Histopathology of *Pinus radiata* Seedlings Infected by *Colletotrichum acutatum* f. sp. *pinea*

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ABSTRACT

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Conidia of *Colletotrichum acutatum* f. sp. *pinea* germinated on young primary needles of *Pinus radiata* seedlings within 6 hr at 25 C and 17 klux. Appressoria were formed by the germinating conidia 24 hr after inoculation under a diurnal regime of 14 hr light at 25 C and 10 hr dark at 15 C. Infection pegs from appressoria pierced the cuticle and enlarged into infection hyphae. These spread to all tissues in the needle, leading to collapse of cells. Mycelium extended through the needle into the stem to the vicinity of the vascular tissue. Thereafter fungal growth ceased. Symptoms of infection were: terminal crook, terminal blight, stiffening and thickening of stem, a period of dormancy and stunting. All were not necessarily exhibited in any one diseased seedling. Terminal crook resulted from continued growth of young stem tissue on the side opposite to lateral necrosis near the apex. Terminal blight, consisting of death of complete stem apex, is reported here for the first time. Seedlings 6 mo old and younger were the most susceptible. Infected *P. radiata* stems showed premature secondary thickening associated with increased xylem tissue.

Additional key words: terminal crook disease, forest nurseries.

In 1963, a previously undescribed disease of *Pinus radiata* D. Don seedlings was recorded from Woodhill Forest Nursery near Auckland, New Zealand (14). It became known as "terminal crook disease" and the fungus associated with the disease subsequently was described as *Colletotrichum acutatum* Simmonds f. sp. *pinea* Dingley & Gilmour (11).

Terminal crook disease has appeared in many nurseries in the northern half of the North Island of New Zealand (4) and has become epidemic in several (11). The disease has also been recorded in Australia (1,2,3), Kenya (13), and Chile (23).

The present study of *C. acutatum* f. sp. *pinea* was undertaken to trace the mode of infection of the fungus in needles of *P. radiata* and its subsequent growth into the stem that results in the terminal crook disease. Histopathologies of other *Colletotrichum* diseases have been reported, (eg, those caused by *C. lindemuthianum* [9,18] and *C. gloeosporioides* [5,19,20]) but none to date involving *C. acutatum*.

MATERIALS AND METHODS

C. acutatum f. sp. *pinea* was isolated from diseased *P. radiata* seedlings obtained from a forest nursery in Tokoroa, New Zealand. The fungus was cultured on potato-dextrose agar at 25 C. Conidia were removed from the surface of 10-day-old cultures, washed on a membrane filter and resuspended in water. Conidial concentration was adjusted to 10^6 per milliliter.

P. radiata seedlings were grown individually in U.C. potting mix (21) contained in 50.8-mm (2-inch)-diameter plastic pots that were placed in the glasshouse. One week before inoculation the seedlings were moved to a Canadian Controlled Environment Ltd., Model EF7H, growth chamber. This operated daily for 14 hr at 25 C and 17 klux light intensity, and 10 hr at 15 C in the dark. Seedlings most commonly used were between 2 and 5 mo old; however, older seedlings up to 12 mo of age were also tested for susceptibility to the fungus.

Conidial suspension was applied to the seedlings, especially to the rosette of needles at the apex, by means of a de Vilbiss atomizer. All seedlings were enclosed in clear plastic bags for the first 10 days following inoculation.

Preece's (25) staining method was used to observe conidia and mycelium on needle surfaces. Inoculated whole needles were immersed in 1% periodic acid, washed in distilled water, immersed in decolorized basic fuchsin (25), washed in sulphurous acid, and mounted in distilled water for examination. Appropriate pieces were cut and mounted on slides in clear lactophenol or in lactophenol trypan blue (8).

In addition, the scanning electron microscope (SEM) was used as an additional tool in the study of the fungus on needle surfaces. Five-millimeter pieces of inoculated needles were mounted on brass stubs and placed overnight in a desiccator. Surfaces of the needle pieces were subsequently coated with gold-palladium alloy and examined in a Model JSM-U3 (Japan Electron Optic Co. Ltd., Tokyo) SEM. Beam voltages up to 20 kV were used.

