Penetration and Infection of Orchid Protocorms by Thanatephorus cucumeris and Other Rhizoctonia Isolates

Brian Williamson and Geoffrey Hadley

Postdoctoral Student and Lecturer in Mycology, respectively, Department of Botany, University of Aberdeen, AB9 2UD, Scotland, United Kingdom.

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ABSTRACT

Penetration and infection of protocorms of the orchid *Dactylorhiza purpurella* by several isolates of *Rhizoctonia* were observed by a slide culture technique. Eight isolates, including pathogenic strains of *Thanatephorus cucumeris* (*Rhizoctonia solani*) and orchid endophytes *Tulasnella calospora* and *Ceratobasidium cornigerum,* were all symbiotic to various degrees.

Living epidermal hair cells were the only site of symbiotic infection. All isolates penetrated hairs from single hyphae and induced growth and differentiation of protocorms. Pathogenic infection of protocorms occurred infrequently with all isolates, following random penetration of epidermal cells. Compatible (symbiotic) infections frequently became parasitic at different stages of protocorm development.

Pathogenicity tests showed that only two isolates of *T. cucumeris* infected cotyledons and hypocotyls of crucifer seedlings. These isolates grew along the junctions between host epidermal cells, and penetrated from dome-shaped infection cushions. Phytopathology 60:1092-1096.

The fungi most consistently obtained from roots of chlorophyllous orchids are imperfect fungi of the form-genus *Rhizoctonia,* many of which are known to stimulate growth and differentiation in seedlings of the host or other orchid species (3, 5, 10, 12, 13, 19, 20). The basidial stages of many *Rhizoctonia* isolates associated with orchid roots have been produced in culture by Warcup and Talbot (29) (Warcup, *personal communication*). These include *Thanatephorus cucumeris* (Frank) Donk (*Rhizoctonia solani* Kühn). Several fungi with hyphal characteristics of *R. solani* from orchids, nonorchid hosts, or soil are known to be symbiotic with orchid protocorms (11, 12, 13, 20), but have not been tested for pathogenicity on nonorchid hosts. The inference (20) that such fungi may be both pathogenic and symbiotic remains unsubstantiated. Isolates of *T. cucumeris* of proven pathogenicity to nonorchid hosts have not previously been tested for symbiotic activity with orchids.

Penetration of orchid roots or protocorms by rhizoctonias has not been fully described, although penetration from single hyphae has been noted (5, 23). Extensive studies of *R. solani* on many nonorchid hosts have shown that penetration most commonly takes place from dome-shaped infection cushions (1, 6, 7, 15, 18, 22, 24) or lobate appressoria (9, 14). Penetration through stomata (7, 8, 9, 28) or injuries (22) has been reported infrequently, and direct penetration of the epidermis by single hyphae has rarely been observed (22, 24).

This paper reports experiments in which orchid protocorms were inoculated with a selection of rhizoctonias including orchid symbionts and known pathogens of nonorchid hosts. Mode of penetration and degree of compatibility were compared. Seedlings of representative genera of the Cruciferae were inoculated with the same fungi for comparative study of infection structures, and to give some indication of pathogenicity.

MATERIALS AND METHODS.—Strains of *T. cucumeris,* for which there is pathological and genetical information (15, 16, 17, 27), and orchid mycorrhizal fungi known to stimulate postgermination development of one or more species of orchid (19, G. Hadley, *unpublished data*) are listed in Table 1. The fungi were maintained on potato-dextrose agar and transferred to Pfeffer 0.1% dextrose agar (21) prior to inoculation of host tissue.

Slide cultures.—Seed of the north temperate orchid *Dactylorhiza purpurella* (T. & T.A. Steph.) Soó (*Orchis purpurella*) was surface-sterilized by shaking in 5% "Domestos" solution (containing 0.5% w/v available chlorine) and washed twice in sterile deionized water. Seed was dispersed in aliquots of molten Pfeffer 0.1% dextrose agar at 45 C, and the suspension poured as a thin layer in sterile petri dishes. After 2 weeks in darkness at 24 C, most seeds had germinated to produce undifferentiated protocorms, 375 (310-470)μ × 310 (220-400)μ.

Portions of agar, 1 cm^2, containing several protocorms were transferred to sterile microscope slides and inoculated at one edge; a sterile coverslip was then pressed over the agar. Eight slide cultures of each isolate were maintained in moist chambers at 24 C in darkness. Penetration and progress of intracellular hyphae in living material was observed daily for 3 weeks.

