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Transmission of *Alternaria macrospora* in Cotton Seeds

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Abstract

Alternaria macrospora was isolated from seeds only after the natural opening of the bolls and exposure of seeds to an environment in which the fungus was present. The fungus lacks the ability to penetrate the boll wall and reach the seed site. Attempts to isolate the pathogen from seeds of immature bolls at different developmental stages failed. Internal infection by slow injection resulted in seed infection and partial shedding of the injected plant parts which was high in buds and decreased with the ripening to mature bolls. Severity of seed infection was not dependent on either inoculum level, boll physiological age or even if the boll itself was not diseased. Infection of flowers under field conditions caused flower shedding. Naturally infected seeds or inoculated seeds with inoculum levels of 100 spores/ml and above resulted in diseased cotyledons, the incidence of which was, for inoculated seeds, positively correlated with inoculum level. A small difference was observed between cultivars in susceptibility to artificial inoculation at the cotyledon stage. *A. macrospora* survived on commercial cotton seeds and on post-season plants left growing at the field edges. Survival in plant debris under field conditions was minimal and may only have a minor effect on field reinfestation.

Zusammenfassung

Übertragung von *Alternaria macrospora* in Baumwollsamen

Alternaria macrospora wurde nur aus Samen isoliert, nachdem sich die Samenkapseln auf natürlichem Wege geöffnet hatten und die Samen dann in eine Umwelt, in der der Pilz vorhanden war, ausgesetzt wurden. Dem Pilz fehlt die Fähigkeit, die Wand der Samenkapsel zu durchdringen, um an den Sitz des Samens zu gelangen. Versuche, den Erreger von Samen aus unreifen Samenkapseln zu verschiedenen Entwicklungsstadien zu isolieren, schlugen fehl.

Eine innere Infektion durch eine langsame Injektion führte zu einer Sameninfektion und zu einem teilweise Abwerfen der eingespritzten Pflanzenteile, die große Knospen besaßen, und nahm bei zunehmender Ausreife der Samenkapseln ab. Die Schwere der Sameninfektion war weder abhängig von der Dichte des Inokulums, dem physiologischen Alter der Samenkapsel noch davon, inwieweit die Samenkapsel selbst nicht erkrankt war. Unter Feldbedingungen bewirkte die Infektion der Blüten ihr Abwerfen. Natürlich infizierte Samen oder inokulierte Samen mit einer Inokulumdichte von 100 Sporen/ml und darüber führten zu erkrankten Kotyledonen. Für inokulierte Samen war diese Wirkung positiv korreliert mit der Inokulumdichte. Zwischen den Kultivaren wurde ein geringer Unterschied in der Anfälligkeit bei der künstlichen Inokulation im Kotyledonenstadium beobachtet. *A. macrospora* überlebte auf Handelssaatgut der Baumwolle und auf nach der Saison übriggebliebenen Baumwollpflanzen, die an den Feldrändern weiterwuchsen. Das Überleben in Pflanzenresten war unter Feldbedingungen minimal und kann nur einen geringen Einfluß auf den erneuten Befall des Feldes haben.

Alternaria blight of cotton caused by *Alternaria macrospora* Zimm. is one of the main cotton diseases in Israel (HADAS and JAKOBY 1981). This fungus has been detected in many countries, but little information on its survival and transmission is available (C.M.I. 1970). The disease is considered to be a seedling disease (EBBELS 1980). Field observations in Israel have indicated that although it is initiated mainly during the seedling stage, plants are also infected at all stages of growth (BASHI *et al.* 1982, 1983). However, it is not yet clear how seedlings become infected at a time when the inoculum in the field is relatively small. The possibility of the disease being seed-borne and the infected seeds initiating the disease has not been demonstrated.

The purpose of this study was to established whether *Alternaria macrospora* is transmitted by seed and to detect the timing of infection of cotton seeds.

Materials and Methods

Living material and experimental design

Diseased cotton leaves were collected and freeze-dried for long-term survival of the pathogen. The fungus was isolated on synthetic Czapek medium (C.M.I. 1968), supplemented with 250 mg/l chloramphenicol. The fungal colonies developing from the dried leaves were identified as *A. macrospora* by a pathogenicity test (2 plants were sprayed with spore suspensions of 15,000 spores/ml, and incubated under partial mist conditions, 5 sec mist every 30 min at 30 ± 2 °C for 5 to 8 days) and by examination of cultures containing spores so that these could be compared with the description of this species (C.M.I. 1970). Spore formation was induced by exposing cultures, grown for 10 days in the dark on Czapek medium at 30 °C, to 5 min sunlight daily for 6 successive days. Spores were collected from the Petri dishes, washed in 0.06 M phosphate buffer solution (pH 6.0) and resuspended in the same buffer containing 0.05 % agar for use as inoculum.

