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Infection and colonization of bean leaf by *Phaeoisariopsis griseola*

E. O. Monda*†, F. E. Sanders and A. Hick

Department of Pure and Applied Biology, Leeds University, LS2 9JT, UK

Infection and spread of *Phaeoisariopsis griseola* in the leaf of bean (*Phaseolus vulgaris*) were investigated by scanning and transmission electron microscopy. Conidia of *P. griseola* germinated by releasing a germ tube either at one end of the conidia or both ends. The germ-tube growth followed the contours of epidermal cells. The fungus entered the leaf through the stomata and grew mainly intercellularly between mesophyll and palisade cells. The chloroplast envelope and plasma membrane of adjacent cells disintegrated, lost structural integrity and dried out. Hyphae did not penetrate host cells. A stroma subsequently formed on the leaf surface. Conidiophores elongated under favourable conditions forming synnemata with conidia at the tips of conidiophores. Conidiophores also emerged through stomata.

Keywords: angular leaf spot, electron microscopy, *Phaseolus vulgaris*, spore germination

Introduction

Angular leaf spot of bean (*Phaseolus vulgaris*), caused by the fungus *Phaeoisariopsis griseola*, is an important economic disease in several Latin American and African countries. The disease causes yield losses of 40–80% in Columbia (Schwartz *et al*., 1981; Mora *et al*., 1985). Initial disease symptoms on leaves are small grey lesions which are angular in shape. The lesions coalesce, turn brown and cause the yellowing of leaves which fall off prematurely. Lesions may also appear on pods and stems. The fungus usually sporulates on the lower surface of the leaf by dark grey to black synnemata. Pathogenic variation of the fungus has been reported in several countries (Buruchara, 1983; Correa-Victoria, 1987; Monda, 1995).

Phaeoisariopsis griseola is an imperfect fungus in the order Moniliales, family Stilbaceae (Barnett & Hunter, 1972). The fungus was first discovered by Saccardo in Italy who named it *Isariopsis griseola* (Saccardo, 1878). However, Ferraris (1909) described the same fungus as *Phaeoisariopsis griseola* and Ellis (1971) confirmed this name.

Although angular leaf spot is an economically important disease, there has been no ultrastructural investigation of the infection and establishment of *P. griseola* in the leaf. The objectives of this study were to examine the mode of infection of bean leaf by *P. griseola*, and to determine the extent of pathogen invasion of the leaf and the development of fruiting structures.

*To whom correspondence should be addressed.

†Present address: Kenyatta University, Botany Department, PO Box 43844, Nairobi, Kenya.

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Materials and methods

Isolate Kat 1 of *P. griseola* isolated from naturally infected bean leaves in Kenya was used in this study. The isolate was deposited at the International Mycological Institute, UK (reference number IMI 366608). The fungus was cultured on V-8 juice agar (200 mL V-8 juice, 800 mL sterile distilled water, 18 g Bacto agar, 3 g calcium carbonate) at 20–22°C for 10–12 days in darkness. The spore suspension was prepared by pouring 10 mL of sterile distilled water onto the culture and scraping the spores off with a wire loop. The suspension was filtered through three layers of muslin and adjusted by haemocytometer to a concentration of 2×10^4 spores mL^{-1}.

Plant inoculation

A susceptible bean cultivar GLPX 1127(a) was used in this study. The plants were grown in a growth chamber at a temperature of 18–22°C in 15-cm-diameter pots of M2 compost (Levington). Lighting was 12 h per day (light intensity of 370 μmol m^{-2} s^{-1} at 0·8 m from the source) and relative humidity 60–70%. Plants, 19–21 days old, were inoculated by spraying the lower surface of trifoliate leaves with a spore suspension

(2×10^4 spores mL^{-1}) using a Hozelock polyspray. The inoculated plants were covered with polythene bags for 4 days and maintained in the growth chamber at a temperature of 18–22°C.

