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# Transfer of *Alternaria macrospora* from Cotton Seed to Seedling: Light and Scanning Electron Microscopy of Colonization

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*With 6 figures*

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## Abstract

*Alternaria macrospora* is transferred from an infected seed, through the plant stem to the cotyledon and sporulates on its surface. However, at the beginning of the process, there is no difference between resistant and susceptible plants. In later stages, lower levels of fungal mycelium and sporulation were detected on the seedlings of a resistant cultivar. The fungus grew inside the plant from the underground to the upper parts. The mycelia which originated from surface spores could penetrate through stomata and result in leaf colonization. *A. macrospora* sporulated ultimately on plant surfaces whereas the internal leaf tissue was colonized by mycelium only. This study suggests that cotyledon infection is a result of seed infection.

## Zusammenfassung

### Übertragung von *Alternaria macrospora* aus Baumwollsamen auf Keimlinge: Licht- und Rasterelektronenmikroskopie der Besiedlung

*Alternaria macrospora* wächst von einem befallenen Samen durch den Pflanzenstengel zu den Kotyledonen, wo der Pilz auf der Oberfläche sporuliert. Jedoch gibt es am Anfang dieses Vorgangs keine Unterschiede zwischen resistenten und empfindlichen Pflanzen. In späteren Stadien wurden niedrigere Myzel- und Sporulationsdichten in Sämlingen eines resistenten Kultivars festgestellt. Der Pilz wuchs innerhalb der Pflanze von den unter- bis in die oberirdischen Teile. Das Myzel, das von Sporen auf den Blattoberflächen stammte, konnte durch die Spaltöffnungen eindringen und zu einer Blattkolonisation führen. Schließlich sporulierte *A. macrospora* an der Blattoberfläche, obwohl das Blattinnere nur vom Myzel kolonisiert wurde. Diese Studie deutet darauf hin, daß eine Infektion der Kotyledonen aus einem Samenbefall resultiert.

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*Alternaria* blight of cotton, caused by *Alternaria macrospora* Zimm. is one of the main cotton disease in Israel (HADAS and JAKOBY 1981). The disease is considered to be a seedling disease (BASHAN 1984, EBBELS 1980). *A. macrospora* can survive on commercial cotton seeds and on post-season plants left at field edges. It can be transferred by various agents and cause infection of seedlings (BASHAN 1984, 1986a, HALFON-MEIRI and COHEN 1983).

The purposes of this study were to explore how the fungus is transferred from the seed to the seedling and how it colonizes the plant leaf.

## Materials and Methods

### Organisms, growth conditions, isolation methods and pathogenicity tests

These were described in a previous paper (BASHAN 1984).

### Seed inoculation

Seeds were dipped, to about half their size, in a solution containing: 50 spores/ml in 0.06 M potassium phosphate buffer, pH 7.0, supplemented with 0.05% agar. Then they were placed in the dark, on a sterile vermiculite layer in a humidity chamber (relative humidity [R.H.] approximately 100%) for 24 h. Afterwards, seeds were transferred into a dark growth chamber at 60% ± 10% R.H. and subjected daily for 2 h to 100% R.H. After emergence, the growth chamber was set to provide intervals of 14 h light (100 W/cm$^2$ at bench level) and 10 h darkness with the same R.H. intervals.

### Cellulose acetate prints of leaf surfaces

In order to remove the surface inoculated spores, the germinating spores, and the surface mycelium the leaves were covered on both sides with cellulose acetate layers. The fungal material present on the leaf surface was attached to the plastic film in the exact orientation it occurred on the leaf. After removal of the films, the leaf was cleared and stained as described later, revealing the fungus material present inside the leaf. The films were prepared as follows: the leaves were stuck to a clean slide by tape on their corners and were gently sprayed with a 4% (w/v) solution of cellulose acetate in 100% acetone forming a thin film over the leaf surface. After drying for 30 min at room temperature, the process was repeated (DHINGRA and SINCLAIR 1985). Samples (1 cm$^2$) were taken by carefully stripping off the film under a stereoscopic microscope (SMZ-1B, Tokyo, Japan). Unstained leaf prints were observed under a light microscope (Universal, Zeiss, FRG). Then all the remaining layers were stripped and the leaves were subjected to clearing and staining. Preliminary observations revealed that this procedure removed most of the material from leaf surfaces.