Cotton plants (*Gossypium barbadense*) cv. Pima and *G. hirsutum* cv. Acala (considered as susceptible and resistant, respectively, HADAS and JAKOBY 1981) were used in all experiments.

There were 5 to 10 replicates for each experiment and each experiment was repeated 2 to 3 times. Five plants, 30 seeds or 25 bolls or flowers were considered as a replicate.

Infection of seeds following spray inoculation of flowers and bolls at different developmental stages

Flowers and bolls at different developmental stages on field grown plants of cv. Pima and glasshouse grown plants of cultivars Pima and Acala were inoculated (100 plant parts each) by gentle spraying with a spore suspension (12,000 spores/ml) until run-off. Inoculation at the flowering stage and afterwards was carried out on: (a) buds, (b) open flowers, (c) flowers which had been fertilized the day before, (d) bolls smaller than 1 cm and (e) bolls larger than 3 cm. To restrict inoculation to the flowers and bolls, a plastic plate with a hole the size of the peduncle was used to protect the rest of the plant from the spray. Each flower and boll was marked. Inoculation of field grown plants at a similar stage of maturity was performed at night during heavy dew. For the glasshouse experiment plants were grown singly in pots containing 10 kg sandy loam soil of Rehovot under controlled conditions (30 ± 2 °C, 12 h light and 12 h darkness) and inoculated 35 days after emergence following exposure for 3 h to 5 s mist every 20 min. Pots were then transferred to the same controlled glasshouse conditions. Seed infection in the previously inoculated bolls and in bolls formed from previously inoculated buds and flowers was investigated in both open and closed bolls present 50 days after inoculation of field plants and 72 days after inoculation of glasshouse grown plants. Surface disinfection, in bolls was performed by immersing in 98 % ethanol for 5 to 10 s and flaming the surface in a laminar flow hood. Seeds were extracted aseptically from the bolls, put on Czapek medium and treated as described in the routine spore formation method. Shedding of each inoculated plant part was generally measured 15 days after inoculation.

Seed infection by slow injection into plant parts

Plants of both cultivars, grown in pots under controlled greenhouse conditions, were injected with spore suspension into the following plant part bases in order to detect events occurring in the plant as a result of "internal disease": peduncles of bolls larger than 5 cm in diameter and bolls smaller than 1 cm in diameter; peduncles of open flowers and closed buds; petioles of leaves, and stem apices (3 cm from the tip). To achieve a slow release of the spore suspension into the tissue, a soft rubber tube (3 mm internal diameter, 10 cm length) sealed at one end and attached to a hypodermic needle (27G × 1) at the other end (a clip sealed the needle end) was filled with 1 ml of twice washed spore suspension (100 or 1000 spores/ml) under gentle pressure as indicated by the swelling of the tube. After insertion of the hypodermic needle into the plant part, the clip was removed allowing the suspension to diffuse slowly into plant tissue for 6—7 h.

After inoculation plants were incubated for 10 days in an isolated controlled growth chamber (25 ± 3 °C, 12 h light, 12 h darkness, disinfected with successive solutions of 0.5 % $KMnO_4$ and 11 % NaOCl, thus, avoiding the possibility of external natural infection and thoroughly washed with tap water). Infection was determined by isolation of the fungus in a sample of 30 seeds or in 30 plant parts following surface disinfection.

Isolation of *A. macrospora* from flowers, bolls, peduncles and seeds of field grown plants in the beginning and at the end of the growing season

Isolation of *A. macrospora* from immature naturally infected plant parts after surface desinfection in the beginning of the summer season (60 to 80 days after emergence) was from: (a) large bolls (5—6 cm in diameter) infected in more than 10 spots per boll, (b) large bolls infected in 2 to 3 spots per boll, (c) small infected bolls (1—2 cm in diameter), (d) infected bolls smaller than 1 cm in diameter, (e) flowers which had been fertilized the day before and (f) peduncles of infected bolls. Each sample contained 25 plant parts and 4 seeds were aseptically removed from each boll. In order to detect systemic invasion of the pathogen into peduncles and bolls, naturally infected bolls were collected from 3 commercial fields (3 samples, 30 bolls or peduncles, 4 seeds per boll were tested). At the end of the growing season isolation of the fungus was from: naturally infected closed

small (smaller than 1 cm in diameter) and large (greater than 4 cm in diameter) bolls; naturally infected bolls which had just opened and open bolls; infected peduncles of both sizes of bolls and non-infected closed and open large and small bolls. After surface disinfection followed by aseptic removal of either the seeds from the bolls, or the vascular system from the peduncles, seeds and peduncles were placed on Czapek agar medium and inspected daily.

