Scanning Electron Microscopy of Infection of Scotch Pine Needles by Scirrhia acicola

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ABSTRACT

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Development of germ tubes from conidia on the needle surface and in the stomatal antechamber of Scotch pine was studied by scanning electron microscopy. Germ tubes usually grew appressed to the needle surface and followed contours of the epidermis. Often germ tube growth seemed to be directed specifically toward an individual stoma. In the antechamber the germ tube usually increased in diameter, became thick-walled, melanized, rugose in surface texture, and irregular in general form and outline. It grew in a meandering fashion without branching, or it branched and developed a convoluted form. Penetration between the guard cells and subsequent growth in the substomatal chamber or mesophyll could follow either type of growth in the antechamber but did not always occur. Germ tube behavior patterns may be the result of responses to stimuli emanating from individual stoma.

Brown spot needle blight, caused by *Scirrhia acicola* (Dearn.) Siggers, constitutes a major problem in establishing longleaf pine (*Pinus palustris* Mill.) in the southern states (12), and it is also prevalent in the Southeast on loblolly pine (*P. taeda* L.) (1), although the damage to this species is unassessed. Recently, brown spot disease became serious in Scotch pine (*P. sylvestris* L.) Christmas-tree plantations in Wisconsin and other north central states (8). Practical controls for the disease under some circumstances are known (12,13), but realization of more satisfactory control lies in selection and breeding for resistance, and already some effort has been directed toward this end (2,15). Unfortunately, information on the processes of infection and disease development or on the nature and expression of resistance in the host is sparse. Knowledge of these aspects is important in identification and release of resistant trees through a resistance breeding program. As an aid to the development of reliable progeny testing through artificial inoculation procedures, we undertook a study of needle infection by conidia of *S. acicola*.

Although numerous reports in the literature pertain to the etiology of brown spot needle blight (12), little is known about the mode of infection by this fungus. Wolf and Barbour (16) stated that mycelium first localize in substomatal chambers, but they did not describe penetration of hyphae through stomata. That infection occurs through stomata was indicated by direct observation of plastic film impressions (14) and stripped epidermal segments (6,9) and finally with fluorescence microscopy of germinated spores on pine needles (11).

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Killebrew (6) and Parris and Killebrew (9) also reported observations of an appressorialike, knobby or convoluted structure that develops above the stoma on loblolly pine, but such a structure was not evident in observations by fluorescence microscopy of the fungus on either scotch pine or longleaf pine needles (11).

When this work began, a reliable method for artificially inoculating pines with *S. acicola* was not available. Since then, Kais (4) reported on environmental factors affecting brown spot infection and proposed a method for inoculation in the greenhouse. During our investigations we made numerous inoculations with a variety of methods, but results were largely inconclusive. Germ tube entry into stomatal antechambers had been commonly observed by fluorescence microscopy (11), and this led to further examination by scanning electron microscopy.

MATERIALS AND METHODS

To select segments bearing germinated spores for scanning electron microscopy (SEM), needles were prescreened by epifluorescence microscopy after spores were labeled with a fluorescent brightener (11).

Scotch pine needles of the season's growth collected at random from 2-yr-old greenhouse-grown seedlings and from trees in a Christmas-tree plantation were examined. Seedlings were inoculated by spraying the needles with a water suspension of conidia produced in culture and incubated for 5 days in plastic bags or in a greenhouse mist chamber. Most of the observations, however, were of naturally-infected needles collected at intervals from June into October 1974 from trees in a heavily-infected Christmas-tree plantation in central Wisconsin.

Most needle segments chosen for SEM were fixed in 3% glutaraldehyde in 0.2 M sodium phosphate buffer (pH

7.4), dehydrated in a graded series of ethyl alcohol solutions from 35% to 100%, dried by the critical-point drying method from amyl acetate through liquid CO_2, coated with gold-palladium, and examined with a JEOL JSM-U3 SEM. Observations at 10 kV brought out the most surface detail.