### Leaf clearing and staining

Leaf clearing and staining for light microscopy were performed principally according to the method described by BASHAN *et al.* (1981).

Cotton leaves were cleared for 15 min in a solution containing: chloralhydrate, 30 g; lactic acid, 20 ml and absolute ethanol, 125 ml. After clearing, the leaves were washed twice in fresh solutions and immersed for 15 min in the same mixture supplemented with 50 ml of 1% aniline blue. The stained samples were finally washed with absolute ethanol and observed under a light microscope.

### Scanning electron microscopy (SEM)

Germinating seeds and seedlings were examined by SEM at 24 h intervals during the first six days after inoculation. Ten samples from each treatment were observed. Pieces of seedlings or seeds of approximately 0.5 cm$^2$ were fixed for 5 h in 5% glutaraldehyde solution in 0.2 M cacodylate buffer (pH 7.3) and then washed at 4°C with acetone solutions of 50% and 70% for 0.5 h, another 70% for

10 h and 100% for 0.5 h and once again for 1 h. The samples were then dried in a critical point dryer in a $CO_2$ environment, stuck on stubs, coated with gold and examined under a Philips SEM 505 scanning electron microscope at 30 KV.

## Chitin measurements

The amount of chitin was measured by analyzing glucoseamine according to ELSON and MORGAN (1933).

## Experimental design

All observations were repeated twice in five replicates, using five samples per replicate. Micrographs given are from a representative experiment. Significance is given by $P \leq 0.05$ in Duncan's multiple range test.

