfer (Oxford Instruments) and examined with a JEOL-JSM- 5410 Scanning Electron Microscope at 5 kV. For each fungus, sections were obtained from nine (muskmelon) or six (autumn squash) seedlings chosen at random.

Results

Fungal reisolation

In plants grown in pots in soil infested with *A. cucurbitacearum* the frequency of reisolation ranged from 20–70%, i.e., the fungus was recovered in 3–10

Fig. 2 Light microscopy of transverse root-hypocotyl and stem sections of muskmelon plants growing either in non-infected soil (a,b) or in soil inoculated with *A. cucurbitacearum* (c,d,e,f, g,h). (a) Root-hypocotyl section of a 42 day-old non-infected plant showing the typical green staining of the cellulose in the epidermis (arrow); (b) Stem of a 42 day-old non-infected plant; (c,d) Sections of the root-hypocotyl transition zone from 30 day-old (c) and 42 day-old (d) plants infected with *A. cucurbitacearum* showing a saffranin red-staining layer due to suberin deposition (arrows). The outer cortical layer appears distorted and exhibits flattened cells; (e) The intact vascular cylinder of the root-hypocotyl section of a 30 day-old infected plant; (f) The vascular cylinder of 42 day-old plants showing tyloses in the lumen of some of the xylem vessels (arrows); (g) Stem section of a 30 day-old plant showing a suberised layer in the epidermal and parenchymatic cells, and hypertrophy and hyperplasia (arrows); (h) Stem section of a 42 day-old infected plant showing collapsed and distorted parenchymatic cells and separation of the vascular bundles. ep: epidermis, xv: xylem vessels, ht: hypertrophy, hp: hyperplasia ▶


200 µm
ep
a
200 µm
ep
b
200 µm
ep
c
200 µm
ep
d
200 µm
xv
e
200 µm
xv
f
200 µm
ht
hp
ep
g
1 mm
h

out of 14 reisolation points per plant. *Monosporascus cannonballus* was recovered from the tissues with a frequency ranging from 50–75% (7 to 14 out of 14 reisolation points per plant). In all the plants the fungi were reisolated since the first time samples were taken (day 10). Therefore, the inoculation was considered successful.

Plants infected with *A. cucurbitacearum*

Histological changes in affected tissues of plants grown in infested soil The first visible effect of *A. cucurbitacearum* infection in the muskmelon root-hypocotyl zone was an atypical saffranin red staining of the epidermis and the outermost parenchymatic cells of the cortex due to suberin deposition (Fig. 2c, d), which contrasts with the normal green staining associated with cellulose (Fig. 2a, b, arrows) . This suberised layer was distinguishable 30 days after soil inoculation with the fungus (Fig. 2c, arrow). In 42 day-old root hypocotyls the outer cortical cells also had a slightly distorted, misshapen and flattened appearance (Fig. 2d, arrow). The xylem vessels remained intact in 30 day-old infected plants (Fig. 2e), but tyloses were detected within the xylem vessels in 42 day-old plants (Fig. 2f, arrows).

In the stems of 30 day-old muskmelon plants, a red-staining suberised layer was also present in the epidermis and the outermost parenchymatic cortex cells (Fig. 2 g). The parenchymatic tissues showed both hypertrophy and hyperplasia (Fig. 2 g, arrows), when compared to non-infected tissues (Fig. 2b). In 42 day-old plants both the pith and cortical parenchymatic cells appeared collapsed and distorted, which resulted in the separation of the vascular bundles (Fig. 2 h).