Comparisons of the appearance of epicuticular wax were made on needles prepared for SEM by different methods. Wax on fresh needles given no treatment except the gold-palladium coating immediately before observation was apparently unchanged (Fig. 1). Use of preparative solutions in the critical point drying process resulted in partial to complete dissolution of wax (Fig. 2). Because observation of germ tube form and position in relation to stomatal structures was easier on de-waxed than on natural surfaces and because natural relationships were unchanged, the critical point drying process was used for most preparations.

Cross sections of infected needles that were first observed by SEM were made from selected segments, which were then processed by standard paraffin embedding and sectioning procedures. Segments were removed from the SEM stubs, soaked in three changes of acetone to remove the silver paste and three changes of 100% tertiary butyl alcohol, and finally infiltrated with and embedded in paraffin.

After observation by light microscopy of sections made from SEM-examined needle segments as described, selected sections were prepared for SEM. Cover slips were removed by soaking slides in xylene, and portions of

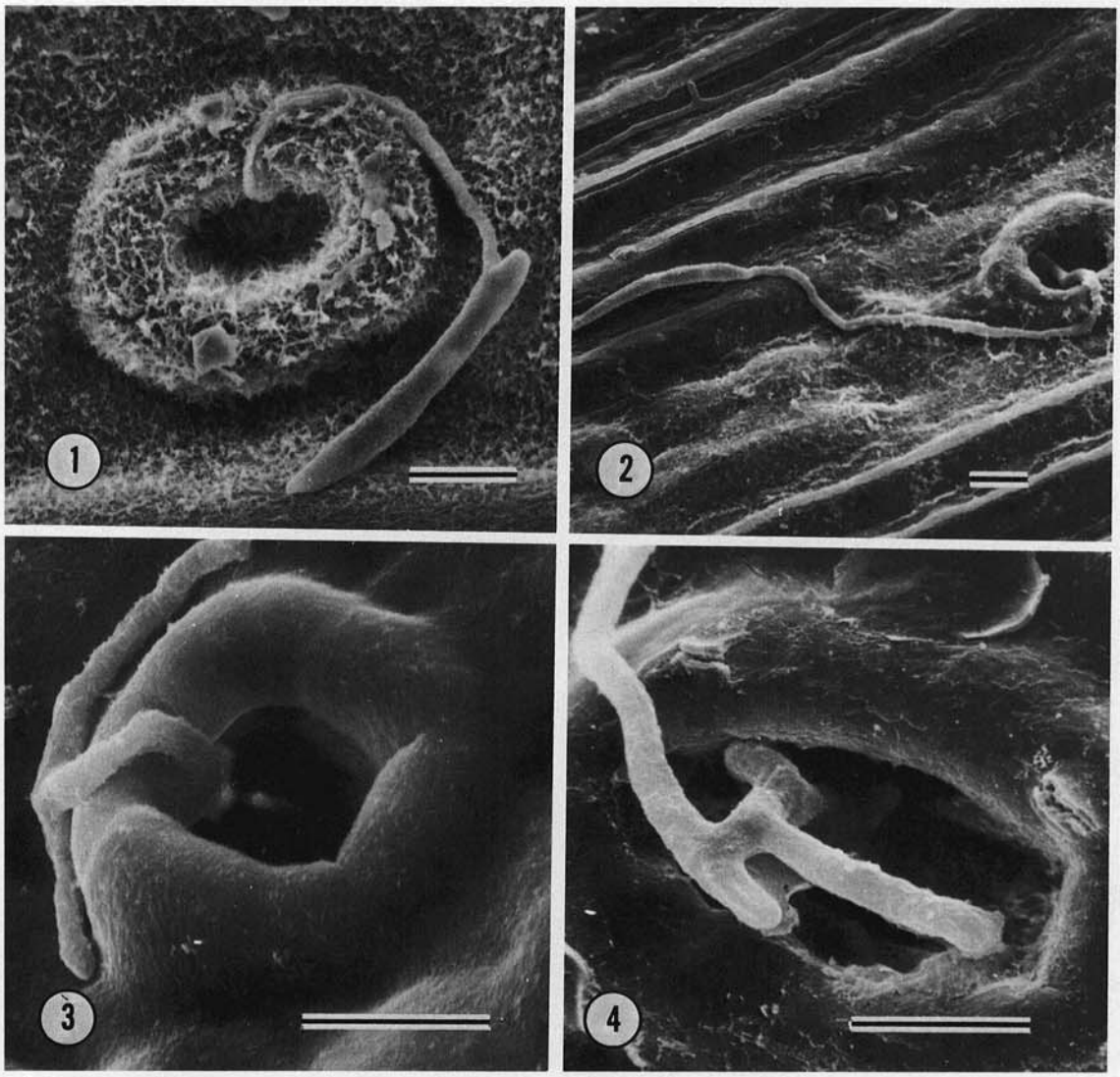

Fig. 1–4. Growth of germ tubes from conidia of *Scirrhia acicola* on needles of plantation Scotch pine trees and penetration of germ tubes into stomata. Scale bars represent 5 μm. **1)** Entry of germ tube into a stoma of a fresh needle on which the epicuticular wax was not disturbed. **2)** Typical germ tube growth on needle surface and entry into the antechamber. **3)** Branching of a germ tube during growth around base of a subsidiary cell and entry of a branch into the antechamber. **4)** Branched development of a germ tube in the antechamber.

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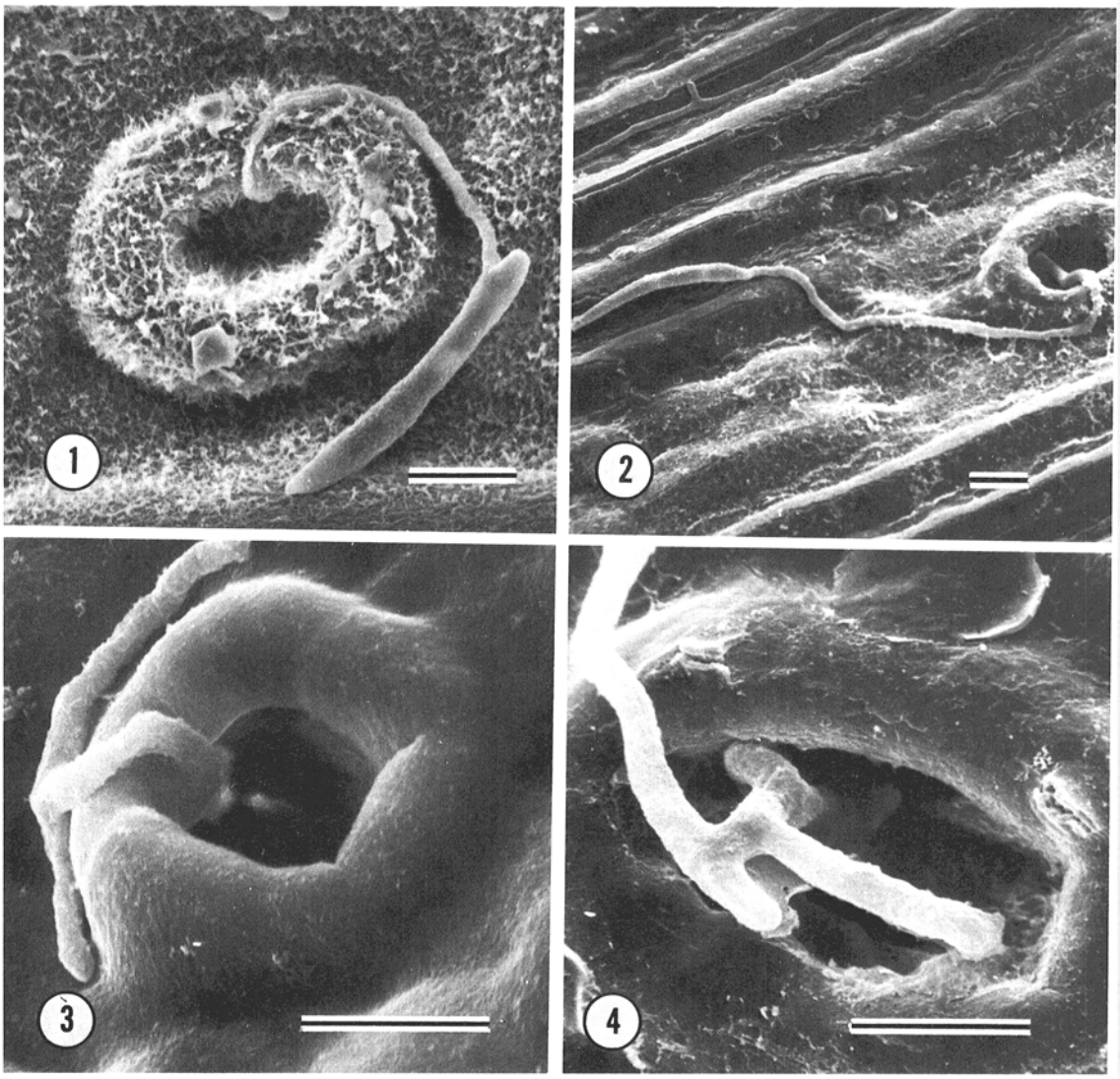