Plant materials were fixed on a regular time schedule and also when the desired stages of infection had developed. Needles and stems were fixed in formalin: acetic acid:50% ethanol (FAA—10:10:100, v/v) for paraffin sections and 3% glutaraldehyde in 0.05 M phosphate buffer for epoxy resin sections. The FAA-fixed materials were dehydrated in an ethanol series, embedded in paraffin, and sectioned at 10–14 μm with a rotary microtome. Sections placed on slides were stained with the periodic acid-Schiff technique (12) and mounted under a coverslip supported and sealed with DPX mountant. The glutaraldehyde-fixed materials were postfixed overnight in 1% osmium tetroxide in 0.05 M phosphate buffer at 4 C, washed in phosphate buffer, dehydrated through a graded ethanol series, infiltrated with propylene oxide through an ethanol-propylene oxide series, and embedded in Epon 812 (6) through a propylene oxide-Epon series. The epoxy resin blocks were cut on a Porter Blum microtome fitted with glass knives. The sections (1–2 μm) placed on slides were stained in toluidine blue (27) for 1–5 min, rinsed thoroughly in water, dried at room temperature, and mounted under a coverslip supported and sealed with DPX mountant.

Photomicrographs were taken on Ilford Pan F, 50 ASA, 35-mm film with a Reichert Photo-Automatic attachment. Drawings were made with the aid of a camera lucida apparatus mounted on a Reichert microscope.

RESULTS

Conidial germination and appressorial formation. One or two germ tubes emerged from any portion of the conidium but most commonly from the side and near the end as shown in Fig. 1A. On

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the surfaces of *P. radiata* needles, conidia germinated about 6 hr after inoculation; however, up to 96 hr were required for maximum germination. Conidia were unicellular, but most became bicellular when germination began (Fig. 1A–C). Germ tubes sometimes produced secondary conidia.

A conidium produced an appressorium directly (Fig. 2) or the appressorium arose from a germ tube usually after limited elongation (Figs. 1B and 3). The appressorium commenced as a