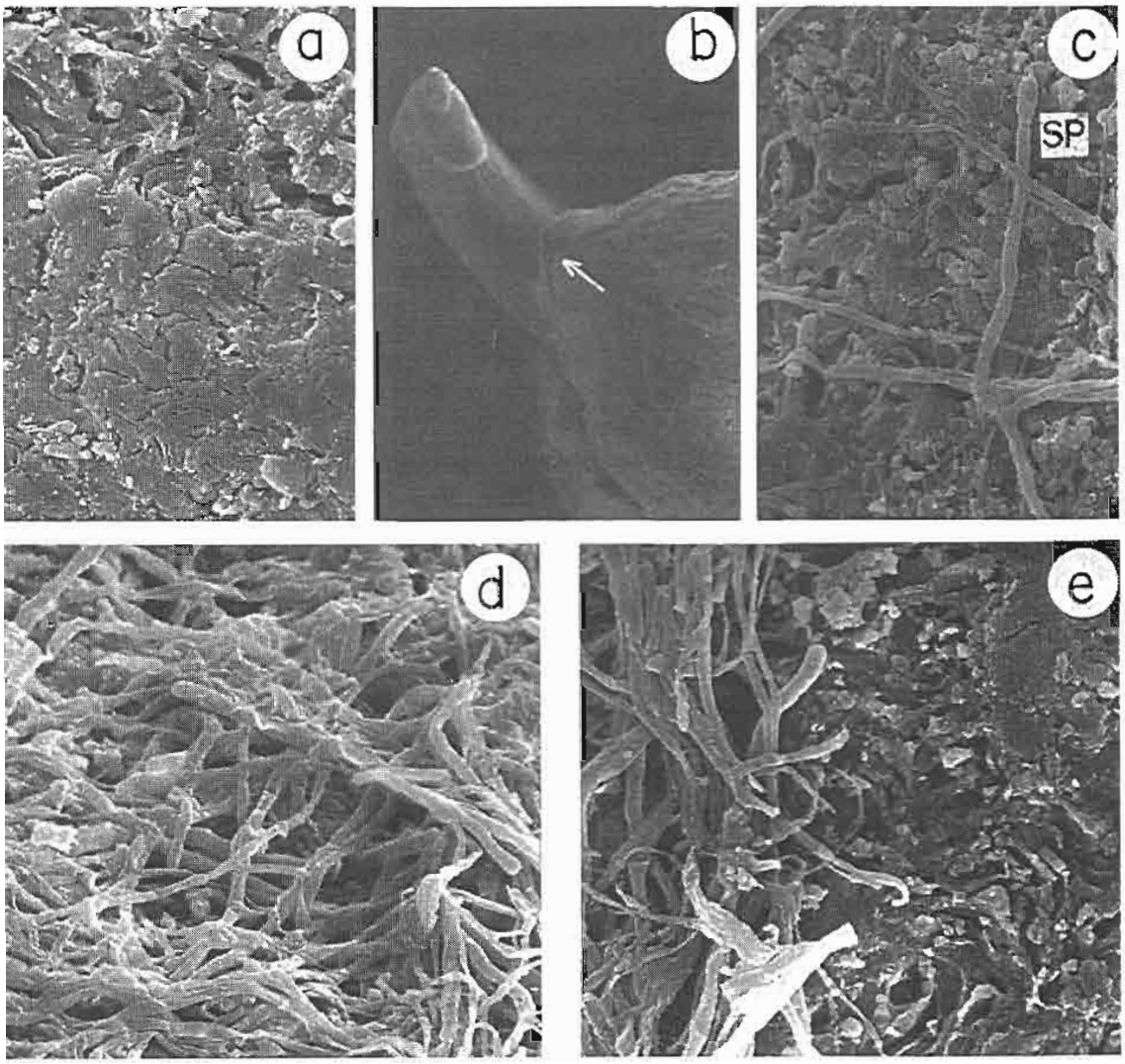


Fig. 1. (a) Scanning electron microscopy of the surface of a healthy cotton seed (× 480). (b) Site of photography (arrow) at the base of the emerging stem (× 32). (c) Germinating spores on seed surface (× 800). (d) Mycelia on seed surface, 48 h after inoculation, in inoculated areas (× 800). (e) Mycelia on seed surface, 48 h after inoculation, in non-inoculated areas (× 800)