Scanning electron microscopy (SEM)

Leaves from inoculated and uninoculated (control) plants were excised at 3, 7 and 12 days after inoculation and cut into 5-mm^2 pieces. They were immediately fixed in 1% osmium tetroxide in 0·1 M phosphate buffer overnight at room temperature (18–20°C). The tissues were washed in the buffer and dehydrated in a graded series of acetone (20–100%). They were dried in a Polaron critical point dryer and mounted on aluminium SEM stubs on which carbon cement (Neubaer Chemikalien, D-48031 Munster, Germany) had been applied. The tissues were sputter-coated with gold–palladium to a thickness of 60 nm in a Polaron sputter coater unit, model E5300 (Polaron Equipment Ltd, Watford, WD1 8XG, UK), and examined with a Camscan series 3/30 BM scanning electron microscope (Camscan Analytical, Waterbeach, CB5 9PY, UK) operating at 10 kV.

Transmission electron microscopy (TEM)

The inoculated and control leaves were cut into 3-mm^2 pieces and fixed in 2·5% (v/v) glutaraldehyde in 0·1 M phosphate buffer (pH 7·0) at room temperature (18–20°C) and postfixed overnight in 1% osmium tetroxide in 0·1 M buffer. After rinsing in the buffer, the tissues were dehydrated in an ethanol series and put into two changes of propylene oxide for 30 min each. The tissues were infiltrated overnight in a 50 : 50 mixture of propylene oxide : Araldite on an Agar specimen rotator (Agar Scientific, Stanstead, CM24 8DA, UK) rotating at 3 rev min^{-1} and then transferred to a 25 : 75 mixture of propylene oxide : Araldite for several hours. The tissues were then transferred to embedding moulds containing neat Araldite which were polymerized at 60°C overnight (Luft, 1961). Ultrathin sections were cut on a Reichert Jung Ultramicrotome with glass knives (Leica UK, Milton Keynes, MK5 8LB, UK). The sections were collected on 100 mesh copper grids and stained with Reynolds lead citrate for 20 min (Reynolds, 1963). The sections were examined in a Jeol 1200EX transmission electron microscope (Jeol UK Ltd, Welwyn Garden City, AL7 1LT, UK).

Light microscopy

Semi-thin sections (1·0–1·5 μm) of resin-embedded material prepared as for TEM were stained for 2 min with 1% toluidine blue in 1% borax at 60°C and examined with a Zeiss Ultraphot photomicroscope (Zeiss, D-7082 Oberkochen, Germany).

Results

Conidial germination and plant penetration

Conidia of *P. griseola* germinated on the leaf surface under moist conditions within 3 days of inoculation by releasing one germ tube either at one end of the conidia or at both ends (Fig. 1a). However, not all conidia germinated, as SEM examination revealed some ungerminated conidia on the leaf surface at 3 and 7 days after inoculation. The germ tubes follow the contours of epidermal cells (Fig. 1b). Germ tubes formed appressorium-like swellings either at epidermal cell junctions or over stomata (Fig. 1b,c) and some hyphae emerged from these swellings. Penetration was through the stoma either by formation of an appressorium-like swelling over the stoma (Fig. 1c) or without forming an appressorium-like swelling at all (Fig. 1d).

Host invasion

After penetration into the leaf, the hyphae grew intercellularly in the air spaces of the mesophyll (Fig. 1e) and palisade tissue (not illustrated). During this early infection period, the host cell wall and plasma membrane were intact at the point of contact with the hypha (Fig. 1f). There was also a close attachment of intercellular hyphae to the host wall and the production of extracellular matrix at the interface (Fig. 1f). Later, the fungal wall thickened and was multilayered with fibrillar extracellular matrix between hyphae (Fig. 2a). Damage was observed in chloroplasts of mesophyll cells where the chloroplast envelope disintegrated between 3 and 7 days after inoculation (Fig. 2b). The host cells were plasmolysed, the plasma membrane disintegrated and the cytoplasma was disorganized (Fig. 2b). Host cells were destroyed as the fungus proliferated, but it was unable to penetrate through the leaf veins (Fig. 2c), possibly due to a lack of intercellular spaces. The fungus also grew in the intercellular spaces of the palisade cells where it destroyed cells. All hyphal growth was intercellular as no hyphae were found within the host cells.