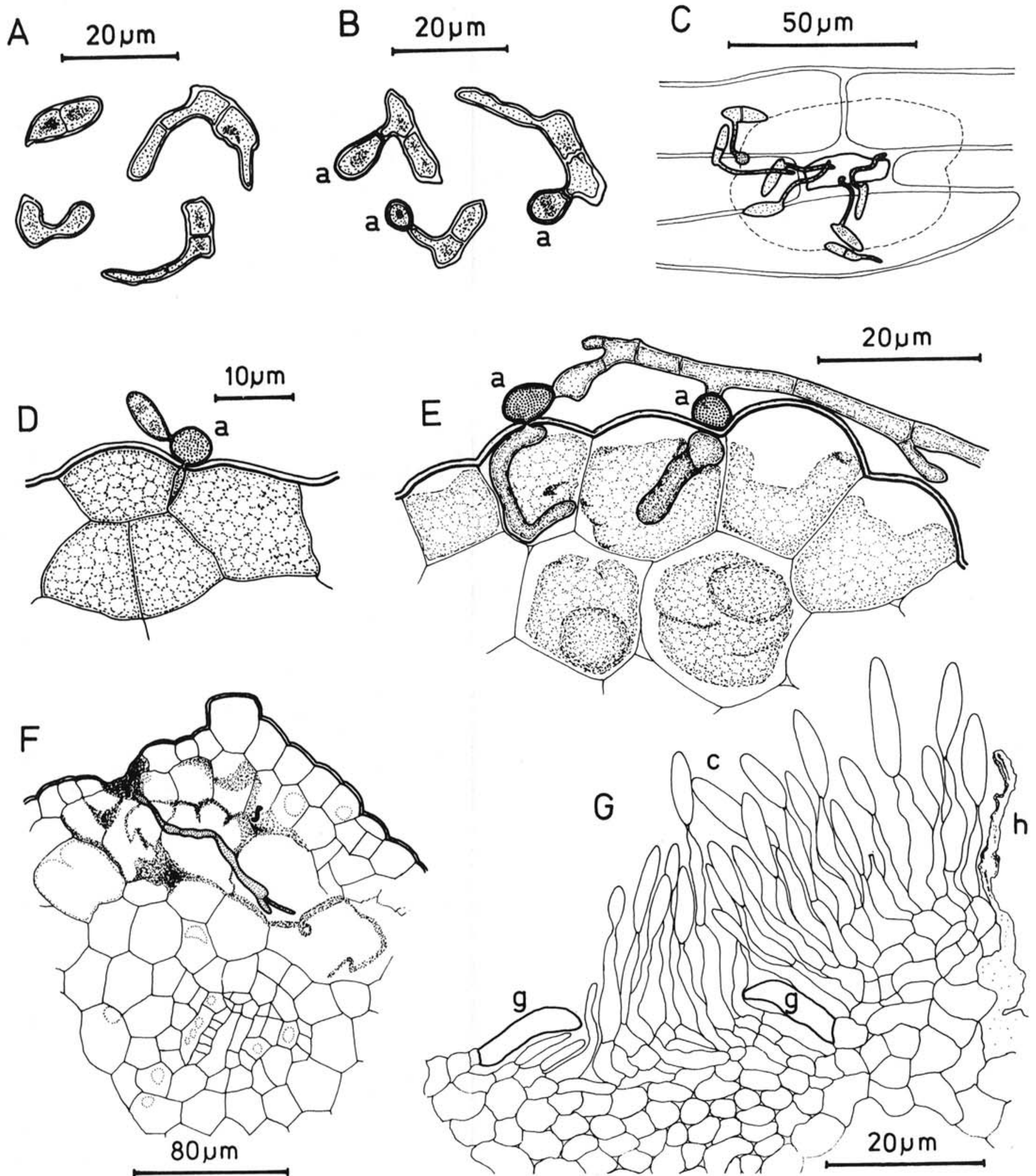


Fig. 1. Camera lucida drawings of stages during germination, infection and sporulation of *Colletotrichum acutatum* f. sp. *pinea*. **A** and **B,** Conidial germination and appressorial formation, respectively, from glass slides; most conidia became septate during germination. **C,** Conidial germination on the surface of a primary needle of *Pinus radiata* in the vicinity of a stomatal pit opening. **D–G,** Transverse sections of portions of infected young primary needles of *P. radiata*. **D,** Intercellular penetration by the infection hypha. **E,** Intracellular penetration by infection hyphae. **F,** Hyphae have penetrated many host cells, causing their collapse. **G,** Fruiting structure, probably from a single stroma, has emerged partly by enlarging a stomatal opening and partly by lifting the epidermis as for an acervulus. Abbreviations: a, appressorium; c, conidium; g, remains of guard cell of a stoma; h, remains of ruptured host tissue.

terminal swelling of the germ tube that soon broadened and developed a thick wall. The swollen appressorium was then separated off by a septum. Germ tubes that did not immediately form appressoria traversed the needle surface and some subsequently formed appressoria. Sometimes a single germ tube produced more than one appressorium. Appressoria were usually formed between 24 and 96 hr after inoculation.

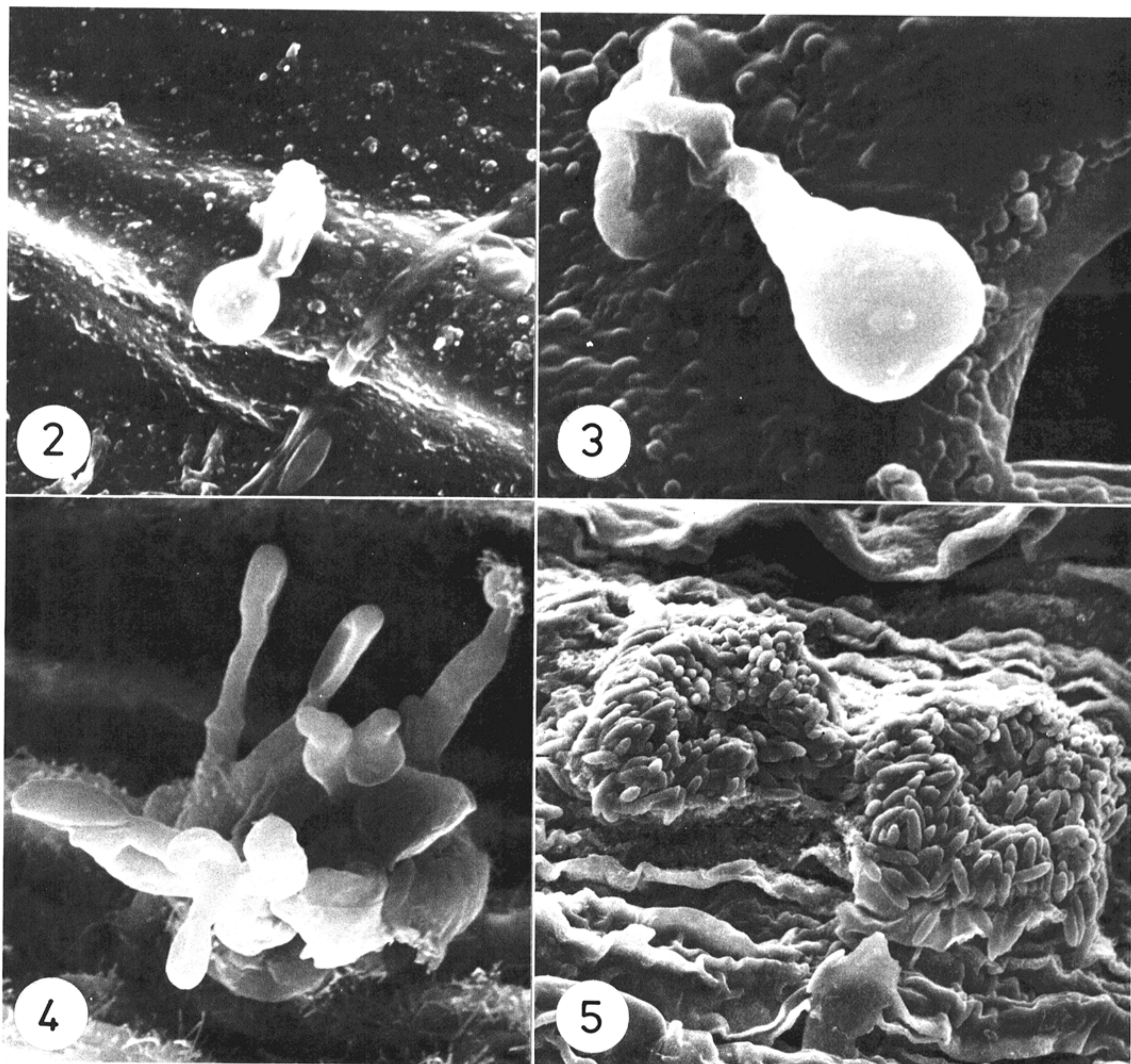
Germ tubes were observed to enter and sometimes grow out of the stomatal pits of the needles (Fig. 1C). Occasionally appressoria were observed within the stomatal pits.