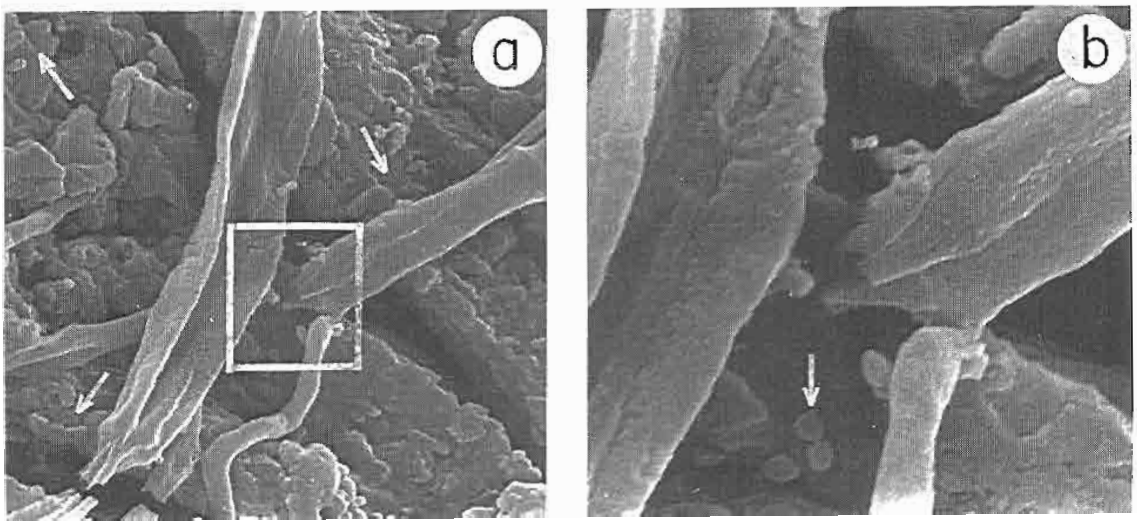


Fig. 2. (a) Penetration of mycelium into the seed (arrows indicate saprophytic bacteria) (× 4,000). (b) Penetration of mycelium into the seed (arrow indicates saprophytic bacteria) (× 10,400)

## Results and Discussion

Mycelium transfer of a phytopathogenic fungus to the upper parts of the plant is a complex event which can occur at least in two modes of action: (a) Internally, by growth of the mycelium inside the germinating seed concomitantly with the young vegetative development; (b) Externally, by growth of the mycelium on the outer surfaces of plant leaves. The mode of fungal transfer of *A. macrospora*, which is seed transmitted (BASHAN 1984, HALFON-MEIRI and COHEN 1983) through the plant is unknown.

Observation of healthy seeds before inoculation revealed neither spores nor cells of saprophytic or pathogenic microorganisms (Fig. 1 a). Immediately after inoculating the seeds with a low concentration of spore suspension, *A. macrospora* spores were rarely seen in either the seeds of the susceptible or the resistant cultivars. Twenty-four hours after inoculation mycelium spreading from sporadic spores was revealed at the base of the emerging stem (Fig. 1 b [arrow] and Fig. 1 C). Thirty-six hours after inoculation the mycelium increased and after 48 h, covered almost the whole photographed site (Fig. 1 d). Uninoculated seed areas were then almost completely covered by mycelia with only few free areas (right side of Fig. 1 e). At that time the mycelium penetrated into the internal seed tissue and a saprophytic population of unidentified bacteria also developed (Fig. 2 a, b). The fungus began to sporulate mainly on cotyledon stems, 72 h after inoculation. One hundred h after inoculation only few spores were formed on the cotyledon leaves (Figs. 3 a, b, c). At this stage there was no difference between the susceptible and the resistant cultivars. This could be explained by the relatively low levels of phenols present in the stems, compared with the leaves (BASHAN 1986 b). No external mycelium was observed on the cotyledons during disease development. The relative resistance of the leaves was temporary — with time, leaves of the susceptible cultivar were infected and those of the resistant