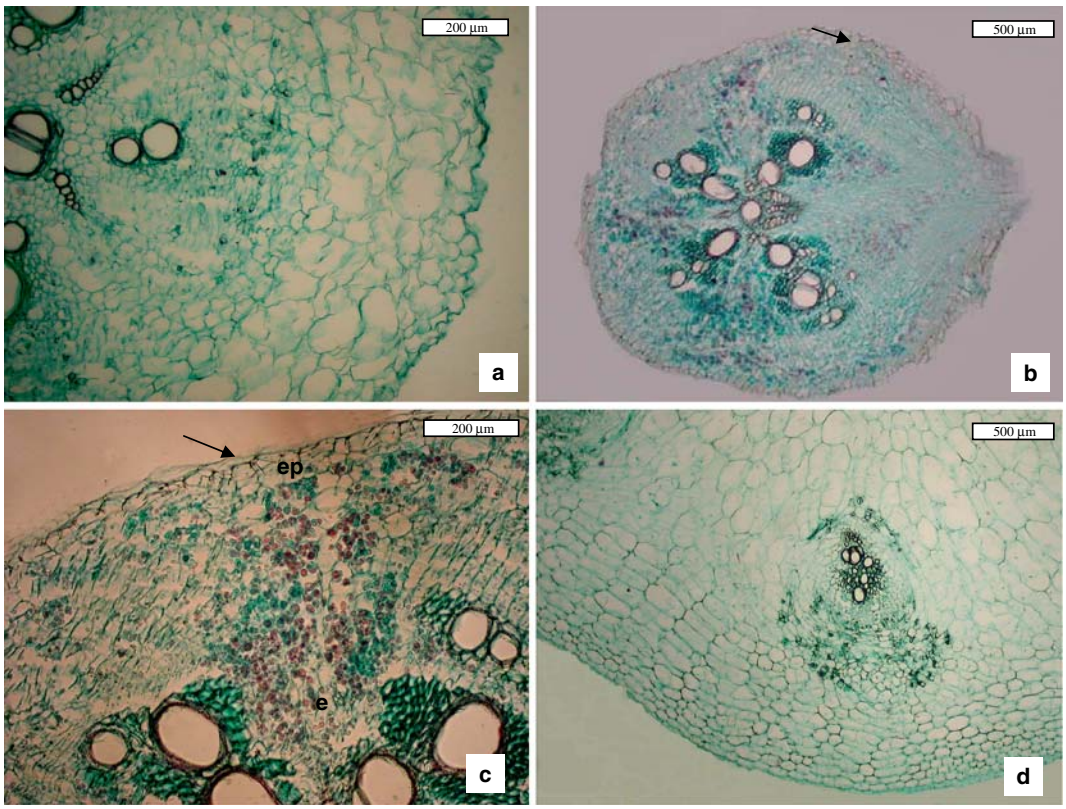


Fig. 3 Light microscopy images of transverse root-hypocotyl and stem sections of autumn squash plants grown in soil inoculated with *A. cucurbitacearum*. (a) Root-hypocotyl sections of a 30 day-old-plant showing the typical appearance of non-infected tissues, with green staining of the cellulose in the epidermal cells; (b) Root-hypocotyl sections of a 42 day-old-plant showing the formation of a superficial red-stained suberised layer in the epidermis (arrow); (c) Magnified view of the suberised epidermis (arrow) of the 42 day-old root-hypocotyl section; (d) Stem section of a 42 day-old plant grown in infected soil with a healthy appearance. ep: epidermis

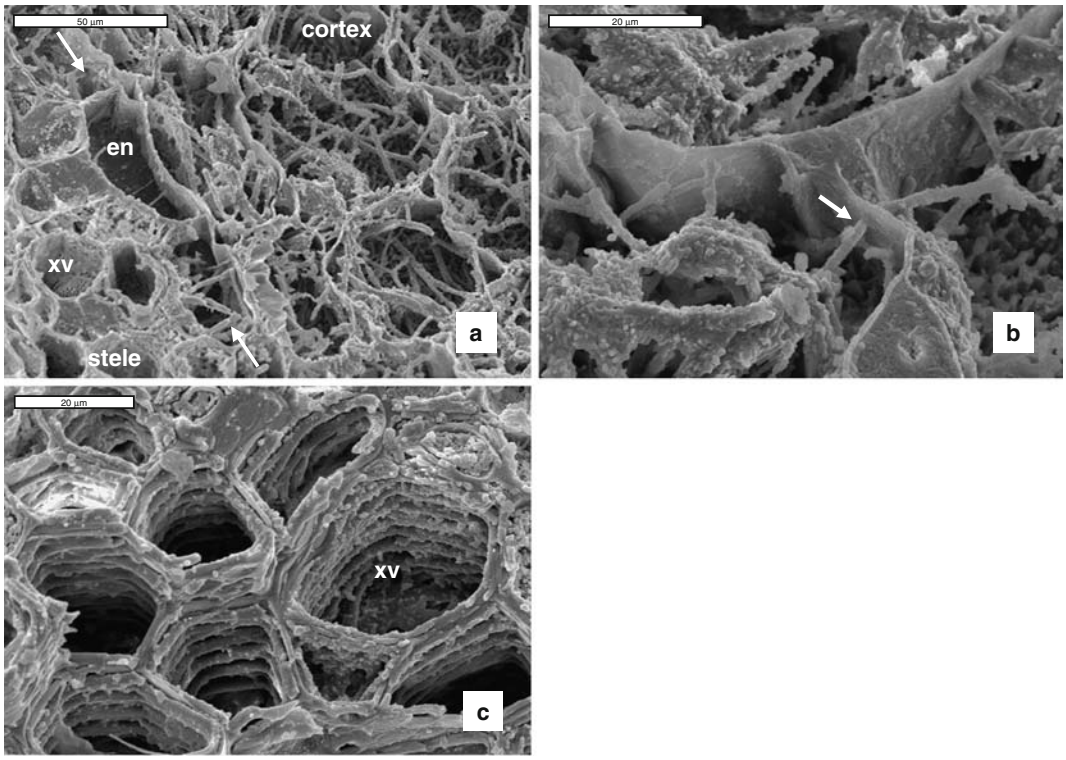


Fig. 4 Scanning electron micrographs of root sections of muskmelon after 8 days of growth in a culture medium infected with *A. cucurbitacearum*. (a) Dense and ramified hyphal network colonisation within the cortical cells. The cells of the endodermis (arrows) showed a lower hyphal density (arrows); (b) Hyphae passing from one cell to another by direct penetration through the cell wall (arrow); (c) A magnified image of the root xylem vessels showing no hyphal colonisation. en: endodermis, xv: xylem vessels

The root-hypocotyl tissues of infested autumn squash seedlings grown in infested soil showed a healthy appearance after 30 days (Fig. 3a). A slightly suberised layer was observed in the epidermis by day 42 (Fig. 3b, c, arrows), some 10 days later than in muskmelon plants. The stems of the infected autumn squash plants did not show any sign of damage throughout the period of study (Fig. 3d).

Colonisation of plant tissues by the fungi in plants grown in Petri dishes After 8 days of culture, the muskmelon roots grown in culture medium infected with *A. cucurbitacearum* showed a dense colonisation of the epidermis and the cortex. The hyphae formed a continuous and ramified network, which extended throughout these two tissues (Fig. 4a, arrows) and was able to penetrate the cell wall (Fig. 4b, arrow). The endodermis appeared to act as a physical barrier to the colonisation of the vascular stele and hyphal density was strikingly reduced inside the cells in the endodermis (Fig. 4a, arrows). At this stage, there was no invasion of the xylem vessels of the root (Fig. 4c). Even though the root tissues were heavily colonised, the hypocotyl samples were intact.