Pathogenicity test.—Seedlings of radish (*Raphanus sativus* L. 'Scarlet Globe'), mustard (*Brassica nigra* [L.] Koch 'White'), and cress (*Lepidium sativum* L. 'Curled') were grown in vermiculite under continuous light for 6 days, then surface-inoculated on cotyledons and hypocotyl with 3-mm agar discs from fungal cultures.

Whole seedlings were examined for the presence of infection structures at daily intervals. Microscopic observation of surface hyphae was facilitated by treat-

TABLE 1. Taxonomic designation, source, and known host reaction of isolates of *Thanatephorus cucumeris* and orchid mycorrhizal fungi

Isolate	Taxonomic designation	Source	Host reaction
16*[a]	*T. cucumeris*	Wheat root, Cungena, S. Australia (N.T. Flentje)	Virulent parasite on roots of many hosts
48*	*T. cucumeris*	Soil, Adelaide, S. Australia (N.T. Flentje)	Parasitic on stems of Cruciferae
82*	*T. cucumeris*	Soil, Cungena, S. Australia (N.T. Flentje)	Nonpathogenic to all hosts tested
87*	*T. cucumeris*	Soil, Moonta, S. Australia (N.T. Flentje)	Parasite on stems of many hosts
Thr1	*Ceratobasidium cornigerum*	*Thrixspermum* protocorms, Malaya (B. Williamson)	Symbiotic with several orchids
Rgr	*Ceratobasidium cornigerum*	*Goodyera repens* root, N.E. Scotland (G. Hadley)	Symbiotic with several orchids
Amo4	*Tulasnella calospora*	*Arachnis* root, Malaya (G. Hadley)	Symbiotic with all orchids tested
T	*Ceratobasidium* sp.	*Dactylorhiza purpurella* root, N.E. Scotland (G. Hadley)	Symbiotic with several orchids

[a] * = Isolates numbered as in the Waite Institute culture collection.

ment in 5% trichloroacetic acid for 2 hr at 20 C, followed by bleaching overnight in dioxan:propionic acid (19:1) at 60 C (4) before staining and mounting.

RESULTS.—*Infection of D. purpurella protocorms.*—Hyphae grew radially from inocula without any indication of chemotactic growth toward protocorms. Where random contact with epidermal hairs occurred, the hair was bypassed and growth of the hypha continued. Compatible penetration of living hairs first occurred 4 days after inoculation; hyphae swelled slightly on contact before perforating the host cell wall (Fig. 1, 2). After penetration, hyphae enlarged to their former diam and grew intracellularly, forming septa and occasional branches. Cytoplasmic streaming in infected hairs continued during hyphal invasion and subsequently. The hyphal apex was surrounded by cytoplasmic particles. All eight isolates penetrated living hairs from single hyphae in this manner. Other interactions, such as lysis of intracellular hyphae while host cell streaming continued or cessation of streaming followed by host cell death and stasis of the fungus, were occasionally seen. In such situations compatible infection was frequently accomplished through another hair on the same protocorm. One penetration could initiate colonization of the cortical parenchyma, but several epidermal hairs were commonly penetrated simultaneously. With all isolates, compatible intracellular invasion was the commonest result of infection, and this subsequently led to symbiosis in a proportion of the protocorms. Within 4 days of infection, hyphal coils (pelotons) became visible in subepidermal parenchyma cells as intracellular starch aggregates diminished, which was the first visible change in host cells following infection. Subsequently many infected protocorms differentiated a shoot meristem and enlarged considerably, indicating the firm establishment of a symbiotic condition (21).

Hairs were frequently invaded from cortical parenchyma cells at advanced stages of infection. The lumen of the hair sometimes became almost occluded by hyphae; nevertheless, streaming was still detectable. Intracellular hyphae often grew out from living hairs, perforating the cell walls by a process similar to that of penetration (Fig. 3, 4). On one occasion, stages of hyphal emergence by isolate 82 were recorded as follows:

1) Zero time: A hypha was observed in contact with the wall of an epidermal hair.

2) 28 Min: Cytoplasm at the hyphal apex appeared vacuolated, and a refractile spot was visible at the point of contact with the host cell wall (Fig. 5).

3) 30 Min: The apex of the emergent hypha was visible and began to expand.

4) 37 Min: The protruding hyphal apex had reached full diam (Fig. 6).

5) 90 Min: Apical extension of the hypha continued, with great cytoplasmic activity (Fig. 7, 8).

6) 24 Hr: The hyphal wall had thickened and a septum with characteristic dolipore was present close to the point of emergence. Cytoplasmic streaming in the host cell continued. A second hypha had also emerged close to the first one (Fig. 9).