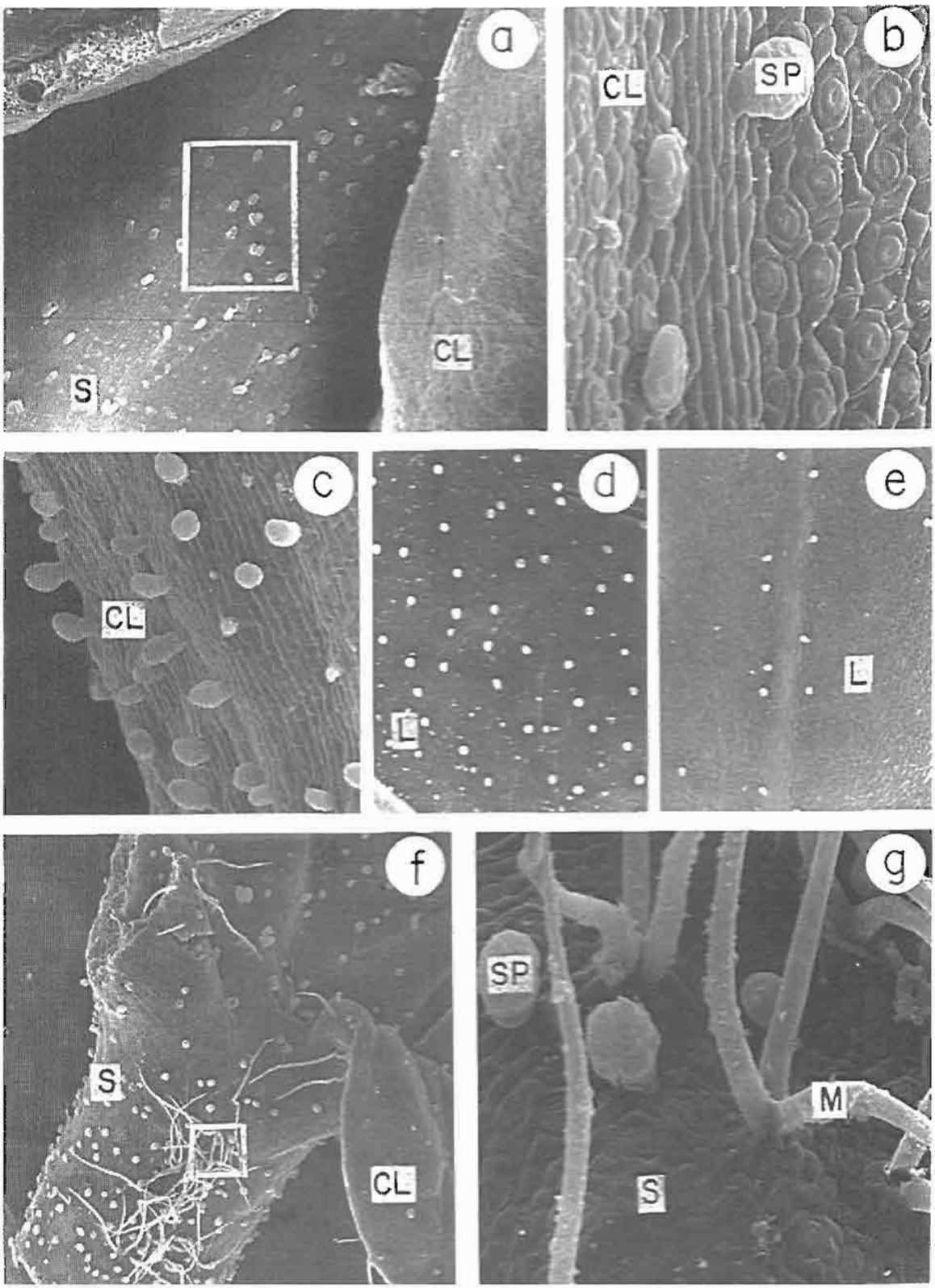


Fig. 3. (a) Sporulation on cotyledon stem (× 64). (b) Sporulation on cotyledon leaf (× 400) 72 h after inoculation. (c) Insert in Fig. 3a shows the location of Fig. 3c (× 240). (d) Sporulation on susceptible cotton leaves cv. 'Pima', 120 h after inoculation (× 64). (e) Sporulation on resistant cotton leaves cv. 'Acala', 120 h after inoculation (× 48). (f) Secondary infections in a susceptible plant (× 48). (g) Insert in Fig. 3f shows the location of Fig. 3g (× 520)

cultivar continued to show small amounts of both internal mycelium and negligible sporulation. One hundred and twenty h after inoculation there was an intensive sporulation on cotyledon leaves of cv. 'Pima', susceptible, (2200 spores/cm$^2$ leaf area), whereas only light sporulation was observed on cotyledon leaves of cv. 'Acala', resistant, (300 spores/cm$^2$ leaf area) (Fig. 3 d, e). At the same time secondary infections developed in the susceptible cultivar originating from internal mycelium of infected stems (Figs. 3 f, g). However, spores were almost invisible on the surfaces of the severely necrotic area in cv. 'Pima', whereas the surrounding tissue contained large numbers of spores (Fig. 4 a, b). In both cultivars it was noticed that the fungus grew from the seeds to the foliage. Sporulation in the underground portion of the cotyledon was very limited (Fig. 4 c) while intensive sporulation occurred on plant foliage (Fig. 4 d). This facilitated fungal spore distribution (BASHAN 1986 a) which resulted in secondary infections observed only in the susceptible cultivar.