Transmission of disease from infected seeds to seedlings

Disease transmission to seedlings was studied using inoculated seeds and also naturally infected seeds of cultivars Pima and Acala. Seeds to be inoculated were surface disinfected by dipping in 1 % NaOCl solution for 5 min followed by 3 successive washings in sterile distilled water and then soaked in spore suspensions of 10—10,000 spores/ml for 60 min.

Inoculated or naturally infected seeds were then sown (1 cm depth) in autoclaved vermiculite (30 seeds per replicate, 5 replicates for each inoculum level) and germinated in partial mist conditions (5 sec mist every 30 min, only at night, 25 ± 3 °C) for 10 days. Symptom appearance was recorded and, if necessary, isolation of the fungus from the cotyledons was attempted.

Survival of *A. macrospora* in post-season plants and plant debris

Four months after harvesting and ploughing, plant material (138 samples) was collected from commercial cotton fields in 3 regions in Israel (Bet-Shean Valley, Sharon and Rehovot regions). Open bolls, closed bolls and stems were taken from buried plants, dead plants on the soil surface and living plants still growing at the edges of the fields. Plants touching the soil were 50—70 % degraded. After removal of seeds from the bolls, half the samples were surface disinfected with ethanol as previously described and half left untreated. Each plant portion was then weighed and homogenized for 3 min in 50 ml sterile 0.06 M phosphate buffer (pH 6.0), cooled under an ice bath using an Omni-mixer (Sorvall) disinfected between portions by flaming with alcohol. The homogenates were serially diluted in the buffer and spread on Czapek medium with a glass rod. Plates were incubated at 30 °C for 120 h and numbers of colonies of *A. macrospora* recorded.

Presence of *A. macrospora* in commercial seed samples

At the end of the growing season, 11 seed samples (6 of cv. Pima and 5 of cv. Acala) were collected from growers' stores. Five lots (250 g) of each sample were vigorously shaken in 200 ml sterile 0.06 M phosphate buffer (pH 6.0) for 2 h. The seeds were then removed, the suspension filtered through cheese-cloth and centrifuged at 10,000 g for 10 min. The pellet was resuspended in buffer, serially diluted and 0.1 ml aliquots placed on dishes of Czapek medium and spread with a glass rod. After incubation at 30 °C for 120 h, the number of *A. macrospora* colonies on each dish was recorded.

Results

Infection of seeds following spray inoculation of flowers and bolls at different developmental stages

Seed infection occurred only when the boll was naturally open by the time of sampling (Fig. 1). The physiological stage at which the boll was infected had no effect on seed infection. When the entire plant was visibly diseased, probably as a result of secondary infections throughout the season, seeds were also infected at the end of the season (even if in some cases the boll itself was not infected). No difference was noted in timing of the natural