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the slide bearing selected needle sections were cut while saturated with xylene with a rotary glass cutter or diamond marker to fit onto SEM stubs. Sections were then processed through changes of 100% ethyl alcohol into amyl acetate, dried by the critical point method, mounted on SEM stubs, and coated with gold-palladium.

RESULTS

The patterns of spore germination and germ tube development were similar on needles from inoculated seedlings and plantation trees, and all illustrations are of needles from naturally-infected plantation trees.

During spore germination on needles, usually the first and often the only germ tube grew from a terminal cell (Fig. 2). On agar media, however, a germ tube commonly grew from every cell of three- or four-celled conidia. Germ tubes as long as 280 μm were measured on artificially-inoculated needles, although most were less than 100 μm, and such extensive growth was not common on plantation trees. The germ tube usually grew appressed to the needle surface and followed the contours of the epidermis (Fig. 2). The germ tube was smooth to rugulose (Fig. 3), whereas the spore surface was markedly verrucose (Fig. 5, 6).

Germ tube development on the needle surface varied,

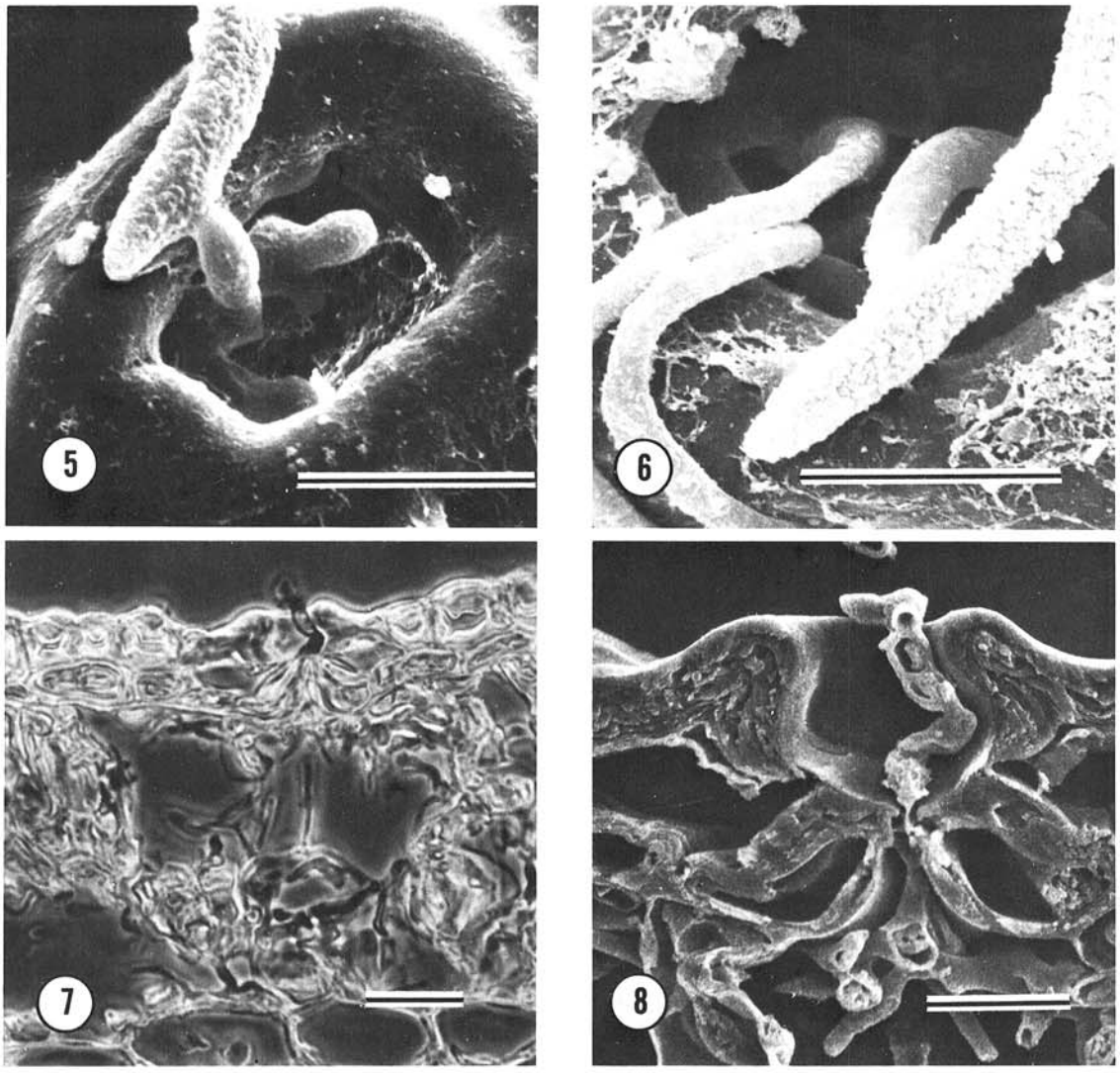