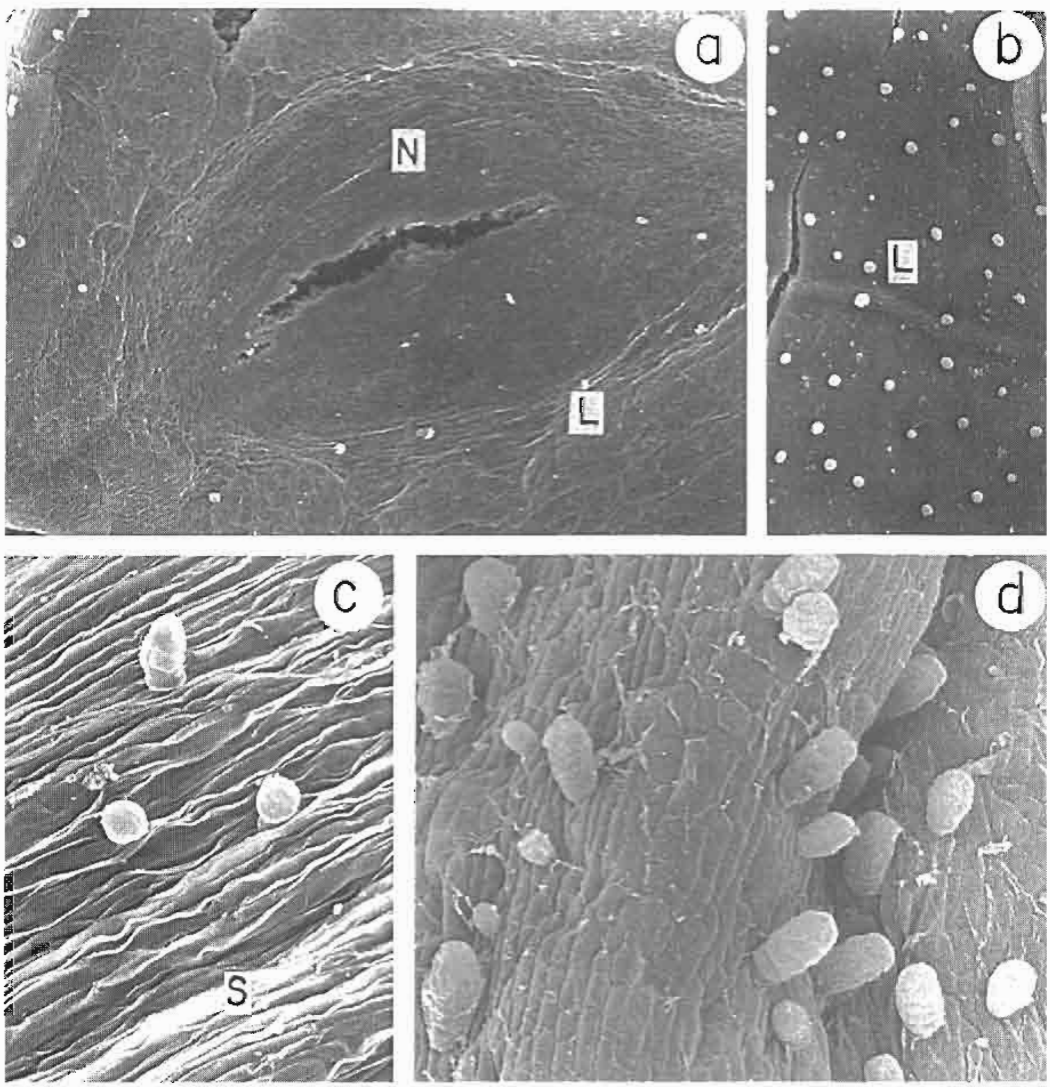


Fig. 4. (a) Sporulation in the necrotic spot in a susceptible plant (× 48). (b) Sporulation close to the necrotic spot (× 48). (c) Sporulation at area of emergence from soil (× 320). (d) Sporulation 3 cm above soil surface (× 320)