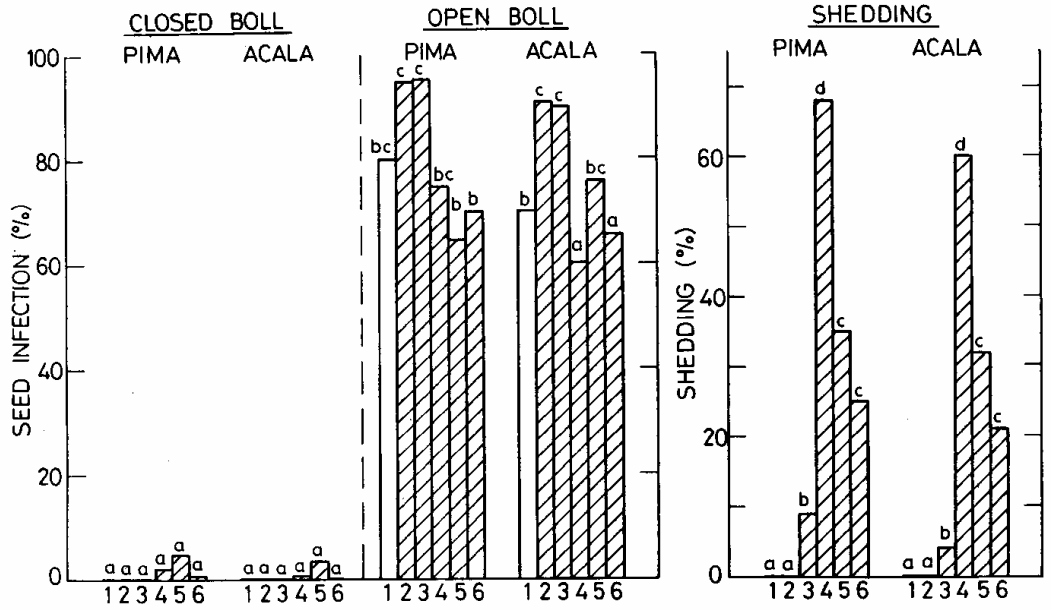

Fig. 1. Effect of inoculation with *Alternaria macrospora* at different stages on seed infection and shedding. 1. not inoculated; inoculation of: 2 — large bolls; 3 — small bolls; 4 — flowers which had been fertilized the day before; 5 — open flowers; 6 — buds. Data for field grown and greenhouse grown plants was combined. Histograms followed by different letters in each sub-figure differ significantly at $P \leq 0.05$

opening of bolls of diseased and healthy plants. A high level of seed infection in non-inoculated control plants was observed in the field, demonstrating the role of natural inoculum causing secondary infections. When plants were grown in a disinfected isolated growth chamber, thus avoiding the possibility of such infections, the seeds were not contaminated with the pathogen either at the time of natural opening of the bolls or later. Infection of flowers caused flower shedding.

Seed infection by slow injection into plant parts

Preliminary observations and isolations did not show internal boll infection by *A. macrospora* in commercial surface infected closed bolls.

No visible symptoms appeared in any of the injected plant parts 10 days after inoculation. However, the fungus could be isolated from both shed and non-shed parts after surface disinfection. The main phenomenon observed after injection was the shedding of the injected parts (Table 1). This was high in buds, decreased where bolls had been inoculated and was least with inoculated leaves and growing tips. The high inoculum level caused the most marked effects. Bolls internally infected had infected seeds. Where comparisons were possible, the percentage of infected seeds in non-shedding bolls was much lower than that of the shed bolls. Severity of seed infection was not dependent on either inoculum level or boll developmental stage. Internally infected bolls were usually degenerative compared with non-internally infected bolls and a high percentage of them (88 %) did not open at the end of the season. The phenomena of seed infection and degeneration of internally infected bolls were

Table 1
Seed infection and shedding following slow injection of *A. macrospora* into various plant parts

Inoculated plant part	Shedding %			Infected seed %				
					100 spores/ml		1000 spores/ml	
	Sterile water	100 spores/ml	1000 spores/ml	Sterile water	in shedding plant parts	in non-shedding plant parts	in shedding plant parts	in non-shedding plant parts
Open flowers	0	40a	70c*	0	ND	80b	ND	82b
Closed buds	0	90b	95d	0	ND	85b	ND	85b
Large bolls	0	0	20b	0	80a	25a	85a	20a
Small bolls	0	0	25b	0	90a	20a	75a	25a
Leaves	0	0	10a	0	ND	ND	ND	ND
Growing tips	0	0	10a	0	ND	ND	ND	ND

* Numbers followed by different letters in the same column differ significantly at $P \leq 0.05$. Results are the average of three experiments.
ND — not determined.

observed also in commercial cotton fields. However, it must be emphasized that the percentage of naturally internally infected bolls was low (2.5 and 0.5 % of bolls in 2 separate samplings of 250 bolls).

Isolation of *A. macrospora* from flowers, bolls, peduncles and seeds of field grown plants in the beginning and at the end of the growing season

Isolation of the fungus from the surfaces of all plant parts in the beginning of the growing season (see list in Materials and Methods) was relatively easy (82 % ± 8 of samples from each part contained propagules of the fungus). However, it was not possible (4 experiments) to isolate the fungus from the inner plant parts even though the immature seeds were incubated on the growth medium for 20 days.

From all plant parts at the end of the growing season (see list in Materials and Methods) only seeds from open bolls or recently opened bolls were infected (95 % and 88 %, respectively). However, visible surface infection (more than 10 spots per boll) was observed in all bolls.

Transmission of disease from infected seeds to seedlings

Seedlings became infected with inoculum concentrations of 100 spores/ml and above (Fig. 2). However, some of the disease symptoms were not typical and confirmation of disease could be obtained only by isolating the fungus from each of these seedlings.