Fig. 5–8. Development in the stomatal antechamber of germ tubes from conidia of *Scirrhia acicola* on needles of plantation Scotch pine trees. Scale bars represent 5 μm. **5)** Branched and convoluted growth of the germ tube from a spore on the edge of a stoma. The netlike strands are remnants of partially dissolved and greatly altered epicuticular wax deposits that coated walls of the antechamber and surface of the subsidiary cells. **6)** Multiple entry and convoluted growth of germ tubes in the antechamber. **7–8)** Phase contrast and SEM views of the same needle cross section at the point of penetration by a germ tube into the substomatal chamber. The germ tube was compressed to a narrow infection hypha between the guard cells. The germ tube in the antechamber has become multiseptate, thick-walled, and melanized in contrast to the typical hyaline growth on the surface.

and the pattern seemed much too irregular to be considered "directed growth" in response to surface features of the substrate, as described by Dickinson (3) and Lewis and Day (7). Growth usually was more or less random at various angles to the epidermal ridges but was generally in a relatively straight or gently curving line (Fig. 2).

Growth of germ tubes on needles of plantation trees varied in relation to possible stomatal attraction. Some grew as if, at some stage in their development, they had responded directly to an attractive stimulus from a stoma (Fig. 1,2). Others showed no evidence of stomatal attraction, some even growing across the edge of a stomatal aperture without entering. Multiple entries were seen occasionally (Fig. 6).