Light microscopy of the colonization process of *A. macrospora* in leaves implied that leaf colonization may be a result of either growth of the mycelium in the intercellular spaces of leaves without internal sporulation or from the germination of surface spores (Fig. 5 a). Penetration into the tissue occurred through open stomata (Fig. 5 b). When the leaf was fully colonized by the fungus some mycelium broke out to the outer surfaces through the stomata (Fig. 5 c). Removal of surface propagules by cellulose acetate film revealed that *A. macrospora* sporulation occurred ultimately on the leaf surface and that mycelium was concentrated inside the leaf tissue (Fig. 5 d).

Hydrolyzing the chitin fragment of the diseased plants and measuring glucoseamine revealed that there were differences in the behaviour of the fungal material. The amount of fungal material, which is higher in stems compared with leaves of both susceptible and resistant plants, did not change in stems for the first

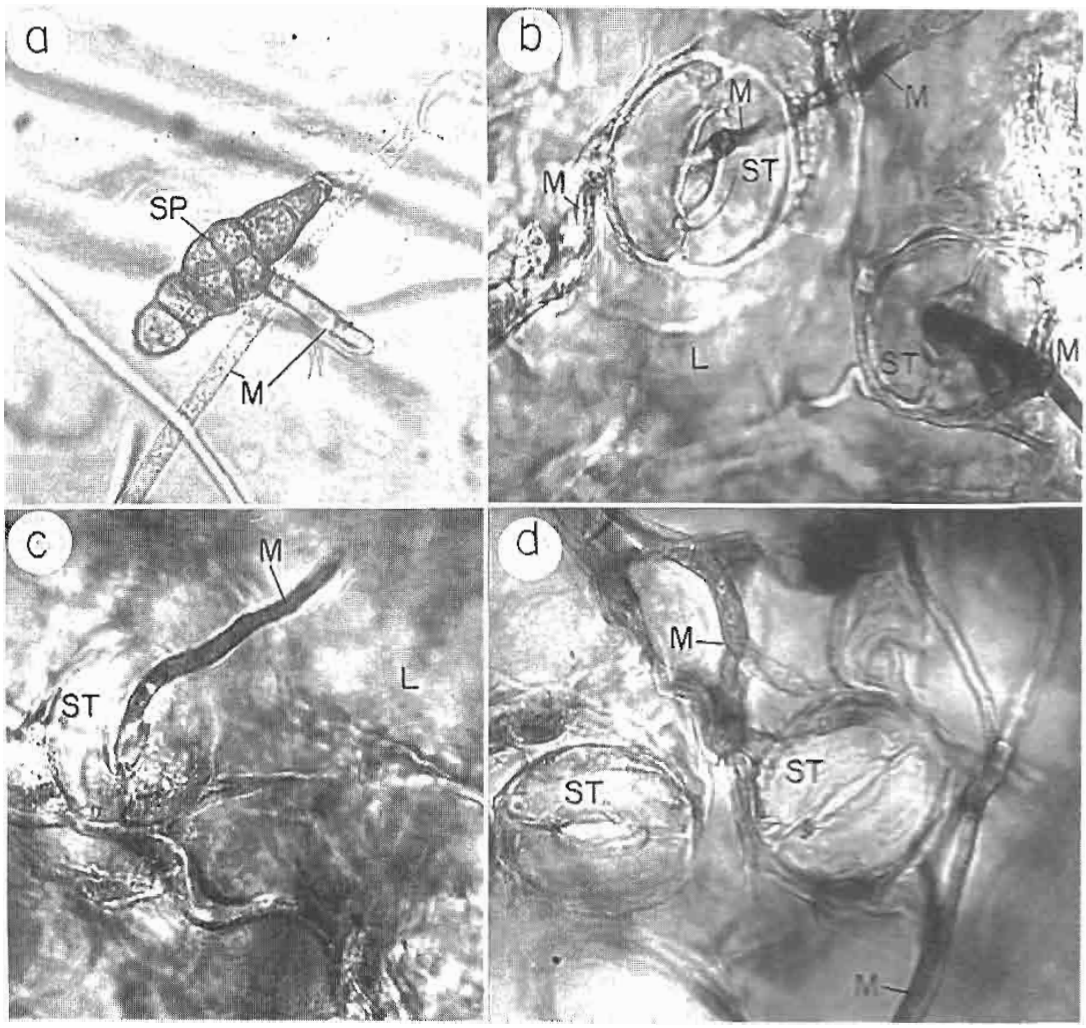


Fig. 5. Light microscopy of the colonization of susceptible cotton leaves, cv. 'Pima', by *A. macrospora*.

(a) Germinating spore of *A. macrospora* (× 700). (b) Penetration of mycelium through open stomata (× 700). (c) Emergence of internal mycelium to leaf surface through open stomata (× 700). (d) Colonization of intercellular spaces by mycelium. The surface propagules were removed by a cellulose acetate film (× 700)