Appressorial germination and needle penetration. Appressoria were thick walled, brown in color and each usually had a small, pale zone when viewed from above. They were not stained by decolorized basic fuchsin and not dislodged easily from the needle surface. No obvious mucilaginous sheath (or envelope) was distinguished. Mature appressoria were more or less rounded or club shaped (Figs. 1B, 2, and 3).

The pale zone in the appressorium, as seen under the light microscope, was probably the germination pore. From such a pore the infection peg pierced directly through the cuticle of the needle. There was no evidence that the infection peg exerted any swelling or chemical action upon the cuticle. After penetrating the cuticle, the infection peg enlarged into the infection hypha, which penetrated either between (Fig. 1D) or directly into (Fig. 1E) epidermal cells and developed to normal hyphal size or larger. Many instances of penetration at the bases of trichomes (epidermal hairs) were observed. Conidia tended to collect at the bases of trichomes.

Development of fungus within the needle. Once the infection hyphae gained entrance through the cell wall they appeared to adhere closely to the cell wall or to directly invade the cytoplasm (Fig. 1E).

The hyphae later penetrated other cells of the epidermis and inner tissues. Cell collapse appeared to occur after the hyphae penetrated them. Hyphae branched and traversed further into the



Figs. 2–5. Scanning electron micrographs of the surfaces of young primary needles of *Pinus radiata* showing *Colletotrichum acutatum* f. sp. *pinea*. **2,** Appressorium arising directly from a conidium now partly collapsed (×2,000). **3,** A short germ tube from a collapsed conidium terminates in an appressorium, which is alongside a stomatal pit opening (×6,000). **4,** Conidiophores, with attached conidia, emerging from a stomatal opening (×2,600). **5,** Two acervuli (×725).

needle. Approximately 24 hr after penetration the mycelium had spread rapidly in all directions and needle tissue at this time had a typically water-soaked appearance around the point of infection. This macroscopically visible symptom soon developed into a definite lesion that progressively extended up and down the needle. Stages of fungal development in the primary needle are shown in Fig. 1D–G. Mycelium invaded and killed all tissues of the needle. Tissues soon lost their identity. At the later stages of infection, the host cell walls were penetrated rapidly by mycelium advancing through cells without exhibiting the bending and adhering phenomena observed during the earlier progress of the infection hyphae.