Sporulation structures

After the destruction of host cells, the fungus formed a stroma (Fig. 3a) which pushed through either the stomata or epidermal wall to the leaf surface (Fig. 3b). Conidiophores emerged from the stroma and elongated to form a synnema (Fig. 4a). The conidiophores also emerged through the stomata (Fig. 4b). The synnemata were formed in association with lesions on the leaf delimited by the veins and conidia were formed at the tip of the conidiophore (Fig. 4c).

Discussion

The germ tubes of *P. griseola* grew on the leaf surface

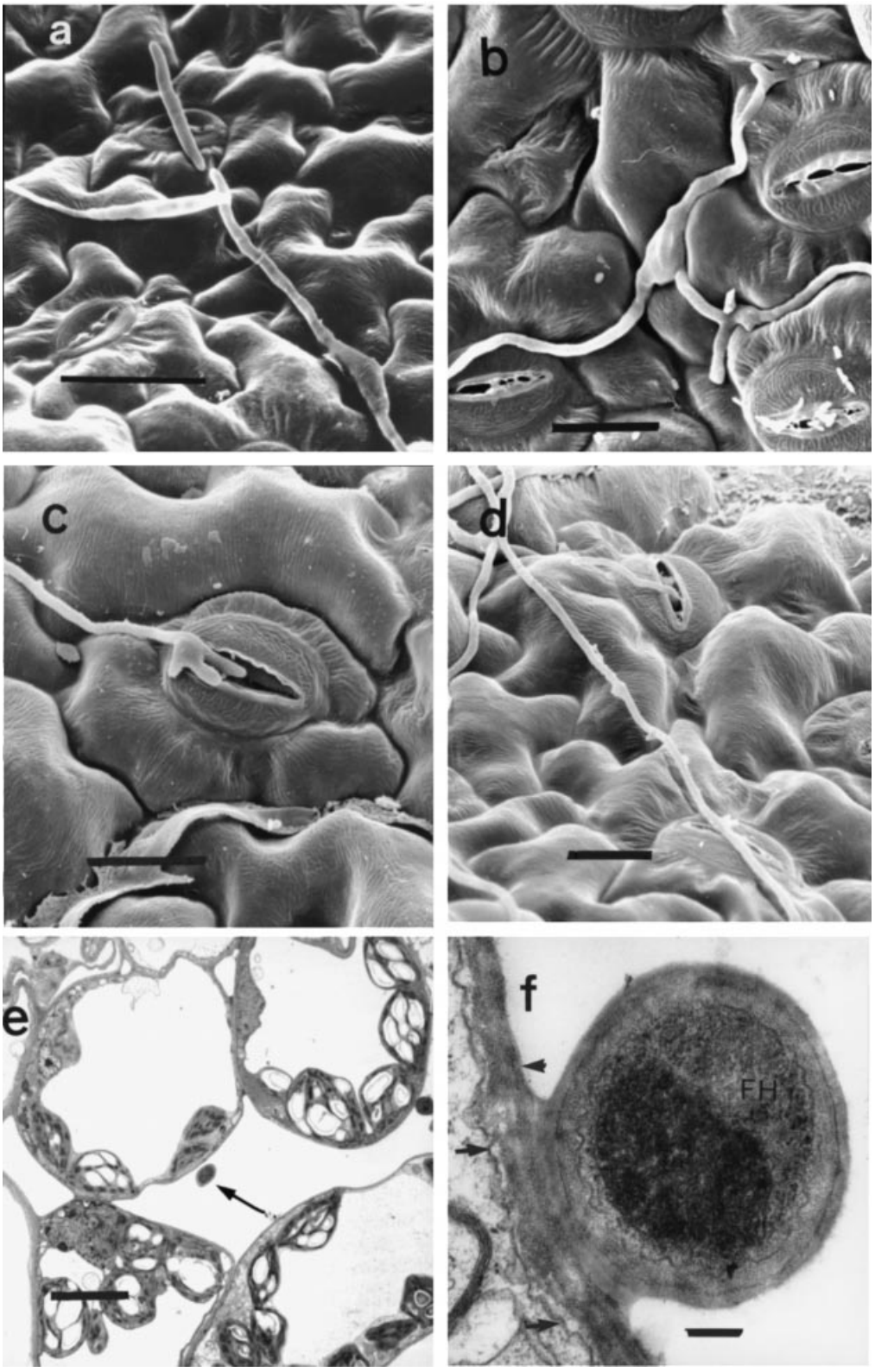

Figure 1 (a) Germinated conidia of *P. griseola* on the lower surface of a bean leaf at 3 dpi. (b) An appressorium-like swelling at the cell junction; note the three germ tubes emerging from the structure. (c) An appressorium-like structure at a stoma. (d) Penetration of leaf through a stoma; note the germ tube passed a closed stoma (a–d, bar = 10 μm). (e) Transmission electron microscopy (TEM) of mesophyll cells with hypha (arrow) in intercellular space at 7 dpi (bar = 5 μm). (f) Hypha attached to mesophyll cell between 4 and 7 dpi; note intact cell wall (arrowhead) and plasma membrane (arrow) of host and the close attachment of hypha to host wall (bar = 200 nm). dpi = days postinoculation; FH = fungal hypha.

