

3. *PHOMA GLOMERATA*, *P. EUPYRENA* AND *P. CAVA* ON SEEDS OF BLACKTHORN (*ACACIA MELLIFERA* SUBSP. *DETINENS*) AND THEIR PATHOGENICITY ON BLACKTHORN SEEDLINGS

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ABSTRACT

Blackthorn seeds from different regions of South West Africa and the north-western Cape Province were examined for seedborne fungi. *Phoma glomerata* occurred on seeds from all regions, whereas *P. cava* and *P. eupyrena* were detected only on seeds from a farm near Okahandja where natural stands of blackthorn were killed by dieback. The fungi colonized the hypocotyl and radicle. Only *P. glomerata* grew systemically in the hypocotyl and radicle, and caused distinct lesions on seedlings. As infection at the base of the trunk and upper taproot is an outstanding feature of blackthorn dieback, it is concluded that seed transmission plays an important part in transferring inoculum to the appropriate tissue for successful infection.

INTRODUCTION

Dieback, a decline disease of blackthorn [*Acacia mellifera* (Vahl) Benth. subsp. *detinens* (Burch.) Brenan] which has killed thousands of hectares of bushes and trees in the northern parts of South West Africa, is a disease of complex abiotic and biotic origin (Part 1, 2). Four fungi *Phoma glomerata* (Cda) Wollenw. & Hochpf., *P. eupyrena* Sacc., *P. cava* Schulz. and *Cytospora chrysosperma* Pers. ex Fr. were shown to be the primary causal organisms (Part 1, 2). However, aspects of the etiology of the disease are still unclear.

From observations that *P. glomerata* and *P. cava* can infect seedlings naturally and that both fungi can grow systemically in plants (Part 2), it might be suspected that these organisms are seedborne and therefore early inhabitants of blackthorn plants. This possibility was investigated in the following experiments.

MATERIALS AND METHODS

Blackthorn seed stocks

Blackthorn seeds collected from various regions (Fig. 1) in South West Africa (November 1985) and the north-western Cape Province of the Republic of South Africa (December 1986) were examined.

Fungi on blackthorn seeds

Untreated seeds were germinated by plating on water agar (WA) (15g agar/1) in petri dishes (5 seeds/dish, 400 seeds/treatment) and incubated on a laboratory bench at 25±2°C. Fungi developing on testas were identified. Fungal hyphae developing from the seeds were transferred to potato-dextrose agar (PDA) (200g potato, 20g glucose, 15g agar/1) and the organisms identified after 8-9 days' incubation at 25°C in the dark. After 10-12 d seedlings were examined for disease symptoms. Samples

were also taken from radicles for histological studies.

Seed inoculation

Isolates of *P. glomerata*, *P. eupyrena*, *P. cava* and *C. chrysosperma*, obtained from blackthorn bushes showing severe dieback, were grown on PDA at 25°C under intermittent light (black light, 12 h cycles) to promote pycnidium formation. After 3 wks spore suspensions were prepared by adding 5 ml sterile distilled water to plates, dislodging mycelia and pycnidia with a glass rod and homogenizing the fungal structures in a Waring blender. Homogenized suspensions were filtered through layers of sterile muslin. The spores were counted with a haemocytometer and suspensions diluted to approximately 4.5 x 10⁵ spores/ml.

Petri dish inoculations: Seeds were treated for 30 min in a water bath at 50°C to reduce seedborne fungi, cooled in a laminar flow bench and inoculated by dipping for 1 min in a spore suspension. Control seeds were dipped in a sterile distilled water. Treated seeds were dried on sterile filter paper in a laminar flow bench. Four wells with diameters slightly larger than those of the seeds were cut in WA in petri dishes and the treated seeds placed into the wells. This prevented fungi from colonizing the WA rapidly and causing unwanted seedling infection from the WA. After 10 days' incubation at 25±2°C on a laboratory bench, seeds and seedlings were examined for disease symptoms and samples for histological studies taken from radicles.