The cortex and the epidermis of autumn squash roots grown in infected culture medium presented a high-density network of hyphae (Fig. 5a). This dense network consisted of hyphae of varying thickness (Fig. 5a). The hyphae grew both intercellularly and intracellularly (Fig. 5a, arrows) and were able to negotiate the cell wall (Fig. 5b, arrow), as they did in muskmelon roots. In contrast, the inner root tissues were seldom colonised by the pathogen's hyphae. Nevertheless, a few hyphae were occasionally visible in the lumen of the xylem vessels of autumn squash roots (Fig. 5c, d arrows). As described above for muskmelon, the endodermis showed less dense hyphal network

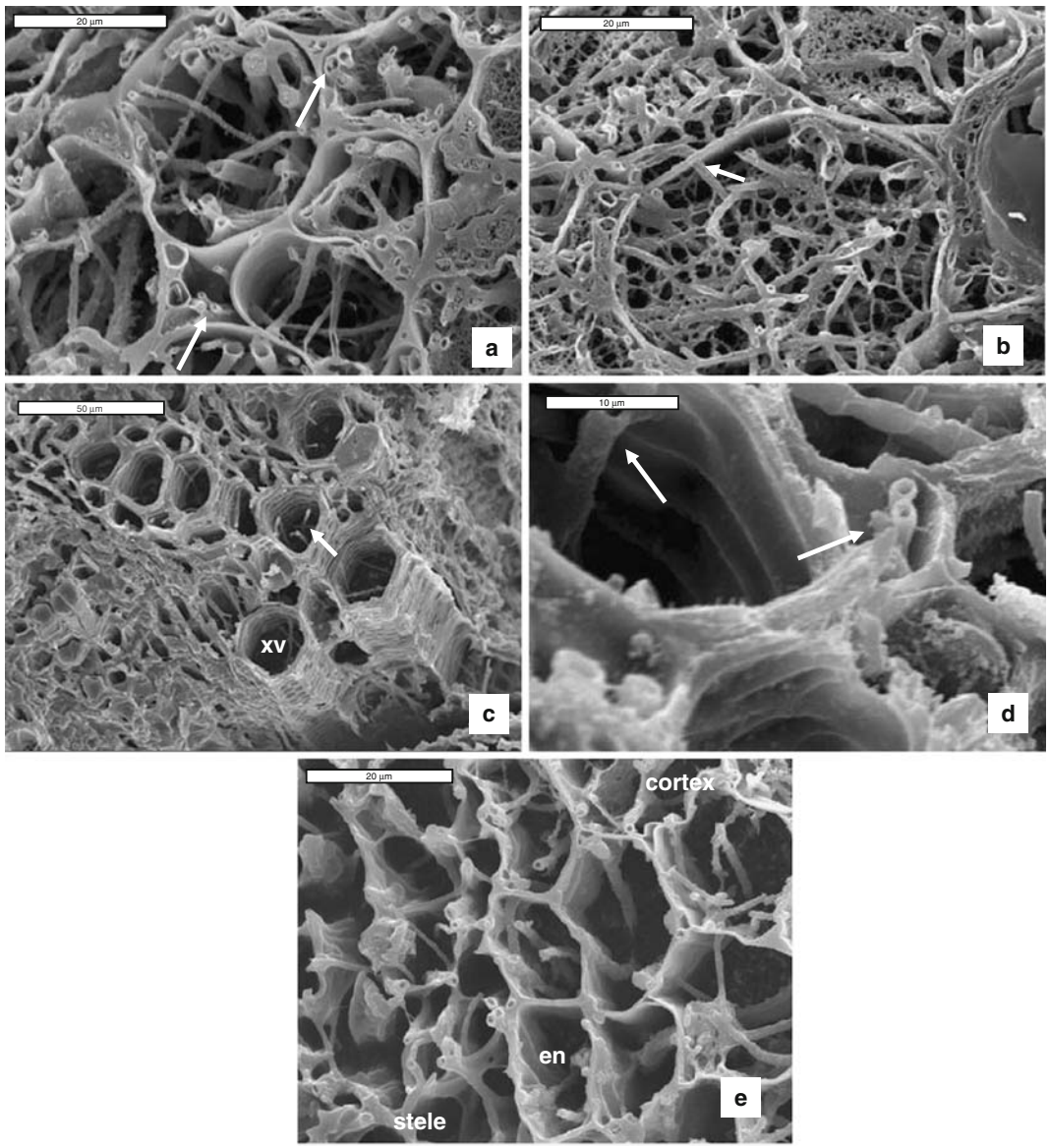


Fig. 5 Scanning electron micrographs of root sections of autumn squash after 8 days of growth in a culture medium infected with *A. cucurbitacearum*. (**a**) Dense and ramified hyphal network colonisation of the cortex by both intercellular (arrow) and intracellular (arrow) hyphae; (**b**) Hyphae passing from one cell to another by direct penetration through the cell wall (arrow); (**c**) Xylem vessels with a few hyphae growing inside (arrow); (**d**) Magnified view of some xylem vessels confirming the presence of hyphae (arrows*;* (**e**) Endodermis (arrows) showing lower hyphal density than the cells of the cortex. en: endodermis, xv: xylem vessels

colonisation than the parenchymatic cortex cells (Fig. 5e, arrows).