The fungus frequently sporulated soon after mycelium had become established throughout surface layers of host cells. Hyphae aggregated into thick intraepidermal or subepidermal stromata. Conidiophores frequently emerged from stomatal openings (Figs. 1G and 4) or forced their way through the epidermis and/or cuticle, forming on acervulus (Figs. 1G and 5) containing a slimy mass of conidia. Conidia were cut off from the conidiophores in basipetal

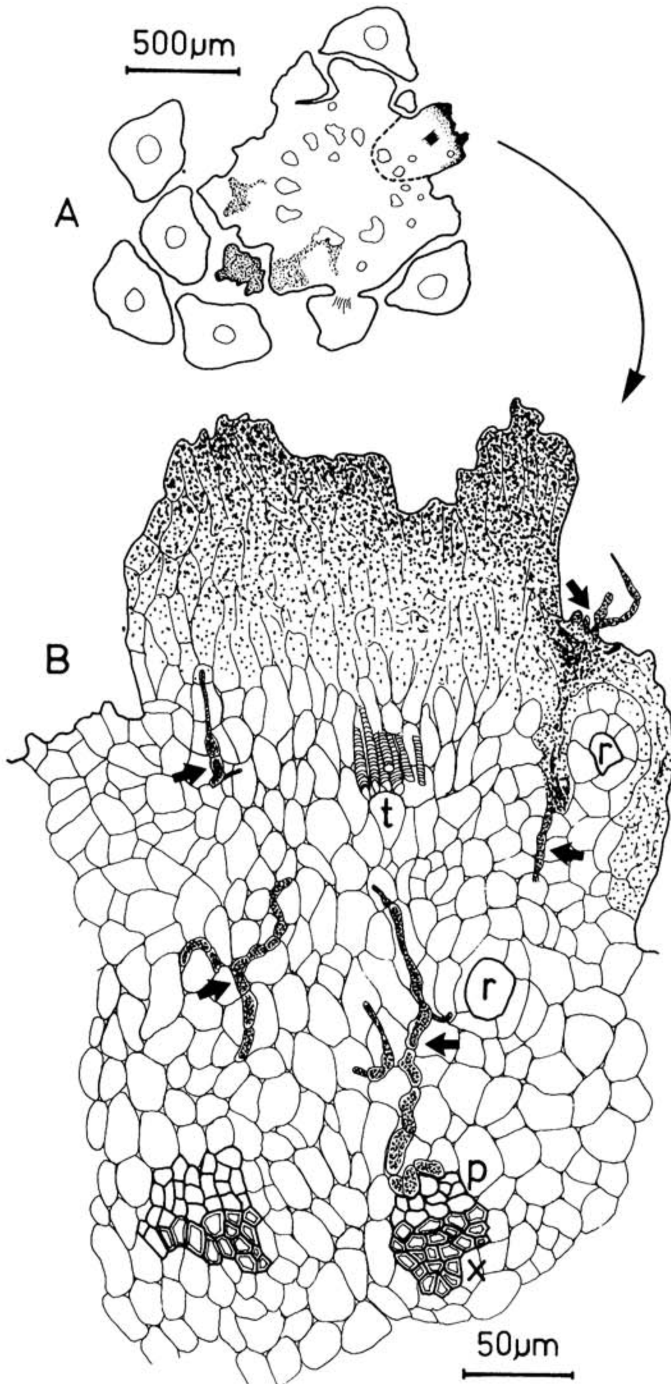
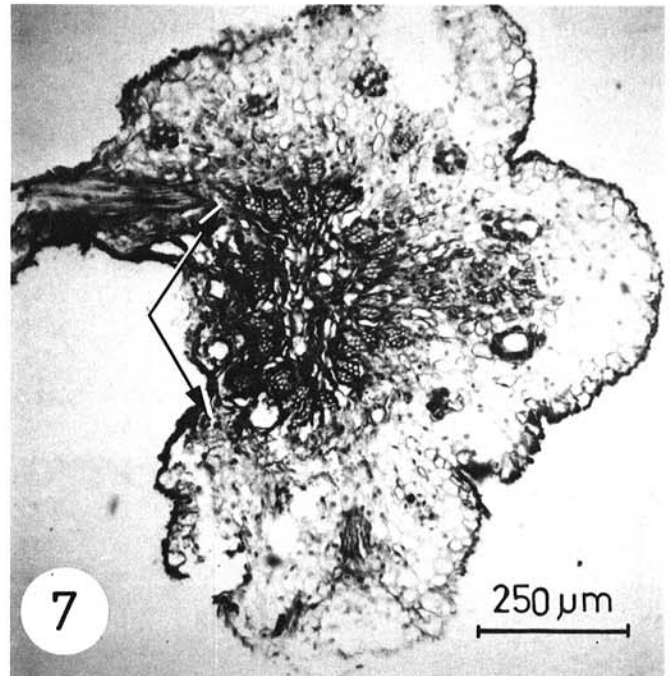
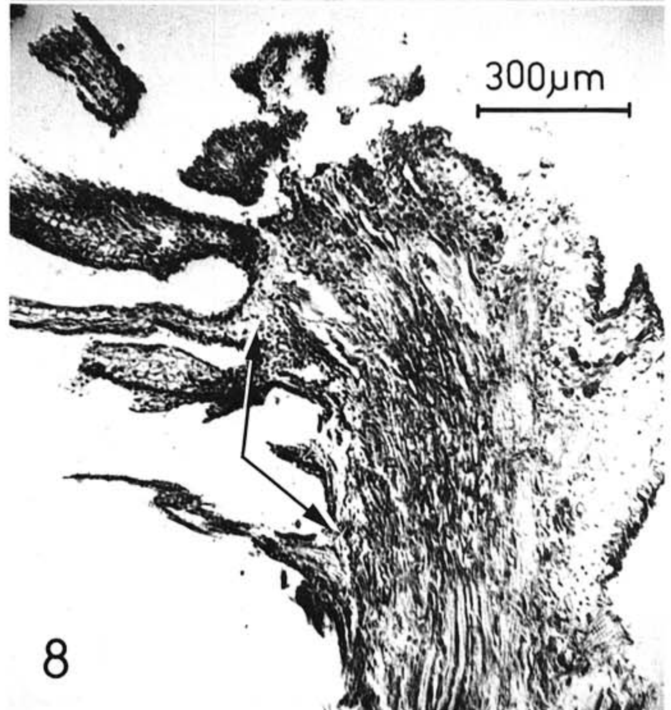