Glasshouse inoculations: Seeds were disinfested with hot water, inoculated as above, planted in seed trays in a steam-disinfested sand-perlite mixture (4:3) and kept in a glasshouse at 20-25°C (night-day). The seedlings were watered regularly and fertilized once a month with Chemicult. After 10 wks they were removed from seed trays and examined for the presence of lesions and pycnidia. Seedlings were surface disinfested for 1 min in 70% ethanol, the hypocotyl and upper taproot plated on PDA and incubated at 25°C in the dark. Fungi growing from tissue were identified after 10-14 d.

Histology

Samples were fixed in a formalin-acetic acid-alcohol (FAA) mixture (Berlyn & Miksche, 1976), dehydrated in tertiary butyl alcohol (Berlyn & Miksche, 1976) in a Shandon automatic tissue processor and embedded in Paraplast. Embedded material was sectioned at 10-12 µm with a rotary microtome and the sections fixed to slides with chrome alum. Sections were stained with safranin orange/fast green (Sass, 1951) or safranin orange/picro-aniline blue (Cartwright, 1929) and mounted in Entellan.

RESULTS

Fungi on blackthorn seeds

Fungi developing from the different seed lots are recorded in Table 1. *P. glomerata* was the predominant organism developing on the testas and occurred on seeds collected from all the regions. It was isolated at relatively high frequencies from seeds obtained from Okahandja, Bray and Vryburg. *P. eupyrena* and *P. cava* occurred only on seeds obtained from the Okahandja region.

Rupture of the testa was first recorded after 2 d. At that stage hyphae were mainly seen in the hilum area. After 4 d the entire surface of the testa was covered with hyphae. After 10 d radicles of seedlings with testa colonized by *P. glomerata* were discoloured dark brown and some became necrotic. Restricted necrotic spots occurred on the cotyledons of some of the seedlings. Abundant pycnidia occurred on the testas and were found indiscriminately on the discoloured and necrotic radicles. Radicles infected by *P. eupyrena* and *P. cava* were discoloured light brown but lesions appeared superficial.

Germination of seeds from the different seed lots, incidence of discoloured radicles and fungi isolated are given in Fig. 2. Germination was not affected by the incidence of seedborne *Phoma* spp. present on seeds. Seeds obtained from Okahandja, Bray and Vryburg, which showed the highest incidence of seedborne *Phoma*, had the highest incidence of discoloured radicles. *P. glomerata* was the predominant fungus isolated from discoloured radicles. Seedlings with the testa covered by other organisms showed no disease symptoms.

Petri dish inoculations

The different organisms had no distinct effect on seed germination but caused lesions of various types on cotyledons and radicles (Table 2). Lesions on cotyledons were circular, restricted and sunken whereas those on the radicles were elongated and superficial. In the case of *P. glomerata*, radicles eventually became necrotic. Some of the seeds that remained ungerminated were extensively colonized. However, closer inspection showed insect damage of the embryo. No fungal growth or lesions were detected on uninoculated seeds 10 d after inoculation.

Glasshouse inoculations

Seedlings started to emerge 3 d after seeds were planted. The testa either remained under the soil or was clearly visible at soil level in contact with the hypocotyl. Some pycnidia had developed on testa after 19 d. Small restricted lesions were seen on some cotyledons attached to seedlings inoculated with *P. glomerata*.

The effect of the different organisms 10 wks after inoculation is shown in Table 3. No organism caused a reduction of seedling emergence or stand. When lifted, a light brown discolouration was evident on hypocotyls and the upper taproot of most of the seedlings inoculated with *P. cava*, *P. eupyrena* and *C. chrysosperma* and on some of the uninoculated seedlings. More prominent, though superficial lesions were caused by *P. glomerata*. In several cases testas were still attached to the seedlings. Fungi corresponding with those used for inoculation were reisolated from these testas. *P. glomerata* was recovered from one testa attached to an uninoculated seedling.

Seedlings inoculated with *P. glomerata* had the highest incidence of discoloured hypocotyls and taproots. Pycnidia of the fungus occurred primarily on hypocotyls.