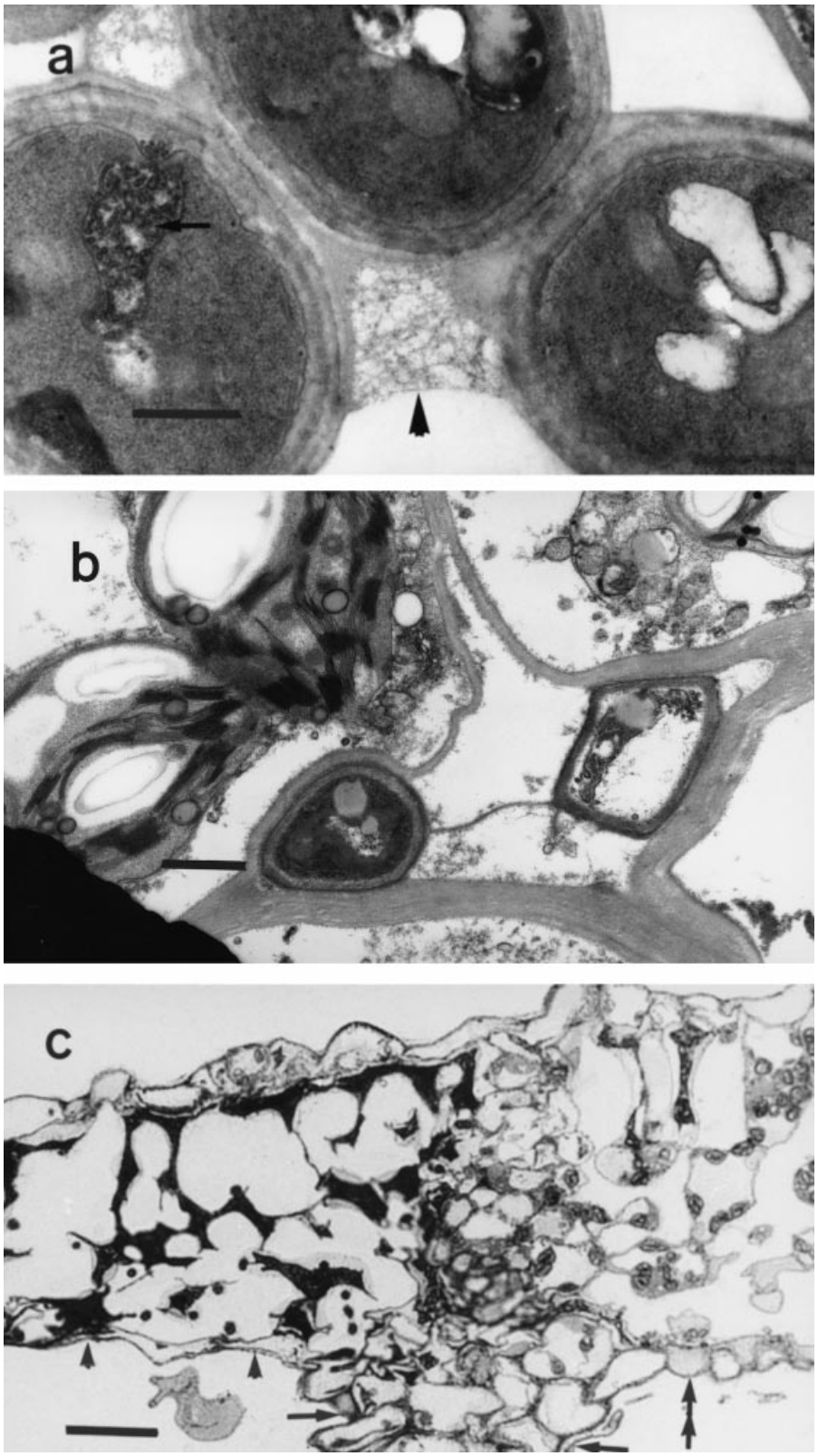

Figure 2 (a) Hyphae in intercellular space; note the apparent fibrillar material (arrowhead) and multilayered fungal wall (bar = 500 nm). (b) Two hyphae in intercellular space; note close attachment of hyphae to host wall, absence of chloroplast envelope and host plasma membrane (bar = 1 μm). (c) Light microscopy of transverse section of infected leaf at 12 dpi; note leaf vein region (arrow), damaged disorganized area on left of leaf vein (arrowhead) and undamaged area on right of leaf vein (double arrowhead) (bar = 20 μm).

extensively without penetrating the leaf. This could be attributed to unfavourable conditions for penetration as plants were exposed to a continuous high relative humidity for 4 days. This type of regime possibly does not exist in nature. In *P. personata*, the causal agent of late leaf spot disease of groundnuts, infection is enhanced by alternate wet and dry periods (Wadia & Butler, 1994).