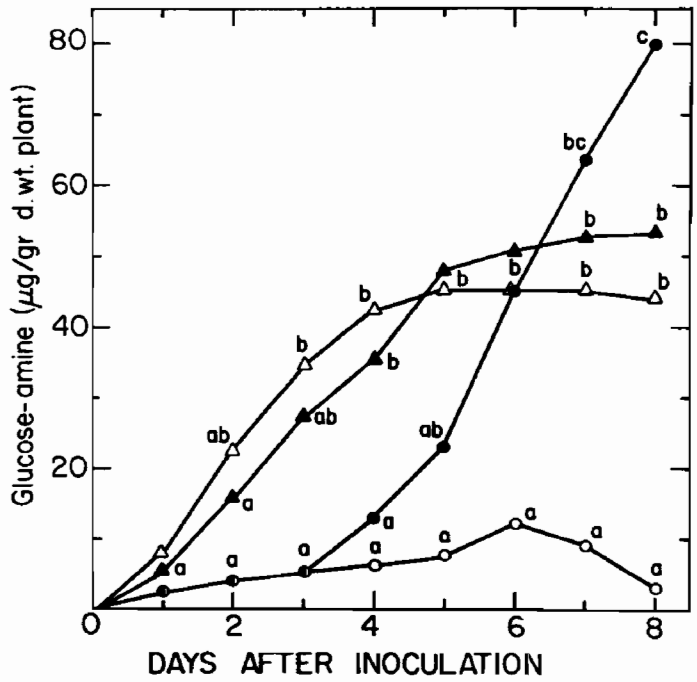


**Fig. 6.** Changes in fungal material in resistant and susceptible plants during disease development from inoculated seeds. ● — susceptible leaves; ○ — resistant leaves; ▲ — susceptible stems; △ — resistant stems. Points on the graph followed by different letters differ significantly at $P \leq 0.05$

six days of disease development. A slight increase in fungal material was observed in susceptible plants later on. The amount of glucoseamine in the leaves was very low for the first three days after inoculation. Afterwards the amount of fungal material increased sharply in susceptible leaves but remained constant or even decreased in the resistant ones (Fig. 6).

By analyzing data from this study and from others (BASHAN 1984, 1986a, 1986b, BASHI 1983, BASHI *et al.* 1983, HALFON-MEIRI and COHEN 1983) it can be concluded that the fungus has a potential ability to pass from infected seeds to seedlings, and that diseased seedlings, found early in the season, originated mainly from infested seeds and not from plant debris *via* air-borne spores.

**Abbreviations:**

S — cotyledon stem; CL — cotyledon leaf; M — mycelium; N — necrotic area; SP — *A. macrospora* spores; L — plant leaf; ST — stomata

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