Fig. 6. Camera lucida drawings of a transverse section of a diseased *Pinus radiata* seedling, about 1 cm below the stem apex. The stippled areas of the host indicate cells that have collapsed following colonization by the fungus. **A,** Stem with surrounding needles. A sector of the stem delimited by dotted lines is enlarged in B. **B,** Sector of stem showing deformed and collapsed cells at the needle-stem margin. Hyphae (arrowed) extend through the cortex as far as the phloem. Abbreviations: r, resin canal; t, leaf trace; p, phloem; and x, xylem.





Figs. 7–8. Photomicrographs of sectioned apices of "crooked" *Pinus radiata* stems to show extent of necrosis (arrowed) associated with infection by *Colletotrichum acutatum* f. sp. *pinea*. **7,** Transverse section of stem to show collapse of portions of the cortex and vascular tissue. **8,** Longitudinal section of stem showing collapsed tissues that have resulted in the formation of a "crook."

succession. On the average, fruiting bodies were first formed on the surfaces of needles 96 hr after inoculation.

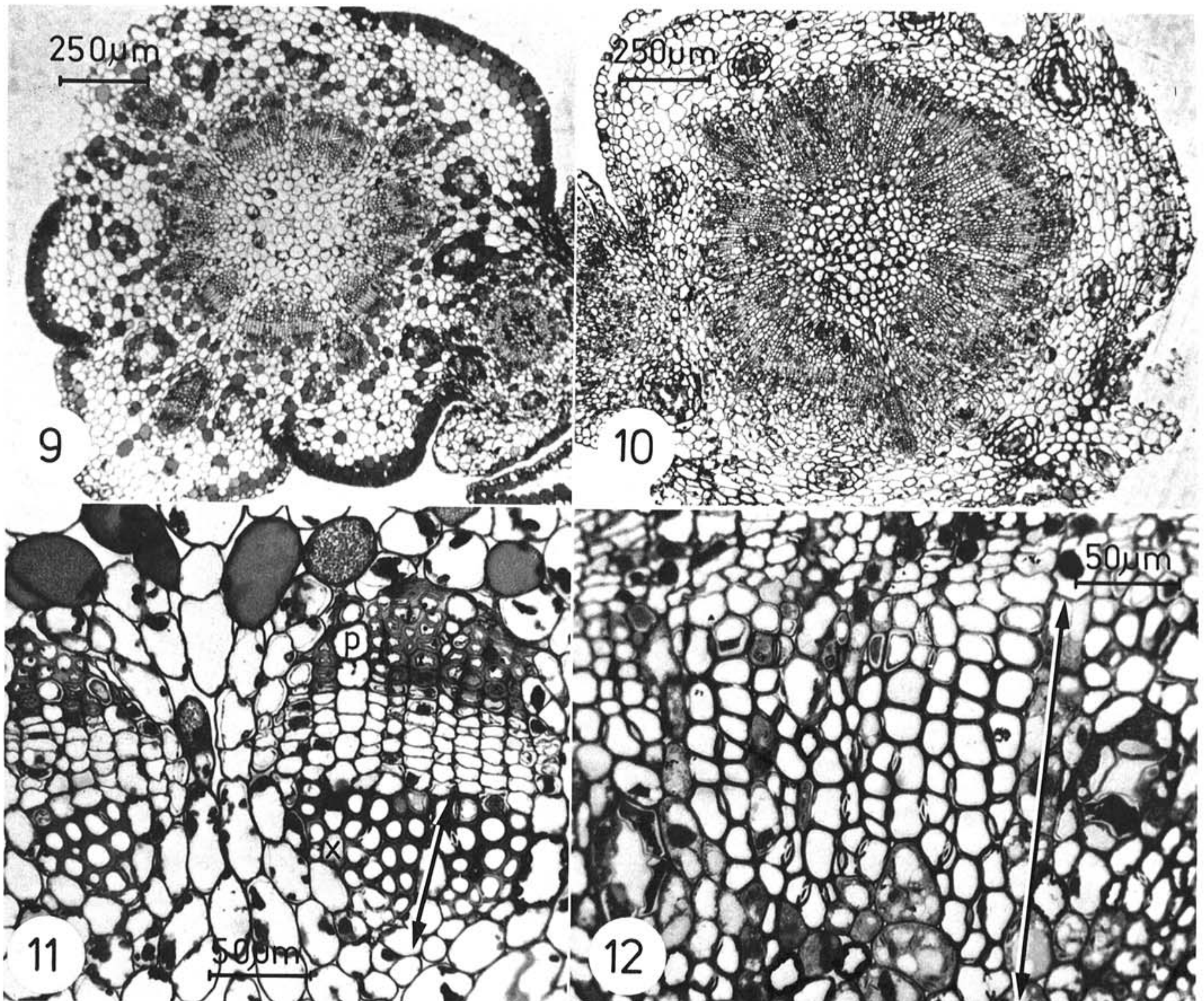
The fungus entered young primary needles, old primary needles, and secondary needles, but it was only in the young primary needles that the fungus was able to advance any significant distance and reach the stem. The rate of fungal growth decreased progressively with increasing age of the needles. In old needles few cells were penetrated by the pathogen, which caused at most a localized lesion.