In the root system of the two host species which were studied, colonisation by *A. cucurbitacearum* hyphae occurred centripetally towards the stele with little acropetal spread, as demonstrated by the observation under the electron microscope of serial longitudinal and transverse sections of infected roots. No hyphae were present in the hypocotyls after 8 days of incubation. The limited acropetal spread of the infection observed was restricted to the root.

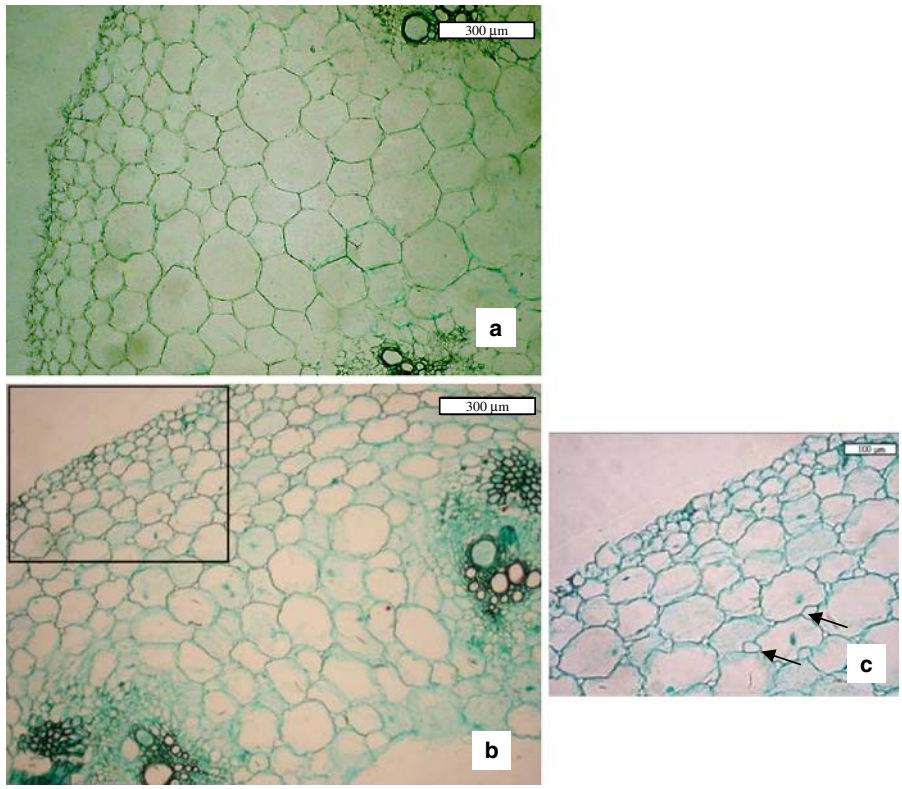


Fig. 6 Light microscopy images of transverse stem sections of 30 day-old muskmelon plants grown in either non-infected (a) or *M. cannonballus* infected soil (b,c). (**a**) Partial view of non-infected stem section. The cells in the cortex are tightly flanked with small intercellular spaces; (**b**) Stem sections from an infected muskmelon plant with no morphological changes as compared to non-infected plants, except for the presence of what appeared to be intercellular spaces; (**c**) Magnified view of (**b**) showing some details of the aerenchyma-like tissue in the cortex (arrows)

Plants infected with *M. cannonballus*

Histological changes in plants grown in infested soil Neither muskmelon nor autumn squash roots grown in soil infested with *M. cannonballus* displayed any histological changes caused by the infection during the period studied. The epidermis, cortex and vascular tissues were alike and remained symptom-free, in the case of both non-infected and infected plants.

No hyphae were observed in stem sections from infected muskmelon plants (Fig. 6b), and the epidermis and cortical tissues were similar to those of the control plants (Fig. 6a), but for a significant increase in the size of the intercellular spaces (Fig. 6c, arrows)

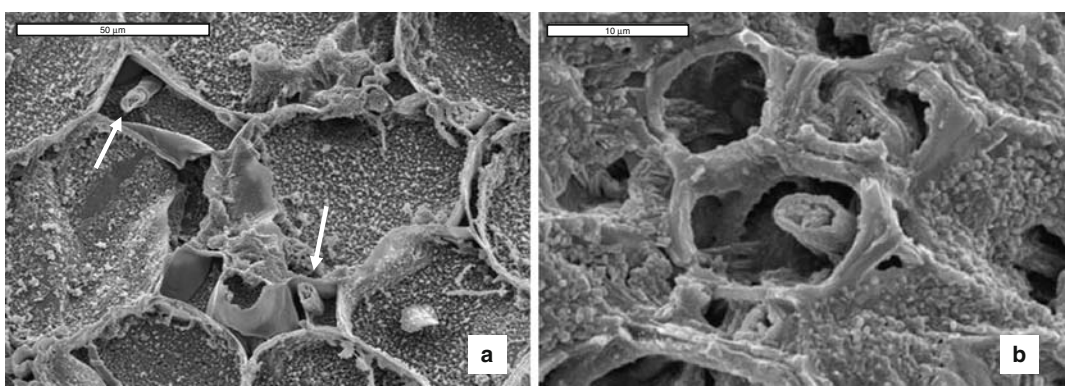


Fig. 7 Scanning electron micrographs of tissue sections of muskmelon after 8 days of growth in a culture medium infected with *M. cannonballus*. (**a**) Transverse lower hypocotyl section showing hyphal colonisation of the parenchymatic cells (arrows); (**b**) Root section showing hyphae inside the lumen of the xylem vessels