Small differences were observed between the two cultivars in disease susceptibility (Fig. 2). There were direct positive correlations between disease incidence and inoculum levels between 10 and 1000 spores/ml ($r = 0.92$ for

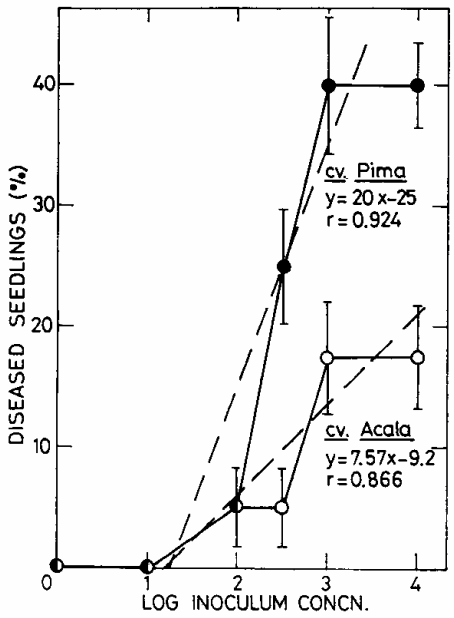

Fig. 2. Relationship between seed inoculum level and seedling disease. Bars represent standard error; — — — regression line

cv. Pima and r = 0.86 for cv. Acala). When plants were maintained under the same conditions for an additional 10 days 79 % and 84 % of Acala and Pima plants, respectively, were infected, probably because of secondary infections.

In an additional trial, disease transmission occurred from seeds of both cultivars obtained from naturally surface-infected open bolls. With the cv. Pima 23.2 % of the seeds produced diseased seedlings and with cv. Acala 18.4 %.

Table 2
Survival of *A. macrospora* in bolls and stems from post-season plants, and dead plants on the soil surface or buried in the soil

Plant part	Condition of plant when found	Mean number *A. macrospora* propagules/g tissue		Infected seeds (%)
		natural	disinfected	
Open bolls	Post season plants	1200b*	42b	22c
	Buried plants	23a	0a	0.5a
	Plants on soil surface	42a	18b	8b
Closed bolls	Post season plants	1050b	0a	0a
	Buried plants	63a	0a	0a
	Plants on soil surface	51a	0a	0a
Stems	Post season plants	1420b	0a	ND
	Buried plants	0a	0a	ND
	Plants on soil surface	10a	0a	ND

* Numbers followed by different letters in the same column differ significantly at $P \leq 0.05$.

ND — not determined.

Survival of *A. macrospora* in post-season plants and plant debris

Populations of *A. macrospora* survived on the surface of the growing plants (Table 2). Contamination of seeds occurred only when bolls had opened. The fungal population in plant debris whether on the soil surface or buried after ploughing was markedly lower than in growing plants left at the field edges.

Presence of *A. macrospora* in commercial seed samples

Of the 6 Pima seed batches 3 were contaminated with *A. macrospora* at a mean rate of 303 ± 60 propagules/250 g seeds; whereas only 1 batch of Acala was contaminated with 212 ± 40 propagules/250 g seeds.

Discussion

Alternaria macrospora is one of the most important diseases of cotton seedlings in the tropics. It is particularly prevalent in areas of central Africa cleared from deciduous woodland (EBBELS 1980), as well as in many other countries, mainly in the Far East. It is of less importance in the USA (CHOPRA and SHARMA 1976, FULTON *et al.* 1960, HADAS and JAKOBY 1981, LING 1944, RANE and PATEL 1956, RUSSELL and HINE 1978, SCIUMBATO and PINCKARD 1974).

This study has demonstrated that the disease can be initiated from seeds infected either naturally or artificially, and that the fungus can not penetrate directly through the boll to the seed site. The fungus was capable of infecting the seeds following internal injection of plants with inoculum, but the boll usually degenerated. The main timing of seed infection was after the natural opening of the bolls when the fibres became exposed to the field environment. The main phenomenon following flower infection was shedding, especially in buds previously inoculated by injection into their peduncles. However, as buds ripened the susceptibility to shedding decreased and mature bolls were seldom shed.

Although survival of *A. macrospora* in plant debris, claimed to be the source of inoculum for the next season by BASHI *et al.* (1982), was demonstrated, this appears likely to have only a minor role in field reinfestation. Only plants that remained growing in the field after harvest time had large numbers of propagules. In Israel cotton fields are extensively ploughed at the end of the season and survival of the fungus in buried or partially buried cotton plant debris was found to be minimal. In addition it was very rare to find non-degraded plants on the surface of the soil in the middle of the rainy season. Thus, the importance of survival of the pathogen in decomposing plant materials seems less likely.

In a limited survey the pathogen was found contaminating commercial seed samples. Such inoculum seems likely to be the cause of primary outbreaks of this disease in the field. More information is, however, required on the

levels of infection of *A. macrospora* in seed stocks and on the development of the disease in the field.

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