Lesions on young primary needles usually led to death of all the more distal portions when the vascular tissue was invaded. A similar situation did not arise on older primary and all secondary needles because the lesions were small and the central steles were not infected.

Individual needles often had multiple infections. Also, many needles on a single seedling apex could be infected. All gradations of infection occurred, from one needle to an entire terminal or lateral rosette of needles being lesioned or killed. When the stem apex or apical rosette of needles was killed a "blight" resulted.

Development of fungus within the stem. No primary lesions were observed on stems. Infection progressed from lesions on the bases of young primary needles into the stem (Fig. 6A,B). Hyphae that entered the stem tended initially to be confined to the elongated cells that bridged the needle and stem and to the stem cortical and medullary ray cells. As was frequently observed in needles, hyphae spread with greater rapidity in the direction of least cell wall interruption; ie, along the longer axis of the cell. In all studies of infected stem apices, the fungus advanced ahead of any observable host cell disorganization. Hyphae then invaded vascular bundles and the parenchymatous cells around them. The fungus also invaded some pith cells, usually by way of medullary ray parenchyma cells between the vascular bundles. Soon after this stage there was a dramatic cessation of all fungal growth.

Symptoms after stem invasion. Figures 7 and 8 illustrate transverse and longitudinal sections of host tissues that have collapsed as a result of fungal infection. Tissues in the infected zone of a stem collapsed while those in the uninfected regions apparently kept growing. The region of a stem directly below the terminal bud is one of active cellular division and differentiation; hence, unaffected tissues easily outgrow the infected. This causes a shoot to "crook" in the direction of the infection (Fig. 8). The terminal bud of an infected plant was not necessarily affected; however, when the site of fungal entry into the stem was very close to the apex the terminal bud was infected and killed, especially when there were multiple invasions from needles close to the apex.



Figs. 9–12. Transverse sectional comparisons of healthy and diseased 14-wk-old *Pinus radiata* stems, about 2 cm below the stem apices. **9,** Healthy stem with primary vascular bundles. **10,** Diseased stem sectioned below a crook 16 days after this symptom was first observed, showing abnormal secondary thickening with pronounced increase in amounts of xylem tissue. **11,** Xylem (x) and phloem (p) elements from the healthy stem section in Fig. 9. Extent of xylem arrowed. **12,** Xylem elements from the diseased stem section in Fig. 10, same scale as Fig. 11. Extent of xylem arrowed.

Another condition was frequently encountered, especially in younger seedlings. Many zones of infection occurred around the stem as a result of many needles becoming infected simultaneously. In such instances, entire apical portions of seedlings were killed, with dieback extending up to 2 cm. Such plants usually had no crook. The lower unaffected portions of these blighted plants remained green, and there was stimulation of lower buds to grow, usually after a period of dormancy.