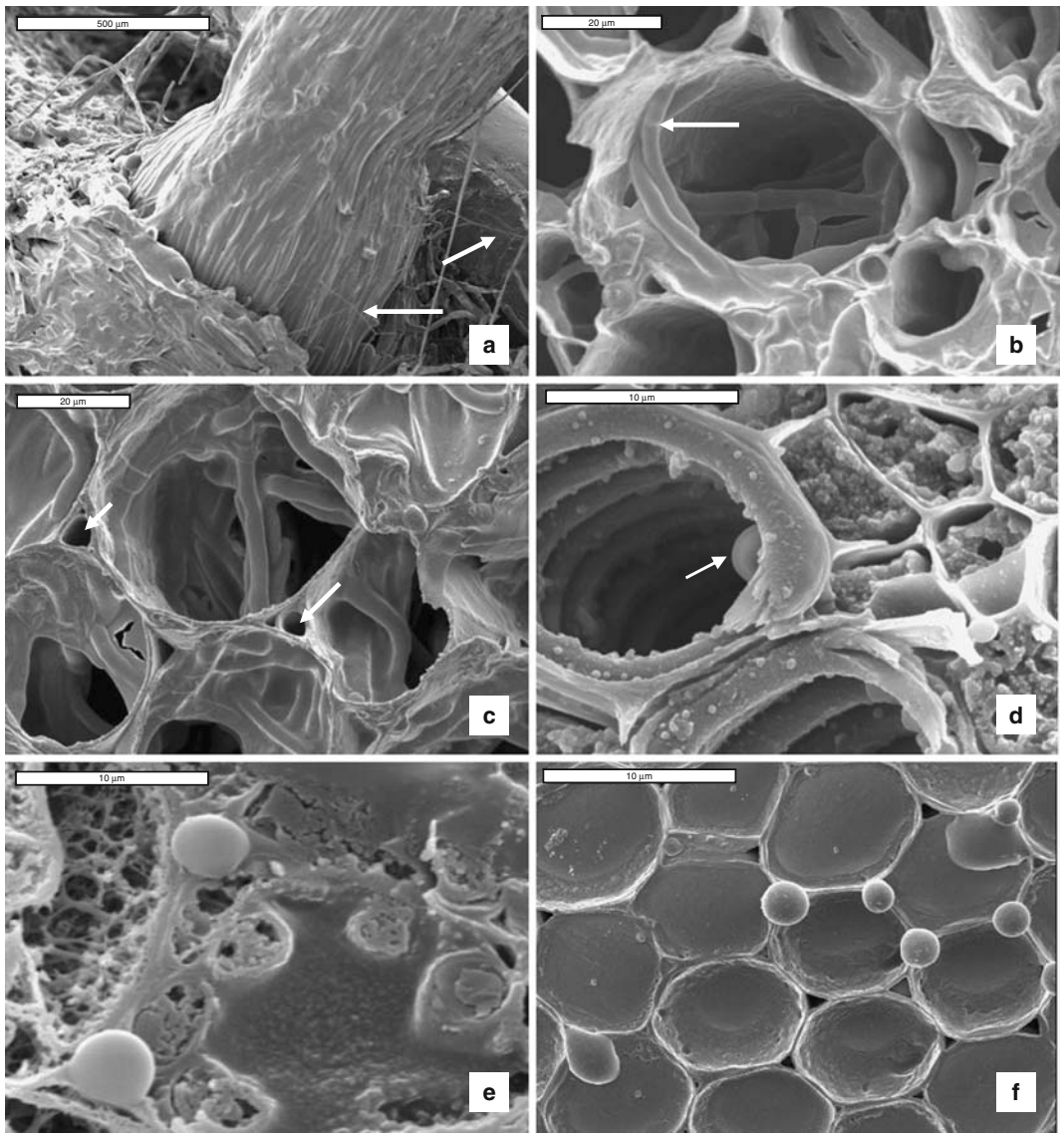


Fig. 8 Scanning electron micrographs of tissue sections of autumn squash after 8 days of growth in a culture medium infected with *M. cannonballus*. (**a**) Hyphae apparently penetrating the root tissues via the point of emergence of the lateral roots (arrows). (**b**) Septate hyphae (arrow) growing and adhered to the inner wall of a parenchymatic root cell; (**c**) Intercellular spaces of cortical root cells presenting sectioned hyphae inside them (arrows); (**d**) A magnified view of a xylem vessel showing a tylose which has formed in its lumen (arrow); (**e**) Spherical structures of different sizes emerging from the intercellular spaces in the cortex of the roots; (**f**) similar spherical structures emerging from the intercellular spaces of the parenchymatic cells of the pith in the hypocotyl

compared to non-infected control stem samples of the same age (Fig. 6a), resulting in the formation of aerenchyma-like tissue. This increase in the size of intercellular spaces was also observed in the lower stem (hypocotyl area) of infected autumn squash plants, but the upper stem was identical in appearance to that of non-infected plants.

Colonisation of plant tissues by the fungi in plants grown in Petri dishes After 8 days in culture in a medium infected with *M. cannonballus*, both the roots and the hypocotyls of muskmelon plants were colonised by the fungus (Fig. 7). The hyphae had extended both intra- and intercellularly (Fig. 7a), and inside plant vessels (Fig. 7b).