Noncompatible (i.e., parasitic) infection was seen occasionally with all isolates. Hyphae grew extensively over the host epidermis, particularly along the junctions between epidermal cells. Penetration took place at numerous sites from single hyphae without prior elaboration of infection cushions. Epidermal and subepidermal parenchyma cells were rapidly invaded by hyphae that never developed pelotons. All protocorms infected in this manner became moribund within 5 days. Intense hyphal growth in necrotic protocorms frequently produced barrel-shaped chlamydospores. Uninfected or symbiotically infected protocorms adjacent to parasitised ones seemed unaffected by the latter.

Parasitism sometimes occurred after a prolonged phase of compatibility or symbiosis. Apparently normal symbiotic protocorms became permeated by hyphae

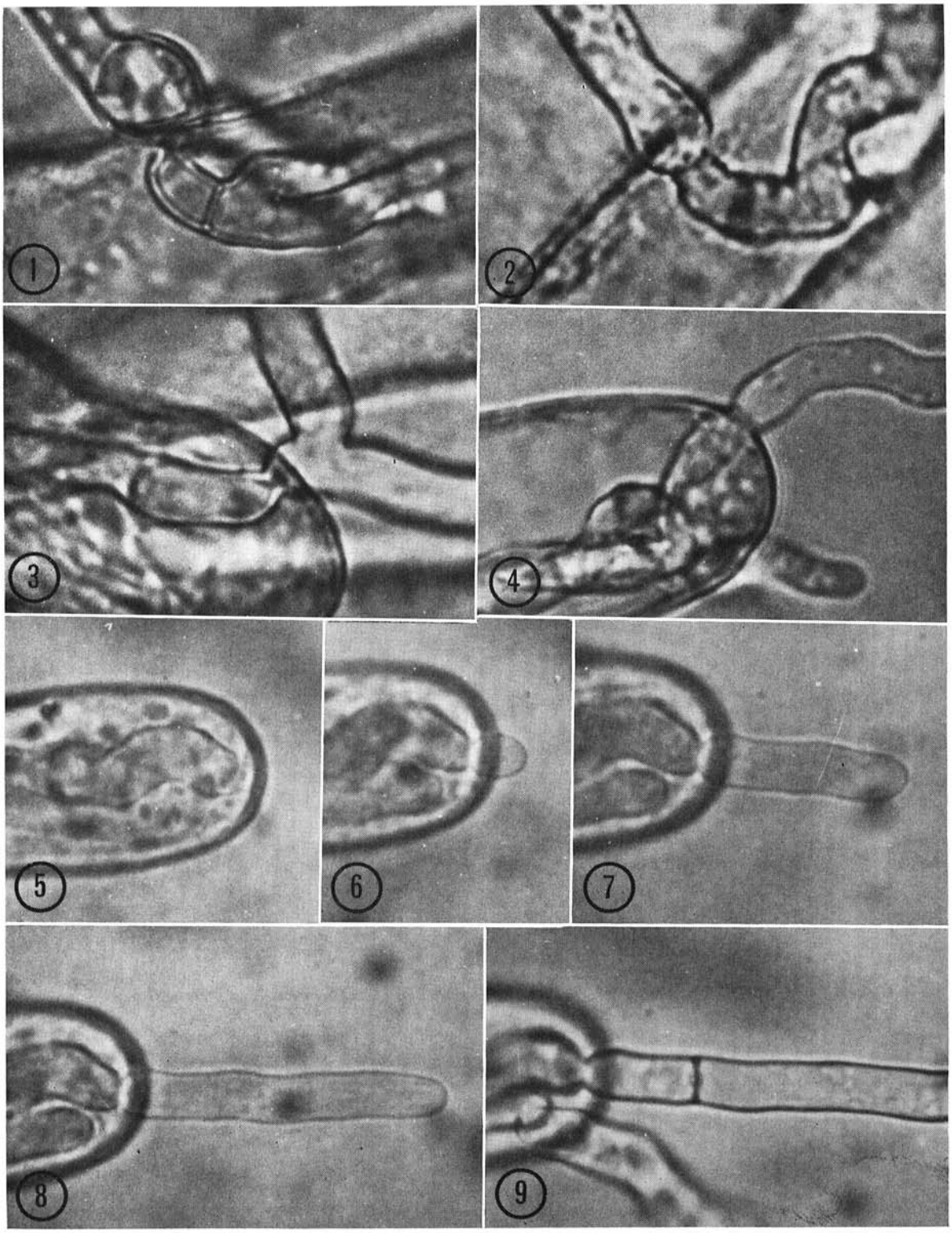

Fig. 1-9. Symbiotic infection of *Dactylorhiza purpurella* by pathogenic strains of *Thanatephorus cucumeris*. **1, 2)** Penetration of epidermal hairs by single hyphae of isolates 48 and 87, respectively (×1,500). **3, 4)** Emergence from hairs by the same isolates (×1,500). **5-9)** Serial photographs of emergence from a living hair by isolate 82. **5)** 28 Min (from first observation), wall perforation; **6)** 37 min, lateral expansion of emergent hypha; **7, 8)** 76 and 87 min, respectively, apical extension continues; **9)** 24 hr, formation of septum and vacuolation of hypha (×1,000).

which grew from the peloton-filled basal parenchyma cells and aggressively invaded all other tissues, killing the host.

The most stable symbiotic relationship occurred with isolates T, 48 and 82, while isolates 87, Amo4, and Thr1, although actively symbiotic, showed a trend to postcompatibility parasitism. Isolate 16 infrequently became symbiotic, and was parasitic in most infections. With isolate Rgr, little symbiosis or parasitism was seen, most protocorms being free of infection. Regardless of the isolate, some protocorms remained uninfected and healthy throughout the experiment, while others became moribund without any evidence of infection.