Dormancy in crooked and blighted plants lasted from one to a few months, after which either a subterminal or a lateral bud became dominant. Crooked plants with unaffected apical buds sometimes reverted to upright terminal growth after about 1 mo.

Both crooking and blighting have been observed in lateral branches as well as the main apex. All infected stems invariably became stiff and thick within a month of crooking or blighting. In contrast, comparable healthy plants, when decapitated, initiated lateral bud growth without dormancy and without stem stiffening and thickening. Artificial crooking was induced physically in several seedlings by wounding laterally with a sterile needle just behind the stem apex. Again there was an absence of stem stiffening and thickening.

A comparison of the vascular elements, especially xylem, in healthy and infected 14-wk-old stems is shown in Figs. 9–12. Ten seedlings were grown under identical conditions and were of similar height and stem width. Five were inoculated. Sixteen days after the crook was first observed, the diseased stems had twice as many xylem elements along the stem radii as did the healthy stems (Figs. 11 and 12). Similar pairs of infected and healthy plants were compared for lignification by staining with the lignin-specific stain, phloroglucinol (16). Xylem in infected stems stained deep red while the color in healthy xylem was very faint.

When *P. radiata* seedlings 7 mo and older were inoculated, neither the crook nor blight condition was expressed in infected seedlings. However, the stem did rapidly thicken and stiffen, and there was also an obvious stopping or slowing down of apical growth (stunting effect) equivalent to the apical dormancy of crooked plants.

Infected plants of *P. radiata* outgrew the disease with age. Likewise, uninfected plants became resistant after about 9 or 10 mo.

DISCUSSION

Our results show a number of similarities and contrasts with other well-studied diseases caused by *Colletotrichum* species. Unicellular conidia of *C. acutatum* f. sp. *pinea* frequently became bicellular on germination. This was also reported for *C. lindemuthanium* (9), *C. gloeosporioides* (10), and *C. graminicola* (24,26).

No obvious sheath or envelope was seen around appressoria of *C. acutatum* f. sp. *pinea*. While Mercer et al (22) mentioned a slime layer that attached the appressoria of *C. lindemuthianum* to epidermal cell walls of *Phaseolus vulgaris*, Politis and Wheeler (24), however, in their ultrastructural studies of *C. graminicola*, made no mention of any mucilaginous coat or sheath around the appressorium.

The pale zone in the appressorium of *C. acutatum* f. sp. *pinea* is interpreted as the site of infection peg production. Marks et al (19,20) reported a distinct pore in the appressorial wall of *C. gloeosporioides*. Politis and Wheeler (24), with *C. graminicola*, likewise regarded the area beneath the infection peg, where the dense cell wall of the appressorium tended to "disappear," as the appressorial germ pore.

After entry, *C. acutatum* f. sp. *pinea* advanced rapidly in young primary needles. In older primary needles, hyphae advanced very slowly. The basis of resistance in older needles is not known. Susceptibility of seedlings diminished greatly after 7 mo. Busch and Walker (7) in their studies of cucumber anthracnose suggested that the "true basis of resistance may well be biochemical with morphological response a purely secondary reaction."

Sporulation of the fungus was by conidiophores emerging through stomatal openings (Fig. 4) and in acervuli. Stomatal openings were enlarged, usually along their axes, apparently due to pressure from the mycelium and conidiophores. This type of sporulation was more frequent than the acervulus type.

Downward progress of the fungus in the stem was negligible. On the other hand, upward growth through younger stem tissue sometimes reached and killed the apical bud. In any case, despite apparent lack of further extension of growth by the fungus, there was continuing host-parasite interaction that resulted in stem stiffening and thickening associated with morphological changes below the site of infection.

The entry of the fungus into a pine seedling stem produced one or more of the following symptoms or conditions: "crooking" with or without death of apical bud; "blighting" with death of apical bud; "stiffening" and "thickening" of the stem; and usually a period of dormancy in all buds leading to stunting of the seedling. All have been described previously (14) with the exception of the terminal blight condition, which is reported here for the first time.

Infection of *P. radiata* stems with *C. acutatum* f. sp. *pinea* appears to affect two physiological functions of a type mediated by plant regulators such as auxins, gibberellins, cytokinins, and inhibitors (15,17,28). That it is the host-parasite interaction that leads to dormancy of apical and lateral buds on the one hand, and precocious secondary thickening on the other, is indicated by the lack of such responses to decapitation and mechanical (needle) wounding of comparable healthy plants.

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