The organism was consistently recovered from discoloured hypocotyls, but less frequently from discoloured taproots. No pycnidia were found on seedlings inoculated with the other fungi. *P. cava* was isolated at a low frequency whereas *P. eupyrena* and *C. chrysosperma* were not recovered from any seedling.

Vertical splitting of the epidermis of the hypocotyl in response to increasing stem growth was evident on virtually all the seedlings. Hypocotyl surfaces therefore consisted of peridermal as well as living epidermal areas.

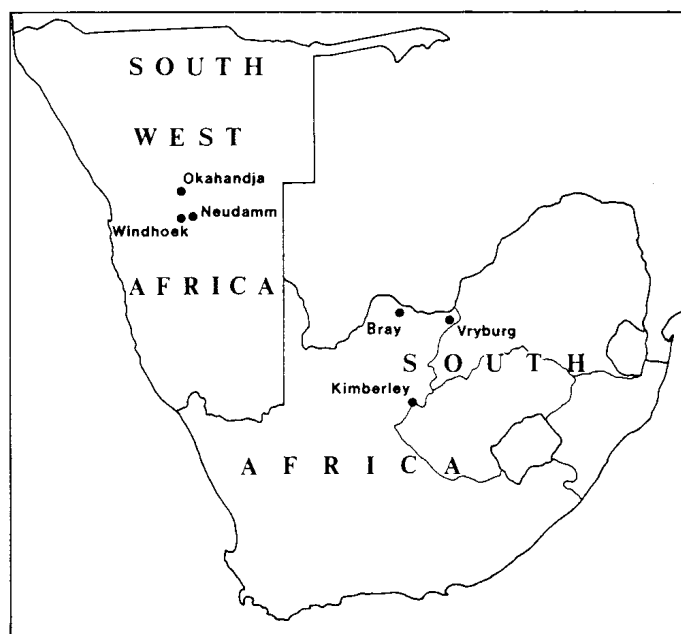


Fig. 1. Localities in South West Africa and the north-western Cape Province of the Republic of South Africa where blackthorn seed were collected for studies on seed transmission.

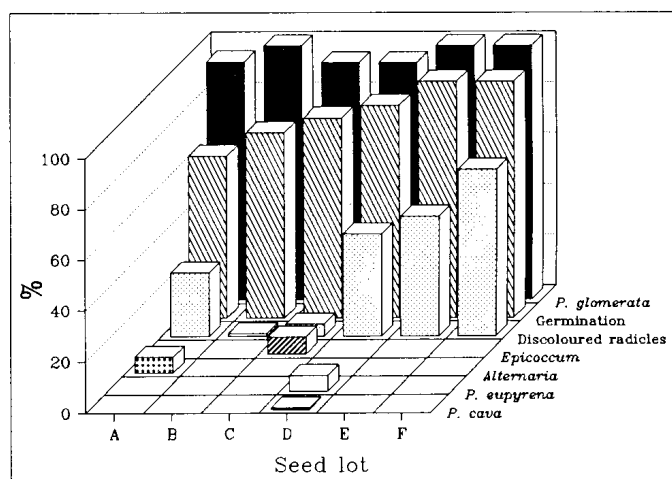


Fig. 2. Germination, discoloured radicles and the incidence of different fungi isolated from discoloured radicles of blackthorn seeds after 10 d incubation at 25°C. Seed lots were obtained from Kimberley region (A), house garden in Windhoek (B), Sonnleiten near Windhoek (C), Mittendorf near Okahandja (D), Vryburg region (E) and Bray region (F).

Table 1 — Seeds of different blackthorn seed lots^a showing fungal growth after 10 d incubation at 25°C:

Organisms	Germinated seeds (%)						Ungerminated seeds (%)					
	Seed lot						Seed lot					
	A	B	C	D	E	F	A	B	C	D	E	F
<i>Phoma glomerata</i>	40.6	4.5	0.9	74.2	52.7	26.6	57.0	4.7	2.5	28.6	42.9	25.0
<i>P. eupyrena</i>	2.7	0	0	0	0	0	0	0	0	0	0	0
<i>P. cava</i>	0.3	0	0	0	0	0	0	0	0	0	0	0
<i>Penicilium</i> sp.	2.1	4.1	2.3	1.1	2.2	3.1	4.6	4.7	1.2	0	0	8.3
<i>Alternaria</i> sp.	4.5	0	0.9	1.1	0	4.7	4.6	0	0	0	0	2.8
Other fungi ^b	16.1	7.6	21.9	0	16.1	25.0	30.8	3.5	14.8	14.3	14.2	30.6
No growth	33.7	83.8	74.0	23.6	29.0	40.6	3.0	87.1	81.5	57.1	42.9	33.33