Penetration and pathogenicity on crucifer seedlings.—All isolates had grown superficially on cotyledons and hypocotyls 2 days after inoculation, but with most isolates intimate contact with the host surface was infrequent. No stomatal penetration was seen. Intimate contact with epidermal cells was observed for isolates 48 and 87. These fungi grew particularly along the junctions between epidermal cells and anastomosed frequently. Complex, dome-shaped infection cushions closely appressed to the host were produced by proliferation and aggregation of adjacent branches (Fig. 10, 11, 12, 13). Hyphae arising from beneath infection cushions penetrated the adaxial or abaxial epidermis of cotyledons and initiated intercellular and intracellular infections of mesophyll tissues. Water-soaked lesions formed, and necrosis rapidly extended to all tissues.

All seedlings inoculated with isolate 48 were completely parasitized; isolate 87 successfully infected only cress and mustard, although it also formed infection cushions on radish. All isolates other than 48 and 87 grew sparingly over the epidermis at random and not along the cell junctions. Anastomosis occurred infrequently, and no infection was seen on any of the seedlings.

DISCUSSION.—There is considerable evidence that infection by *T. cucumeris* (*R. solani*) is associated with the development of dome-shaped infection cushions or lobate appressoria prior to penetration of the host (7, 9, 14, 18, 22). The type of infection structure may be correlated with the source of the isolate (8). Evidence presented here suggests that surface factors characteristic of a particular host may have a greater influence on the mode of penetration than previously thought.

The eight fungi tested include unspecialized strains of *T. cucumeris,* which infect roots (isolate 16) or stems (isolates 48, 87), one nonpathogenic strain (isolate 82), and *Ceratobasidium* and *Tulasnella* strains known to be orchid symbionts. Regardless of source, host specificity, and identity, each isolate penetrated epidermal hairs of orchid protocorms from single hyphae. Isolates 48 and 87 also infect crucifers, penetrating the host from dome-shaped infection cushions. Clearly the host is involved in the processes controlling the development of infection structures.