Branching of a germ tube on the needle surface was not common but occurred occasionally, usually in association with a stoma during the course of growth around the base of the subsidiary cell (Fig. 3).

After entering the stomatal antechamber, the germ tube developed according to one of two characteristic patterns. In one type it continued growth more or less irregularly without branching (Fig. 2). In a second type it branched, became convoluted, often increased in diameter, became melanized, thick-walled, and rugose in surface texture, and was more irregular in general form and outline than on the needle surface (Fig. 4, 5). Subsequent infection was possible from either type but did not always follow.

Observations of the fungus in the stoma and after it penetrated between the guard cells were made on needle cross sections by both light microscopy and SEM. The depth of field provided by SEM allowed an excellent view of germ tube entry into the stoma, development within the antechamber, penetration between the guard cells, and subsequent growth of hyphae in the mesophyll (Fig. 7,8). Light microscopy provided additional information such as septation and melanization of the fungus in the stomatal antechamber.

No true appressorium or appressoriumlike structure was ever observed. The extensive branching and convolution of the germ tube that often developed in the antechamber (Fig. 4, 5) may well have been what Killebrew (6) observed on stripped epidermal segments and misinterpreted as an appressorium.

DISCUSSION

The factors that influence infection of pine needles by *S. acicola* and the reasons for inconsistent results from artificial inoculations still have not been completely explained. The variable behavior of the germ tube after it enters the stomatal antechamber, however, gives some clues to the problem.

The growth patterns on the needle surface in relation to stomata suggest the possibility of stomatal attraction as reported for *Dothistroma pini* by Peterson (10). Possibly some stimulus attracts a germ tube to an individual stoma, but if so, this is not a general phenomenon expressed uniformly by all stomata on a needle.

Branching of the germ tube that occurred at the outer base of the subsidiary cell was first thought to be a response to a thigmotropic stimulus. But as more examples were observed, it became evident that branching usually occurred well after the germ tube changed its direction of growth to continue for some distance around the base of the subsidiary cell. Perhaps branching was then initiated in response to a physical or chemical stimulus that emanated from the stoma at a certain time and was not constantly present. This also might account for a sudden directional change as in Fig. 2. If, when a germ tube was growing at random over or near a stoma, there was no emission of a stimulus, then the germ tube might continue to grow without being attracted into the antechamber, as was observed many times.

The branching and convoluted development of the germ tube in the antechamber and the thickening, melanization, and rugose texture of the cell wall all seem to be responses to a stimulus or a microenvironment that is not encountered on the needle surface.

Penetration of the germ tube between the guard cells with resultant infection of needle mesophyll tissue appears to be a further response either to a different stimulus or to the continued presence of the original stimulus. The presence or absence of this stimulus or the timing of its appearance may govern behavior of the germ tube within the antechamber and ultimate infection of the needle. The importance of degree of opening between guard cells is unknown. Field observations have led to the inference that light may favor infection, and recently Kais (4) found that infection of artificially inoculated needles is increased significantly by light. Perhaps light influences position of the guard cells or favors production of a stimulus that induces germ tube penetration.

Infection of a pine needle by *S. acicola* undoubtedly is influenced by stimuli associated with its physiological state, including age (5). We have observed patterns of germ tube development and subsequent penetration in longleaf pine needles that correlate well with the age-susceptibility relationship reported by Kais (5). We are uncertain whether such a relationship exists with Scotch pine. In our observations of Scotch pine needles collected from June to October, however, in which there was no control of the date of inoculation, we did find indications of a relationship between age and germ tube development in stomata. In June collections most germ tubes remained unbranched in the antechamber, whereas in collections of mature needles after about mid-August, although both branched and unbranched germ tubes were observed, the convoluted form was more common.

Although age or maturation of needle tissue may be one significant factor influencing infection, there seem to be others also. In fact, artificial inoculations of young needles of both longleaf and Scotch pine seedlings did not always result in infection even after numerous stomatal entries. The stimuli that favor infection when the needle tissue is susceptible still remain to be determined.

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