^aSeed collected from: A = Mittendorff near Okahandja; B = Sonnleiten near Windhoek; C = house garden in Windhoek; D = Bray region; E = Vryburg region; F = Kimberley region.

^bSpecies of *Epicoccum*, *Aspergillus*, *Pseudodiplodia*, *Fusarium*, yeasts and sterile fungi.

Table 2 — Effect of *Phoma glomerata*, *P. eupyrena*, *P. Cava* and *Cytospora chrysosperma* on surface disinfested blackthorn seeds^a 10 d after inoculation in petri dishes^b:

Organisms	Germination (%)	Seedlings (%) with lesions on	
		Cotyledons	Radicles
<i>P. glomerata</i>	86.1	25.8	77.4
<i>P. eupyrena</i>	86.1	9.7	67.7
<i>P. cava</i>	86.1	0	58.1
<i>C. chrysosperma</i>	88.9	12.5	46.9
Control	86.1	0	0

^aFour seeds/petri dish, 36 seeds/treatment.

^bIncubated at approximately 25°C on a laboratory bench.

Table 3 — Effect of *Phoma glomerata*, *P. eupyrena*, *P. cava* and *Cytospora chrysosperma* on blackthorn seeds disinfested with hot water 10 wks after being inoculated in a glasshouse^a:

Organism	Emerged seedlings (%)	Seedlings (%) with lesions on		Recovered (%) from	
		Hypocotyls	Radicles	Hypocotyls	Radicles
<i>P. glomerata</i>	92.1	96.6	79.3	100	79.6
<i>P. eupyrena</i>	88.9	53.6	32.1	0	0
<i>P. cava</i>	85.7	13.0	22.5	5	7.5
<i>C. chrysosperma</i>	96.8	63.8	23.4	0	0
Control	84.1	3.9	6.8	0	0

^aSeeds (63/treatment) planted in a sand-perlite mixture, seedlings kept at 20-25°C (night and day temperatures).

Histology

Septate hyphae were observed in the cortex, between the tightly-packed cells of the deeper cortex and sometimes the vascular cambium of radicles from seedlings har-

bouring *P. glomerata* on testas, but with no macroscopic symptoms of infection. In certain cases intercellular hyphae reached the xyleum. In necrotic radicles, massive hyphal invasion of host cortical cells and considerable host cell collapse were noted. Dictyochlamydo spores

occurred in the collapsed epidermis and immediately adjacent cortex. Superficial and semi-immersed pycnidia were also noted.

On seedlings inoculated in petri dishes, hyphae of the different fungi were confined mainly to the epidermal cells. Walls of host cells adjacent to the hyphae appeared thickened and stained an intense red with safranin and fast green. Hyphae of *P. glomerata* were occasionally seen between the large cells of the cortex, whereas penetrating hyphae of *P. cava* were seen in the cortex of only one radicle. No further invasion was noted. Pycnidia of *P. glomerata* were frequently found under the epidermal cells and caused the epidermis to lift. Those of the other fungi were superficial.

DISCUSSION

The present study has shown *P. glomerata*, *P. cava* and *P. eupyrena* to be seedborne and to colonize hypocotyls and radicles of blackthorn seedlings. *P. glomerata* formed abundant pycnidia on the testa of the seeds from all the regions and, as shown previously (Part 1, 2), appears to be ubiquitous on blackthorn. However, it is also able to infect seedlings naturally, causing either superficial or more distinct lesions on the hypocotyl and radicle. This finding confirms a previous observation that *P. glomerata* might be seedborne and could cause seedling death (Part 1).