In symbiotic infections of *D. purpurella* protocorms,

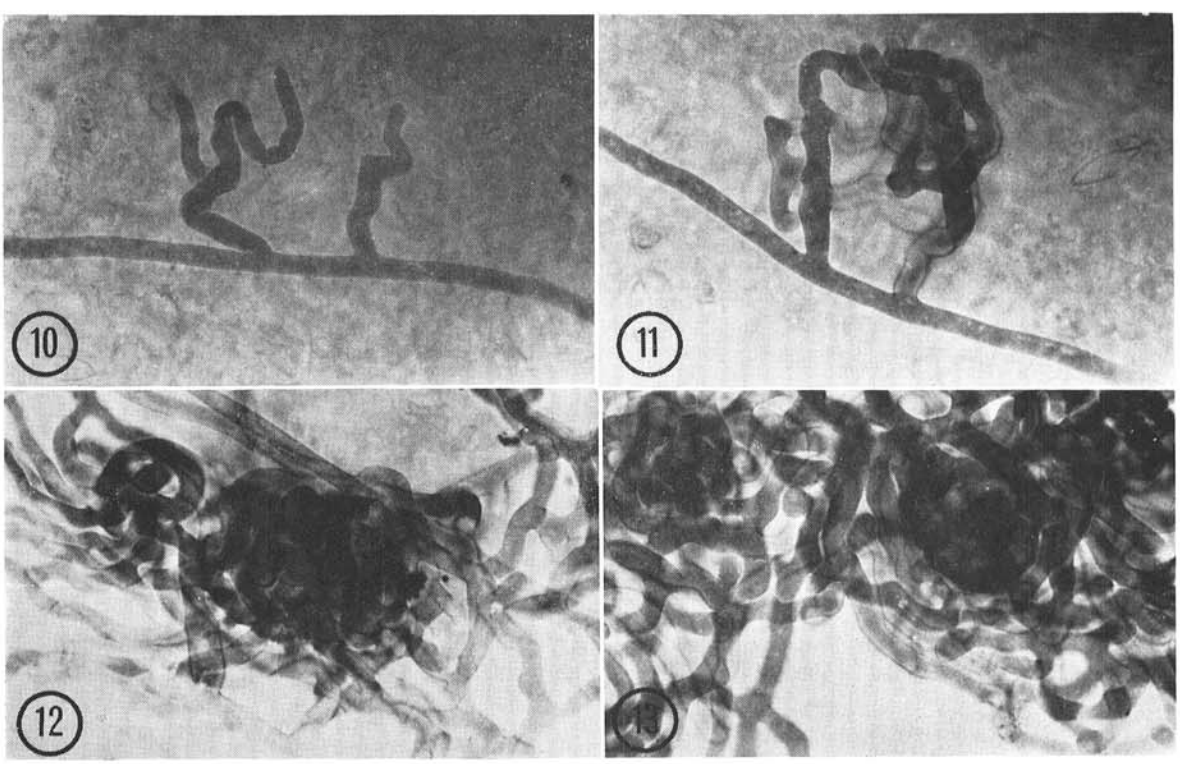

Fig. 10-13. Development of dome-shaped infection cushions on cress cotyledons by *Thanatephorus cucumeris* (isolate 48). **10)** Lateral branches arise in close proximity. **11)** Initiation of cushion development by anastomosis of proliferating laterals. **12, 13)** Cushion increases in complexity prior to penetration (all ×440).

hairs and no other epidermal cells are the site of penetration. There is no hyphal growth over the epidermis except when parasitism occurs. Flentje et al. (16) showed that the development of infection cushions by *T. cucumeris* is in some way controlled by exudates from a susceptible plant, and that other undefined surface characteristics initiate penetration. They report that the metabolites which induce cushion development are probably produced continually by susceptible hosts rather than arising at the time of host-fungus interaction. Exudates may induce development by inhibition of apical growth, as suggested by Robertson (26). Infection cushions are not required for penetration of orchids. Presumably, factors inducing cushion development are absent from orchid exudates and additional surface factors needed for penetration, if any, are only present on epidermal hairs.

The eight fungi tested here all initiate a compatible infection of *D. purpurella* protocorms leading to the establishment of a symbiotic condition in at least a proportion of the population. *T. cucumeris* strains 48 and 87, despite their potential as parasites of crucifer hosts, are no less effective than some isolates from orchids in their symbiotic activity.

This is the first conclusive evidence that strains of *T. cucumeris* pathogenic on other hosts can be symbiotic with orchids. Downie (13) showed that fungi regarded as *R. solani* from potato, wheat, and tomato were symbiotic with *D. purpurella*. However, none of Downie's isolates has been tested for pathogenicity on nonorchid hosts. Similar criticisms may be valid for other strains regarded as *R. solani* which were found by Harvais & Hadley (20) to stimulate differentiation of orchid protocorms. Doubt may also exist concerning the identity of fungi generally described as *R. solani*; e.g., Downie's isolate Csl from wheat may be a *Ceratobasidium* sp. (25).

Evidence suggests that *D. purpurella* is naturally dependent on an endophyte for differentiation (21). Failure of symbiosis leading to parasitism of protocorms is a feature of all populations in culture and may also occur in nature; factors such as high temp, depletion of carbohydrate source, aggressive fungi, and, perhaps, in vitro conditions accentuate the phenomenon (19, 21).

The work described here deals with protocorms and no observations have been made on root infection in *D. purpurella*. Alconero (2) has recently described pathological and compatible (mycorrhizal) infections by *R. solani* in adjacent areas of *Vanilla* roots. Cells containing lysed pelotons were sometimes parasitically invaded, and it seems that postcompatibility parasitism in protocorms may be paralleled in *Vanilla* roots.

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