In the autumn squash seedlings of the same age, grown under identical conditions, the hyphae of the fungus had colonised all root tissues (Fig. 8). Hyphae were found at the point of emergence of lateral roots (Fig. 8a), suggesting that this could be the site of initial infection. However, we did not pursue our findings to demonstrate this possibility. In the tissues, the hyphae were present both within cells (Fig. 8b, c) and within intercellular spaces (Fig. 8c, arrows). The hyphae which adhered to the inner surface of cell walls were septate (Fig. 8b, arrow). In the lumen of the xylem vessels, tyloses were formed as a response to fungal infection (Fig. 8d, arrow). Spherical structures of different sizes were seen emerging from the intercellular spaces of the cortex of the root samples (Fig. 8e) and from the pith parenchyma cells of the hypocotyl (Fig. 8f). No other signs of fungal infection were observed in the hypocotyls of autumn squash.

As described above for *A. cucurbitacearum* infection, in the two species which were studied, root colonisation by *M. cannonballus* proceeded mainly centripetally, there being little acropetal spread.

Discussion

In this study we examined the early colonisation, spread and histological changes produced as a result of infection by two fungi associated with 'root and vine decline', *A. cucurbitacearum* and *M. cannonballus*, in two cucurbit species which differ in their sensitivity to this disease, the highly sensitive muskmelon (*C. melo*) and the autumn squash (*C. maxima*), which is tolerant to the disease. Owing to its tolerance, autumn squash is routinely used as a rootstock for both muskmelon and watermelon in order to control soil-borne diseases, such as Fusarium wilt (Miguel et al. 2004), Acremonium collapse (in watermelon; Armengol et al. 1998) and Monosporascus wilt (in muskmelon; Cohen et al. 2005; in watermelon; Beltrán et al. 2008). Furthermore, research indicates that the symptoms caused by both fungi are more severe in melons and watermelons than in squash (Aegerter et al. 2000). Plant mortality has been reported to be much lower and to occur much later in the season in muskmelon plants grafted onto *Cucurbita* rootstock than in non-grafted plants (Pivonia et al. 2004). The results obtained in the latter study confirmed the higher susceptibility of muskmelon to *A. cucurbitacearum* infection, since in the soil-growing plants symptoms developed earlier and were more intense in this species than in autumn squash (Figs. 2 and 3). On the other hand, no anatomical changes were produced as a result of infection by *M. cannonballus* after 42 days of culture, except for a significant increase in the size of the intercellular spaces in the stems of muskmelon plants (Fig. 6c), a response which may be related to ethylene synthesis caused by the fungal infection (Drew et al. 1979; Zacarías and Lafuente 2008). This finding is consistent with epidemiological studies, which demonstrate that *A. cucurbitacearum* shows a preference for attacking the fine roots of young plants (Biernacki and Bruton 2001), while *M. cannonballus* symptoms occurred at a later stage in development in thickened roots (García-Jiménez et al., 2000). However, the possibility that the type of inoculum we used for *A. cucurbitacearum* (inoculated as conidia) could have accelerated the development of the symptoms in comparison with *M. cannonballus* (inoculated as mycelium) cannot be dismissed.

Our scanning electron microscopy observations showed that both fungi colonised the epidermis and cortex of both muskmelon and autumn squash extensively, and in both species hyphal density decreased at the level of the endodermis. This tissue seemed to act as a barrier to these fungal infections, as has also been reported for *Fusarium oxysporum* (Olivain and Alabouvette 1999; Benhamou and Garand 2001). Inner tissues were seldom colonised, but occasionally hyphae were observed within the inner tissues, even inside the lumen of xylem vessels, both in muskmelon (*M. cannonballus*; Fig. 7b) and in autumn squash (*A. cucurbitacearum*; Fig. 5c, d). This was probably a consequence of the extremely severe infection conditions to which the recently germinated seedlings we used in our study were subjected. Young seedlings have been reported to present low resistance to infections, as their recently formed tissues lack some of the resistance mechanisms which are in place in older plants (Garret 1970). In spite of the colonisation of xylem vessels by the fungal hyphae, non-fungal structures were detected in the hypocotyl vascular tissues. This indicates that these fungi were not spreading systemically through the vascular system of the host. In agreement with our observations, hyphae of *M. cannonballus* have been reported

to penetrate and grow inside vessels, extending from both the protoxylem and the metaxylem of cantaloupe roots, but the fungus did not move systemically through the plant (Waugh et al. 2005).

For the reason stated above, we were only able to observe the early anatomical response to infection caused by *A. cucurbitacearum*. In the soil-grown muskmelon plants, the first symptom we observed was the deposition of suberin, a widespread response to pathogenic infection (Agrios 1996), in the cell layers beneath the epidermis, a phenomenon which occurred after 30 days in culture (Fig. 2c). At the same time hypertrophy and hyperplasia were observed in the root-hypocotyl and stem tissues. These histopathological changes resulted in the formation of corky and necrotic lesions on the hypocotyl and gave a knotted appearance to the infected plants (García-Jiménez et al. 1994; Armengol et al. 1998). These symptoms are similar to those found in other crops in response to attack by other pathogenic fungi, such as *F. oxysporum* f. sp. *lycopersici* (Olivain and Alabouvette 1999), *Synchytrium endobioticum*, *Taphrina deformans* and *Plasmodiophora brassicae* (Šutić and Sinclair 1991). This was followed 12 days later (in 42 day-old seedlings) by the formation of tyloses within the xylem vessels (Fig. 2f), the collapse of the pith and the separation of vascular bundles in the stem (Fig. 2h), a morphological symptom described by Armengol (1997) which demonstrates the colonisation of the lower stem by this fungus. Extensive tylose formation has previously been described in *Monosporascus*-infected muskmelon (Alcántara et al. 1998; Pivonia et al. 2002), but to our knowledge it has not yet been reported in *Acremonium* infection. Although the cause of tylose formation is debatable, these structures reduce water flow in plant vessels and may be the cause of wilting, particularly under conditions where water demand is high (Šutić and Sinclair 1991; Pivonia et al. 1997).