It appears that *P. glomerata* colonizes principally the testa. During germination inoculum close to the hilum develops further into the embryo. In preparatory work (data not shown), seedlings remained uncolonized when testas harbouring pycnidia of *P. glomerata* were removed at an early stage after germination. However, the presence of the fungus in hypocotyls and the tap root of seedlings emerging from hot water-treated seeds indicates internal infection of some seeds.

On naturally-infected seedlings, hyphae of *P. glomerata* occasionally penetrated deeply into the cortex of the radicle but caused virtually no cell damage. This shows that the fungus might grow systemically in blackthorn tissue, as was suggested earlier (Part 2). At least three other closely related species, *P. tracheiphila* (Perrotta, Magnano Di San Lio & Bassi, 1985), *P. tracheiphila* f. sp. *chrysanthemi* (Baker *et al.*, 1985) and *Leptosphaeria maculans* (Nathaniels & Taylor, 1983; Hammond, Lewis & Musa, 1985) also have a systemic phase.

Although *P. cava*, *P. eupyrena* and *C. chrysosperma* colonized the radicle of artificially inoculated seedlings, they did not penetrate any further than the epidermis. Host cells reacted heavily to their presence, as illustrated by the intense red staining of cortical cells bordering the epidermis. Only on one seedling was *P. cava* seen to penetrate into the cortex of the radicle. The fact that they could be killed by surface disinfestation showed that they occurred only superficially on these seedlings. Previous work showed that *P. eupyrena* and *C. chrysosperma* are unable to attack young actively-growing blackthorn seedlings and can enter older plants only through wounds (Part 2).

Fungal isolates used in the study could possibly have lost pathogenicity during culturing or might be less aggressive than those occurring on naturally infected plants. *Phoma* is considered a highly variable fungus (Doren-

bosch, 1970; Boerema & Dorenbosch, 1973; White & Morgan-Jones, 1984) and fresh isolates of *P. exigua* var. *foveata* generally produced larger lesions on potato tubers than stored isolates (Bain, Lennard & Wastie, 1982). In brassica seed crops both virulent and non-virulent strains of *Leptosphaeria maculans* (*P. lingam*) occur. Those in the virulent group caused spreading lesions and a severe dry rot on stem and root tissue of seedlings, whereas those in the second group caused elongated lesions that remained confined to the superficial layers of cells and did not noticeably affect plant vigour (Humpherson-Jones, 1983, 1985; Hammon & Lewis, 1987). The formation of corresponding lesions types by this fungus (this study and Part 2) suggests that *P. glomerata* might behave in the same way.

The occurrence of *P. cava*, *P. eupyrena* and *P. glomerata* on seeds from a region seriously affected by blackthorn dieback might be of great importance to the etiology of the disease. Previous work suggested that *P. glomerata* might act as a primary colonizer predisposing tissue to infection by *P. cava*, *P. eupyrena* and *C. chrysosperma* (Part 2). The susceptibility of the radicle to colonization is apparently of significance to the *Phoma* spp. as the testa is not lifted from the soil during germination. The long attachment to the hypocotyl of cotyledons harbouring *P. glomerata* and the vertical splitting of the epidermis at the hypocotyl provide further means of assisting colonization of the hypocotyl (lower stem). Inoculum of some of the fungi may therefore be ideally situated for infection when tissues are damaged or plants subjected to stress.

Infection at the stem or trunk base and the upper taproot was previously correlated with blackthorn dieback (Part 1).

Other species of *Phoma* are well-known seedborne pathogens and the importance of seed transmission in disease development is well-documented (Neergaard, 1977; Domsch, Gams & Anderson, 1980). Seed transmission also plays an important role in tree diseases like *Marssonina brunnea* on poplar (Spiers & Wenham, 1983) and black seed rot (*Diplodia gossypina*) and pitch canker (*Fusarium moniliforme* var. *subglutinans*) of pine (Miller, 1984; Anderson, 1986).

Although the real influence of the different *Phoma* spp. on seedlings is not clear from this study, the results suggest that seed transmission could play an important role in disease development and help with the spread and survival of the fungi. It will be important to determine to what extent they have to colonize seedlings and if plants should be stressed before disease symptoms will develop.