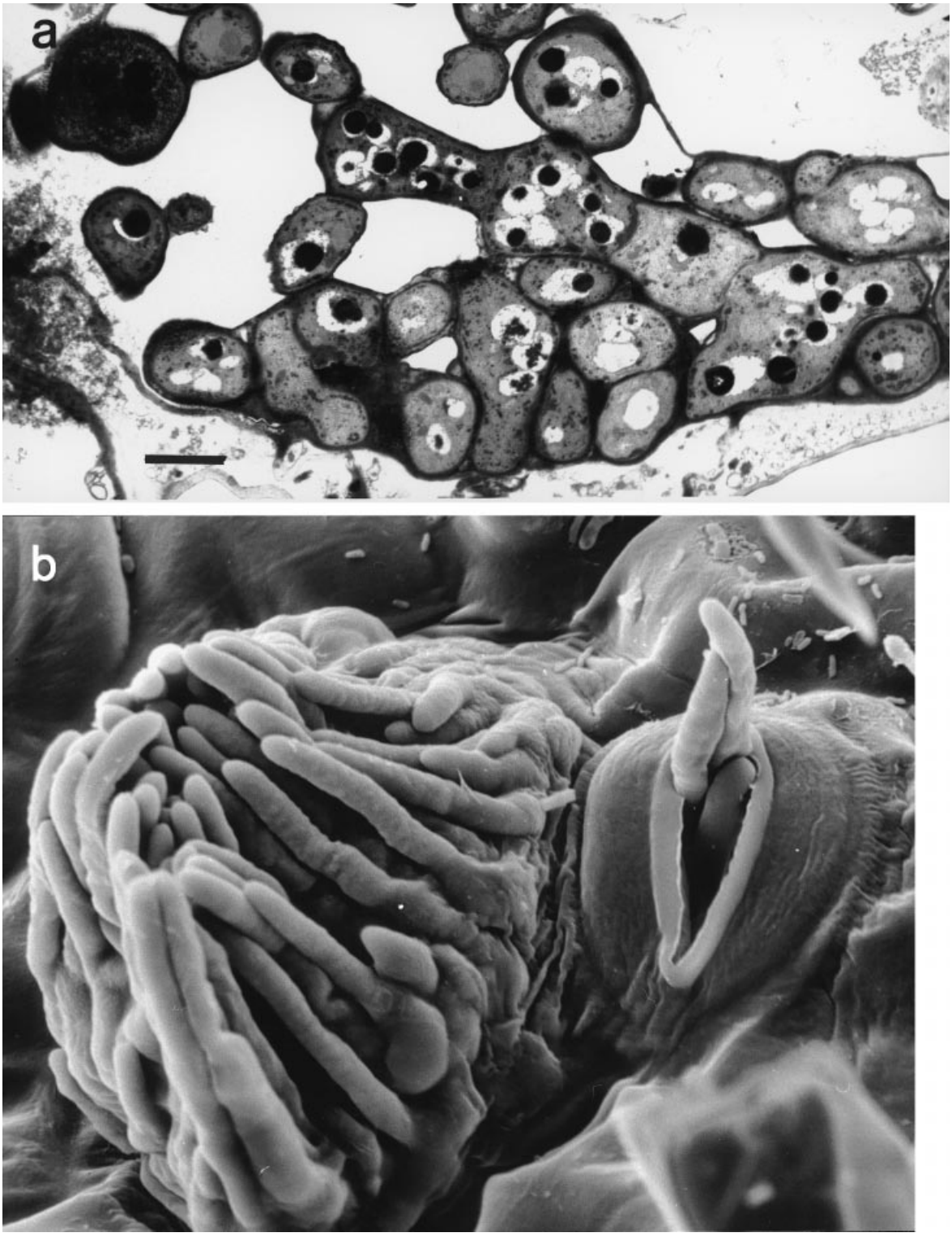

Figure 3 (a) Stroma in the leaf; note complete destruction of mesophyll cells (bar = 5 μm). (b) Conidiophores (arrows) growing from the stroma and also emerging through the stoma (arrow) (bar = 10 μm).