Under the relatively mild infection conditions we used in the potted plant experiment, the autumn squash seedlings showed only modest suberin deposition in the cells beneath the epidermis after 42 days in culture (Fig. 3), which demonstrates slower fungal colonisation than in muskmelon. This result supports the view that cucurbitacins, fungal inhibitors produced by autumn squash and other cucurbit species, may be responsible for the tolerance these plants exhibit to vine decline (Bruton et al. 1998). However, in agreement with previous reports (Mertely et al. 1993; Armengol et al. 1998), we were able to recover both *A. cucurbitacearum* and *M. cannonballus* from the infected autumn squash plants and, in the heavy infestation conditions used in the Petri-dish grown plants, the colonisation of the autumn squash tissues was similar to that observed in muskmelon (Figs 4, 5, 7, 8). Under these unfavourable conditions, the size and water transport capacity of the root system may be a decisive factor in tolerance to wilting, as suggested by Pivonia et al. (1997, 2004), a proposal supported by the behaviour of Cucurbita hybrids (Dias et al. 2004).

Acknowledgements This work was funded by grant AGL2000-1809-C03-01 from the Spanish Ministry of Science and Technology. We would like to thank Dr. J. García-Jiménez, Dr. J. Armengol and Dr. R. Beltrán for their expert guidance on the pathological aspects of this research and Dr. J.L. Guardiola for his critical review of the manuscript. The authors also wish to thank the U.P.V Electron Microscopy Service for providing technical assistance with the S.E.M. observations. We would like to thank the R&D&I Linguistic Assistance Office, Universidad Politécnica de Valencia (Spain) for granting financial support for the linguistic revision of this paper.

References

Aegerter, B. J., Gordon, T. R., & Davis, R. M. (2000). Occurrence and pathogenicity of fungi associated with melon root rot and vine decline in California. *Plant Disease, 84*, 224–230. doi:10.1094/PDIS.2000.84.3.224.

Agrios, G. N. (1996). *Fitopatología*. San José, México: Ed. Uteha.

Alcántara, T., Stanghellini, M. E., Kim, D. H., & Rasmussen, S. L. (1998). Tyloses formation in relation to symptom development of vine decline of melon caused by *Monosporascus cannonballus*. *Phytopathology, 88*(9), S2.

Alfaro-García, A., Armengol, J., Bruton, B. D., Gams, W., García-Jiménez, J., & Martínez-Ferrer, G. (1996). The taxonomic position of the causal agent of Acremonium collapse of muskmelon. *Mycologia, 88*(5), 804–808. doi:10.2307/3760975.

Armengol, J. (1997). Aspectos patológicos, epidemiológicos y culturales de *Acremonium cucurbitacearum* Alfaro-García, W. Gams et J. García-Jiménez. Universidad Politécnica de Valencia. Ph.D. Thesis.

Armengol, J., Sanz, E., Martínez-Ferrer, G., Sales, R., Bruton, B. D., & García-Jiménez, J. (1998). Host range of *Acremonium cucurbitacearum*, cause of Acremonium collapse of muskmelon. *Plant Pathology, 47*, 29–35. doi:10.1046/j.1365-3059.1998.00199.x.

Beltrán, R., Vicent, A., Sales, R., García-Jiménez, J., & Armengol, J. (2005). Population dynamics of *Monosporascus cannonballus* in marsh soils in eastern Spain.

European Journal of Plant Pathology, 113(4), 357–365. doi:10.1007/s10658-005-2930-0.

Beltrán, R., Vicent, A., García-Jiménez, J., & Armengol, J. (2007). Quantification of *Monosporascus cannonballus* ascospores in muskmelon fields in eastern Spain. *Journal of Phytopathology, 155*, 248–250. doi:10.1111/j.1439-0434.2007.01215.x.

Beltrán, R., Vicent, A., García-Jiménez, J., & Armengol, J. (2008). Comparative epidemiology of Monosporascus root rot and vine decline in muskmelon, watermelon and grafted watermelon crops. *Plant Disease, 92*(1), 158–163. doi:10.1094/PDIS-92-1-0158.

Benhamou, N., & Garand, C. (2001). Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by non-pathogenic *Fusarium oxysporum* Fo47. *Phytopathology, 91*, 730–740. doi:10.1094/PHYTO.2001.91.8.730.

Biernacki, M., & Bruton, B. D. (2001). Quantitative response of *Cucumis melo* inoculated with root rot pathogens. *Plant Disease, 85*, 65–70. doi:10.1094/PDIS.2001.85.1.65.

Bruton, B. D., Russo, V. M., García-Jiménez, J., & Miller, M. E. (1998). Carbohydrate partitioning, cultural practices, and vine decline diseases of cucurbits. In J. McCreight (Ed.), *Cucurbitaceae 98 (pp. 189-200)*. U.S.A: American Society of Horticultural Science Press, Alexandria.

Bruton, B., García-Jiménez, J., Armengol, J., & Popham, T. (2000). Assessment of virulence of *Acremonium cucurbitacearum* and *Monosporascus cannonballus* on *Cucumis melo*. *Plant Disease, 84*, 907–913. doi:10.1094/PDIS.2000.84.8.907.