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REFERENCES

- ANDERSON, R.L. 1986. New Method for assessing contaminations of slash and loblolly pine seeds by *Fusarium moniliforme* var. *subglutinans*. *Plant Disease* 70: 452-453.
- BAIN, R.A. LENNARD, J.H. & WASTIE, R.L. 1982. Variation in pathogenicity among isolates of *Phoma exigua* var. *foveata* on potato cultivars differing in resistance. *Potato Research* 25: 113-119.
- BAIN, R.A., LENNARD, J.H. & WASTIE, R.L. 1987. Variation in pathogenicity among field isolates of *Phoma exigua* var. *foveata*. *Annals of applied Biology* 111: 309-315.
- BAKER, K.F. DAVIS, L.H., WILHELM, D.S. & SNYDER, W.C. 1985. An aggressive vascular-inhabiting *Phoma* (*Phoma tracheiphila* f. sp. *chrysanthemi* nov. f. sp.) weakly pathogenic to chrysanthemum. *Canadian Journal of Botany* 63: 1730-1735.
- BERLYN, G.P. & MIKSCH, J.P. 1976. Botanical Micro-technique and Cytochemistry. The Iowa State University Press, Ames, Iowa.
- BOEREMA, G.H. & DORENBOSCH, M.J. 1973. The *Phoma* and *Ascochyta* species described by Wollenweber and Hochapfel in their study on fruit-rotting. *Studies in Mycology* 3: 1-49.
- CARTWRIGHT, K. St. G. 1929. A satisfactory method of staining fungal mycelium in wood sections. *Annals of Botany* 43: 412.
- DOMSCH, K.H., GAMS, W. & ANDERSON, T.H. 1980. Compendium of Soil Fungi Vol 1 & 2. Academic Press, London.
- DORENBOSCH, M.J. 1970. Key to nine ubiquitous soil-borne *Phoma*-like fungi. *Persoonia* 6: 1-14.
- HAMMOND, K.E. & LEWIS, B.G. 1987. Variation in stem infections caused by aggressive and non-aggressive isolates of *Leptosphaeria maculans* on *Brassica napus* var. *oleifera*. *Canadian Journal of Botany* 65: 23-25.
- HAMMOND, K.E., LEWIS, B.G. & MUSA, T.M. 1985. A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathology* 34: 557-565.
- HUMPHERSON-JONES, F.M. 1983. Pathogenicity studies on isolates of *Leptosphaeria maculans* from brassica seed production crops in south-east England. *Annals of Applied Biology* 103: 37-44.
- HUMPHERSON-JONES, F.M. 1985. The incidence of *Alternaria* spp. and *Leptosphaeria maculans* in commercial brassica seed in the United Kingdom. *Plant Pathology* 34: 385-390.
- MILLER, T. 1984. Diseases of female stroboli in southern pine seed orchards. Pages 15-20 in: Proceedings of the Cone and Seed Insects Working Party Conference Working Party S2.07-01. H.O. Yates III, ed.
- NATHANIELS, N.Q.R. & TAYLOR, G.S. 1983. Latent infection of winter oilseed rape by *Leptosphaeria maculans*. *Plant Pathology* 32: 23-31.
- NEERGAARD, P. 1977. Seed Pathology 1. The Macmillan Press Ltd, London.
- PERROTTA, G., MAGNANO DI SAN LIO, G. & BASSI, M. 1980. Some anatomical and morpho-functional aspects of resistance to *Phoma tracheiphila* in citrus plants. *Phytopathologische Zeitschrift* 98: 346-358.
- SASS, J.E. 1951. Botanical Microtechnique. The Iowa State College Press. Ames.
- SPIERS, A.G. & WENHAM, H.T. 1983. The Poplar seed-transmission of *Marssonina brunnea*. *European Journal of Forest Pathology*. 13: 305-314.
- WHITE, J.F. & MORGAN-JONES, G. 1984. Studies in the genus *Phoma*. 4. Concerning *Phoma macrostoma*. *Mycotaxon*. 20: 197-204.