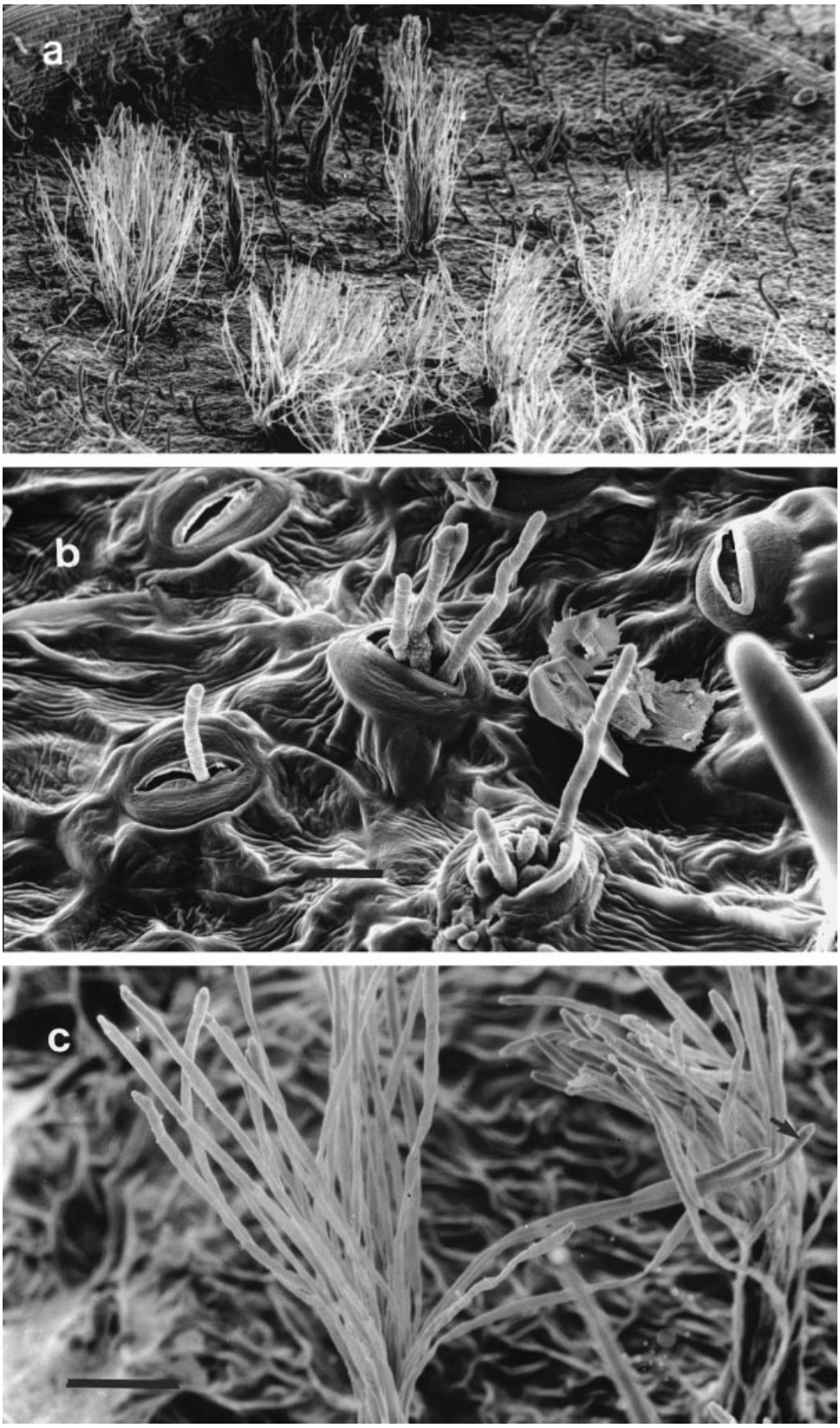

Figure 4 (a) Synnemata (arrow) on the lower leaf surface (bar = 300 μm). (b) Conidiophores emerging through the stoma (bar = 10 μm). (c) Conidia (arrow) at the tip of conidiophore (bar = 30 μm).

During germ-tube extension, appressorium-like structures were formed at the junctions of cells and also over stomata. Wynn (1976) indicated that this type of response was not due to surface topography. These structures have also been observed on groundnut leaves infected by *P. personata* (Wadia & Butler, 1994), *Wikstroemia indica* leaves infected by *P. williamsiae* var. *williamsiae* (Walker & White, 1991) and wheat seedlings infected by *Pseudocercosporella herpotrichoides* (Daniels *et al*., 1991). In the present study, no septum was observed delimiting the appressorium-like structures from the germ tubes or hyphae. It is possible that the function of these structures could be the adhesion of pathogen to the host.

The germ tube did not grow directly towards stomata. This supports the observations by Wadia & Butler (1994) that, in continuous wetness, germ tubes reach stomata by chance but, under intermittent wetness, germ tubes of *P. personata* show tropic growth towards stomata. Abdou *et al*. (1974) also observed no directional growth of germ tubes of *Cercospora personatum* on plants incubated under high relative humidity for long periods. Although *P. williamsiae* var. *williamsiae* penetrates its host directly (Walker & White, 1991), this mode of penetration was not observed in the present study.