Cohen, R., Pivonia, S., Burger, Y., Edelstein, M., Gamliel, A., & Katan, J. (2000). Toward integrated management of Monosporascus Wilt of melon in Israel. *Plant Disease, 84*(5), 496–505. doi:10.1094/PDIS.2000.84.5.496.

Cohen, R., Burger, Y., Horev, C., Potat, A., & Edelstein, M. (2005). Perfomance of Galia-type melons grafted onto Cucurbita rootstock in *Monosporascus cannonballus*-infested and non-infested soils. *The Annals of Applied Biology, 146*, 381–387. doi:10.1111/j.1744-7348.2005.040010.x.

Dhingra, O. D., & Sinclair, J. B. (1985). *Basic plant pathology methods*. Boca Ratón, Florida: CRC Press, Inc.

Dias, R. C. S., Picó, B., Espinós, A., & Nuez, F. (2004). Resistance to melon vine decline derived from *Cucumis melo* subsp. *agrestis*: genetic analysis of root structure and root response. *Plant Breeding, 123*, 1–7. doi:10.1046/j.1439-0523.2003.00944.x.

Drew, M. C., Jackson, M. B., & Giffard, S. (1979). Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptative responses to flooding in *Zea mays* L. *Planta, 147*, 83–88. doi:10.1007/BF00384595.

García-Jiménez, J., Velázquez, M. T., Jordá, C., & Alfaro-García, A. (1994). *Acremonium* species as the causal agent of muskmelon collapse in Spain. *Plant Disease, 78*(4), 416–419.

García-Jiménez, J., Armengol, J., Sales, R., Jordá, C., & Bruton, B. D. (2000). Fungal pathogens associated with melon collapse in Spain. *EPPO Bulletin, 30*, 169–173. doi:10.1111/j.1365-2338.2000.tb00873.x.

Garret, S. D. (1970). *Pathogenic root-infecting fungi*. London, UK: Cambridge University Press.

Johansen, D. A. (1940). *Plant Microtechnique*. New York: McGraw Hill Book Company.

Martyn, R. D., & Miller, M. E. (1996). Monosporascus root rot and vine decline: an emerging disease of melon worldwide. *Plant Disease, 80*(7), 716–725.

Mertely, J. C., Martyn, R. D., Miller, M. E., & Bruton, B. D. (1991). Role of *Monosporascus cannonballus* and other fungi in a root rot/ vine decline disease in muskmelon. *Plant Disease, 75*(11), 1133–1137.

Mertely, J. C., Martyn, R. D., Miller, M. E., & Bruton, B. D. (1993). An expanded host range for the muskmelon pathogen *Monosporascus cannonballus*. *Plant Disease, 77*(7), 667–673.

Miguel, A., Maroto, J. V., San Bautista, A., Baixauli, C., Cebolla, V., Pascual, B., López, S., & Guardiola, J. L. (2004). The grafting of triploid watermelon is an advantageous alternative to soil fumigation by methyl bromide for control of Fusarium wilt. *Scientia Horticulturae, 103*(1), 9–17. doi:10.1016/j.scienta.2004.04.007.

Olivain, C., & Alabouvette, C. (1999). Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. *The New Phytologist, 141*, 497–510. doi:10.1046/j.1469-8137.1999.00365.x.

Pivonia, S., Cohen, R., Kafkafi, U., Ben Ze'ev, I. S., & Katan, J. (1997). Sudden wilt of melons in Southern Israel: fungal agents and relationship with plant development. *Plant Disease, 81*, 1264–1268. doi:10.1094/PDIS.1997.81.11.1264.

Pivonia, S., Cohen, R., Katan, J., & Kigel, J. (2002). Effect of fruit load on the water balance of melon plants infected with *Monosporascus cannonballus*. *Physiological and Molecular Plant Pathology, 60*, 39–49. doi:10.1006/pmpp.2001.0375.

Pivonia, S., Cohen, R., Cohen, S., Kigel, J., Levita, R., & Katan, J. (2004). Effect of irrigation regimes on disease expression in melon plants infected with *Monosporascus cannonballus*. *European Journal of Plant Pathology, 110*, 155–161. doi:10.1023/B:EJPP.0000015341.95455.22.

Pollack, F. G., & Uecker, F. A. (1974). *Monosporascus cannonballus* an unusual Ascomycete in cantaloupe roots. *Mycologia, 66*, 346–349. doi:10.2307/3758370.

Šutić, D. D., & Sinclair, J. B. (1991). *Anatomy and physiology of diseased plants*. Boca Ratón, Florida: CRC Press.

Waugh, M. M., Stanghellini, M. E., & Kim, D. (2001). Scanning electron microscopy of germinated ascospores of *Monosporascus cannonballus*. *Mycological Research, 105*(6), 745–748. doi:10.1017/S0953756201004117.

Waugh, M. M., Ferrin, D. M., & Stanghellini, M. E. (2005). Colonization of cantaloupe roots by *Monosporascus cannonballus*. *Mycological Research, 109*(11), 1297–1301. doi:10.1017/S0953756205003722.

Zacarías, L., & Lafuente, M. T. (2008). Etileno, ácido abscísico y otros reguladores del desarrollo. In J. Azcón-Bieto & M. Talon (Eds.), *Fisiología y bioquímica vegetal. (pp. 445–466) Madrid*. Spain: Interamericana McGraw-Hill.