Phaeoisariopsis griseola grows mainly in intercellular spaces and its spread in the leaf is limited by the leaf veins giving the lesion an angular shape. Damage to host cells was not observed for the first 7 days and there seemed to be a balanced phase of the relationship between host and parasite during this early period. However, after this period, the pathogen became aggressive and destroyed host cells. As cells which were in contact with the fungus, as well as cells away from the fungus, were affected by the pathogen, this could imply the release of a diffusible product by the pathogen which disrupted the chloroplast membrane and host plasma membrane causing cell necrosis and chlorosis. Plasma membrane disorders could be a central event in pathogenesis allowing the fungus access to nutrients from the host. As *P. griseola* is incapable of direct penetration of the host, and as no intracellular hyphae were observed, the damage to host plasma membranes and chloroplast membranes could possibly be attributed to the production of a toxin by *P. griseola*. Some pathogens, such as *Alternaria alternata* (Nishimura *et al*., 1974) and *Helminthosporium victoriae* (Pringle & Scheffer, 1964; Hanchey *et al*., 1968), produce toxins which damage the plasma membrane and cause chlorosis (Keck & Hodges, 1973).

The formation of a stroma after the destruction of host cells permits the fungus to remain dormant until favourable conditions for sporulation prevail. *Phaeoisariopsis griseola* therefore survives unfavourable conditions in infected plant tissues.

Additional studies of *P. griseola*, including its enzymes and toxins, could provide clues about the toxic metabolites and mode of growth of this fungus.

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